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Fraxinellone inhibits inflammatory cell infiltration during acute pancreatitis by suppressing inflammasome activation

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ABSTRACT

Inflammasomes promote the production of pro-inflammatory cytokines, such as interleukin (IL)-1β and IL-18, which are the representative mediators of inflammation. Abnormal activation of inflammasomes leads to the development of inflammatory diseases such as acute pancreatitis (AP). In this study, we demonstrate the inhibitory effects of a new natural compound fraxinellone on inflammasome formation and examine the role of inflammasomes in a mouse model of AP. AP was induced with hourly intraperitoneal injections of supramaximal concentrations of the stable cholecystokinin analogue cerulein (50 μg/kg) for 6 h. Mice were sacrificed 6 h after the final cerulein injection. Blood and pancreas samples were obtained for further experiments. Intraperitoneal injection of fraxinellone significantly inhibited the pancreatic activation of multiple inflammasome molecules such as NACHT, LRR and PYD domains-containing protein 3 (NLRP3), PY-CARD, caspase-1, IL-18, and IL-1β during AP. In addition, fraxinellone treatment inhibited pancreatic injury, elevation in serum amylase and lipase activities, and infiltration of inflammatory cells such as neutrophils and macrophages but had no effect on pancreatic edema. To investigate whether inflammasome activation leads to the infiltration of inflammatory cells, we used parthenolide, a well-known natural inhibitor, and IL-1 receptor antagonist mice. The inhibition of inflammasome activation by pharmacological/or genetic modification restricted the infiltration of inflammatory cells, but not edema, consistent with the results observed with fraxinellone. Taken together, our study highlights fraxinellone as a natural inhibitor of inflammasomes and that inflammasome inhibition may lead to the suppression of inflammatory cells during AP.

1. Introduction

Acute pancreatitis (AP) is a fatal disease associated with high mortality rate [1] and various complications [2]. In general, alcohol abuse and gallstone are typical risk factors of AP [3,4]. Upon AP onset, pancreatic acinar cells are activated to release excessive digestive enzymes and inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. In addition, monocytes such as macrophages and neutrophils infiltrate the inflamed site [5,6]. Although several researchers have attempted to understand the regulatory mechanism underlying AP [7], the development of an effective treatment strategy and the knowledge of the mechanism of AP are still lacking.

One of the promising signaling pathways involved in the pathogenesis of AP is the inflammasome cascade. Inflammasomes comprise three domains, namely, a nucleotide-binding domain, leucine-rich repeat-containing family (NLR), an apoptosis-associated speck-like protein containing CARD (ASC), and caspase-1. After its activation by the cytosolic protein complex of an inflammasome, caspase-1 mediates the secretion of pro-IL-1 and IL-18, which are important factors involved in inflammation induction [8]. In an AP model, NLRP3, ASC, and caspase-1 cascades are reported to be essential for the development of pancreatic inflammation [9]. Moreover, the inhibition of IL-1 signaling by the treatment with IL-1 receptor antagonist (IL-1RA) results in the reduction in pancreatic inflammation and tissue damage [10,11]. Thus, the blockade of inflammasomes or IL-1 signaling may serve as a promising strategy for the effective treatment of AP. How

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Table 1
Effect of fraxinellone on biochemical parameters.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Creatinine (mg/dL)
DMSO	125.67 ± 26.95	35.00 ± 2.65	765.33 ± 55.90	0.18 ± 0.02
0.5 mg/kg	126.00 ± 18.38	35.67 ± 6.35	745.00 ± 27.18	0.19 ± 0.03
1 mg/kg	126.67 ± 15.81	36.00 ± 3.46	730.33 ± 81.12	0.19 ± 0.02
5 mg/kg	127.67 ± 11.55	36.67 ± 0.58	706.33 ± 36.12	0.18 ± 0.03
10 mg/kg	134.33 ± 21.78	35.00 ± 2.65	763.67 ± 39.43	0.18 ± 0.02
50 mg/kg	147.33 ± 11.22*	40.33 ± 1.53*	761.67 ± 31.21	0.22 ± 0.01*

AST, aspartate transaminase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CREA, creatinine; DMSO, dimethyl sulfoxide. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments.
* $P < 0.05$ versus DMSO treatment alone.

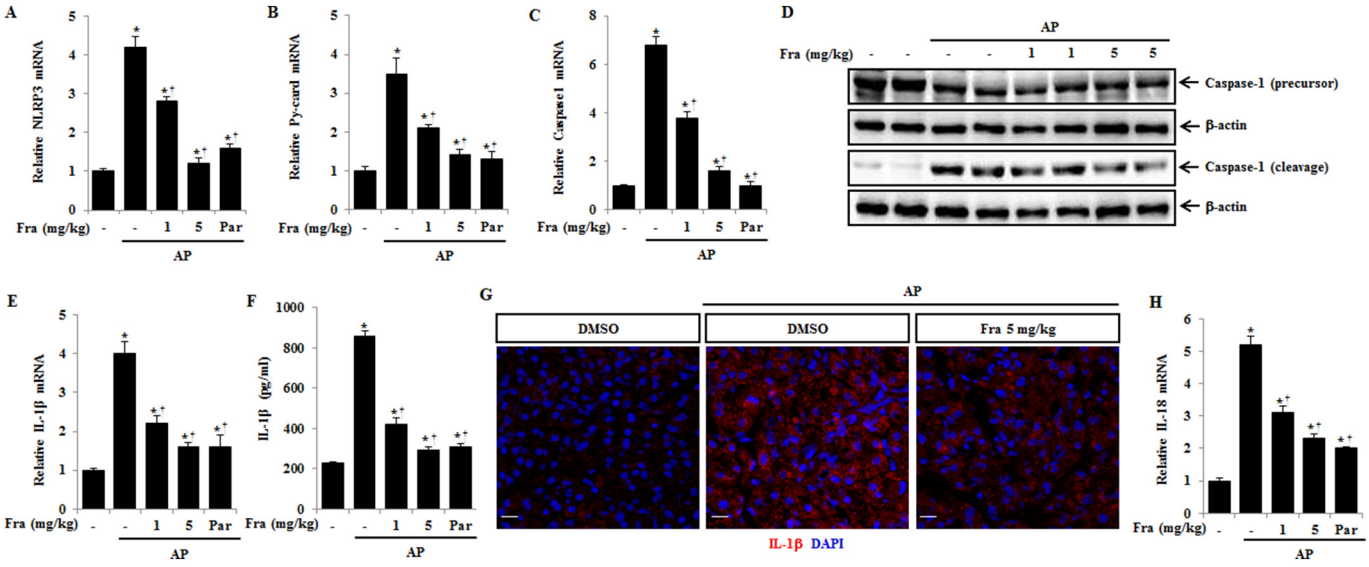

Fig. 1. Effects of fraxinellone (Fra) on inflammasome activation during AP. Mice were pretreated with fraxinellone (1 or 5 mg/kg), parthenolide (25 mg/kg) or DMSO 1 h before the first cerulein (50 μg/kg) injection. Mice were sacrificed 6 h after the last cerulein injection. (A–C) mRNA levels of pancreatic NLRP3, PY-CARD, and caspase-1. (D) Total and cleaved form of caspase-1. β-actin was used as a loading control. (E) mRNA levels of pancreatic IL-1β and (F) circulating IL-1β levels from serum. (G) Immunofluorescence staining of IL-1β in the pancreas. Nuclei were stained with DAPI. (H) mRNA levels of pancreatic IL-18. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. *$P < 0.05$ versus DMSO treatment alone, †$P < 0.05$ versus cerulein treatment alone. Scale bar: 10 μm. "Par" is "Parthenolide" the inhibitor of inflammasomes.

inflammasomes contribute to the development of AP is questionable.

Fraxinellone is a limonoid component isolated from *Dictamnus dasycarpus* [12] and has been reported to exert anti-inflammatory activities related to the inhibition of inflammatory mediators through the suppression of nuclear factor kappa B (NF-κB) in macrophages [13]. In addition, fraxinellone has been demonstrated to possess neuroprotective and vaso-relaxing activities [12,14]. Wu and colleagues recently reported that fraxinellone could attenuate murine colitis via suppression of NLRP3 inflammasome activation [15]. However, the potential role of fraxinellone in AP is yet unknown. Therefore, in this study, we investigated the anti-inflammatory effects of fraxinellone on cerulein-induced AP and the underlying regulatory mechanism. In addition, we examined the role of inflammasomes during AP.

2. Materials and methods

2.1. Materials

Avidin-peroxidase, 3′3′5′5′-tetramethylbenzidine (TMB), cerulein, Tris–HCl, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), potassium chloride (KCl), calcium chloride ($CaCl_2$), HEPES, and fraxinellone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-IL-1β and recombinant IL-1β, were procured from R&D Systems (Minneapolis, MN, USA). The monoclonal antibodies against caspase-1, β-actin, and F4/80 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against neutrophils were purchased from Abcam (Cambridge, UK) and the Easy-Blue™ Total RNA extraction kit was supplied by iNtRON Biotechnology (Sungnam, KyungKi Do, South Korea).

2.2. Animal model

All experiments were conducted in compliance with the protocols approved by the Animal Care Committee of Wonkwang University and were approved by the Institutional Animal Care and Use Committee (IACUC) Certification of the Wonkwang University, South Korea. Several female or male C57BL/6 mice (age: 6–8 weeks, weight: 15–20 g) were purchased from Orient Bio (Sungnam, KyungKi Do, South Korea). IL-1R antagonist mice were purchased from Jackson laboratory (Bar Harbor, ME, USA). The animals were maintained under standardized conditions with an ambient temperature of 23 °C ± 2 °C and a 12 h light and dark cycle for 7 days. Before the induction of pancreatitis, the animals were fed with a standard laboratory chow and had free access to water. Animals were randomly assigned to control or experimental groups. The mice were made to fast for 18 h before the induction of AP. AP was induced by intraperitoneal injections of supramaximal concentration of the stable cholecystokinin (CCK) analogue cerulein (50 μg/kg), administered hourly for 6 h. Control animals were administered saline under the same conditions. For the examination of prophylactic effects, fraxinellone (1 and 5 mg/kg, $n = 6$), parthenolide

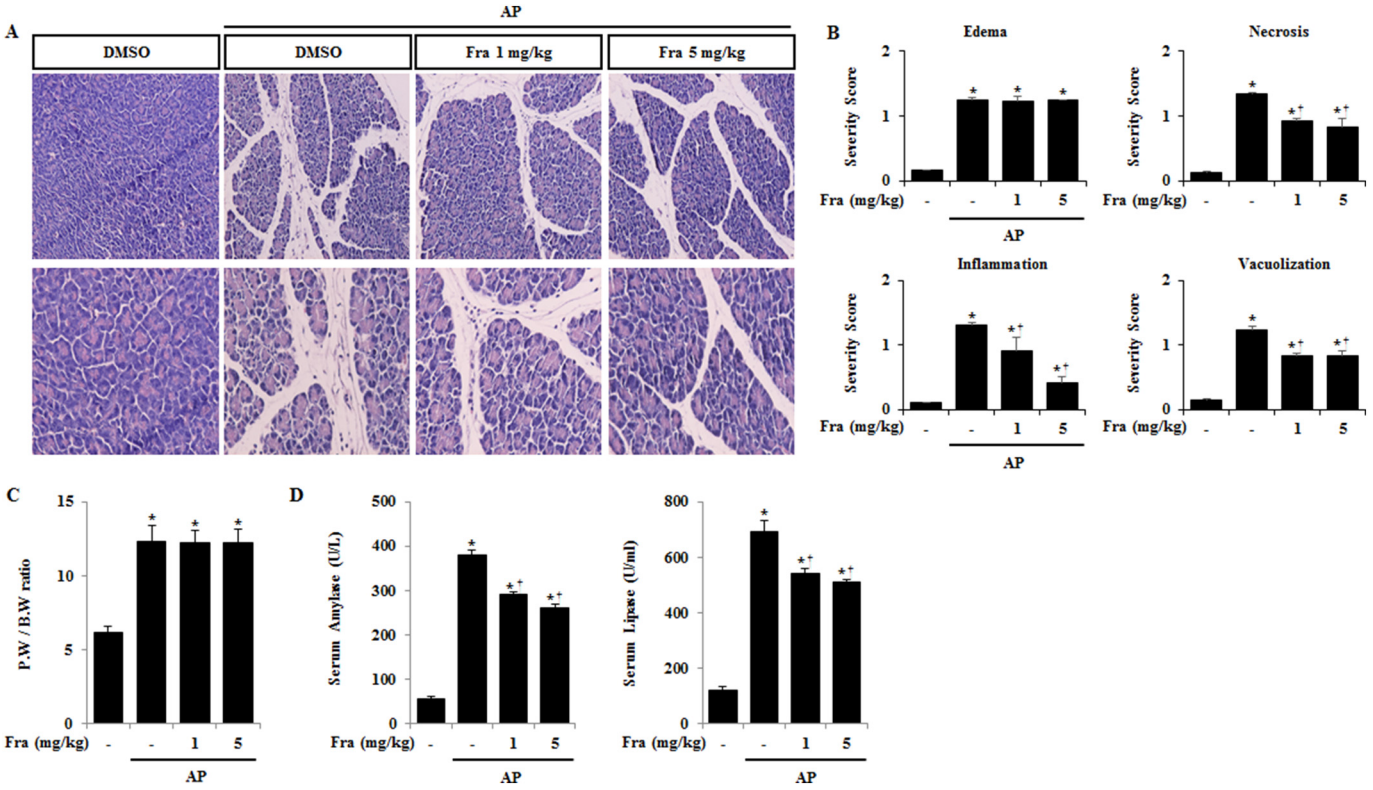

Fig. 2. Prophylactic effects of fraxinellone (Fra) on pancreatic injury during cerulein-induced AP. Mice were pretreated with fraxinellone (1 or 5 mg/kg) or DMSO 1 h before the first cerulein (50 μg/kg) injection. Mice were sacrificed 6 h after the last cerulein injection. (A and B) Representative H&E-stained sections of the pancreas (upper panel, 100× and lower panel, 200× magnification), histological score for edema, inflammation, necrosis and vacuolization. (C) P.W./B.W. ratio. (D) Serum amylase and lipase activities. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. *$P < 0.05$ versus DMSO treatment alone, †$P < 0.05$ versus cerulein treatment alone.

(25 mg/kg, $n = 6$), or dimethyl sulfoxide (DMSO, $n = 6$) were intraperitoneally administered 1 h before the first cerulein injection. For the examination of therapeutic effects, fraxinellone (5 mg/kg, $n = 6$), or DMSO ($n = 6$) were intraperitoneally administered 1, 3, and 6 h after the first cerulein injection. Mice were sacrificed 6 h after the last cerulein injection. Pancreas and blood samples were immediately collected for further examination.

2.3. mRNA expression

Transcription of IL-β, NLRP3, PY-CARD, caspase-1, IL-18, C-C motif chemokine ligand 2 (CCL2), and C-X-C motif chemokine ligand 2 (CXCL2) was analyzed by reverse-transcription polymerase chain reaction (RT-PCR) in mouse pancreatic tissues. Total RNA from pancreas samples were extracted with Easy-Blue™, and the purity of the samples was confirmed by RNA calculator (Gene Quant Pro, Biochrom, Holliston, MA, USA). The total RNA extraction kit was used according to the manufacturer's instructions and reverse transcription of RNA to cDNA was performed using the ABI cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). TaqMan quantitative RT-PCR with an ABI StepOne Plus detection system was performed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). For each sample, triplicate test reactions and a control reaction without reverse transcriptase were analyzed for the expression of the gene of interest and to prevent any variations in the reactions. All PCR data were normalized to the expression level of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT). Forward, reverse, and probe oligonucleotide primers for multiplex real-time TaqMan PCR were purchased from ABI (Applied Biosystems, Foster City, CA, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Levels of protein were analyzed in serum samples with an ELISA. ELISA for IL-1β was performed in duplicates in 96-well plates (Nunc, Roskilde, Denmark) that were overnight incubated with 100 μL aliquots of anti-mouse IL-1β monoclonal antibodies (1.0 μg/mL). The plates were washed in phosphate-buffered saline (PBS) containing 0.05% Tween-20 and blocked with PBS containing 10% fetal bovine serum (FBS) for 2 h. After additional washes, the standards and serum were added to the plates and incubated at room temperature for 3 h. The frozen serum samples were thawed and diluted in PBS. After washing the wells, 0.2 μg/mL of biotinylated anti-mouse IL-1β was added to each well. Incubation was continued at room temperature for 1 h. The wells were washed and treated with avidin-peroxidase for 30 min at room temperature. Wells were washed again, followed by the addition of the TMB substrate. Color development was measured at a wavelength of 450 nm using an automated microplate ELISA reader. Standard curves were obtained for each sample using serial dilutions of recombinant IL-1β.

2.5. Western blotting

Pancreatic tissues were homogenized and lysed on ice, and the lysates were boiled in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], 20% glycerol, and 10% 2-mercaptoethanol). The proteins were separated by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS-Tween-20 (PBST) for 2 h at room temperature and then incubated with primary antibodies overnight at 4 °C. After washing thrice, the membrane was incubated with a secondary antibody for 1 h. The proteins were

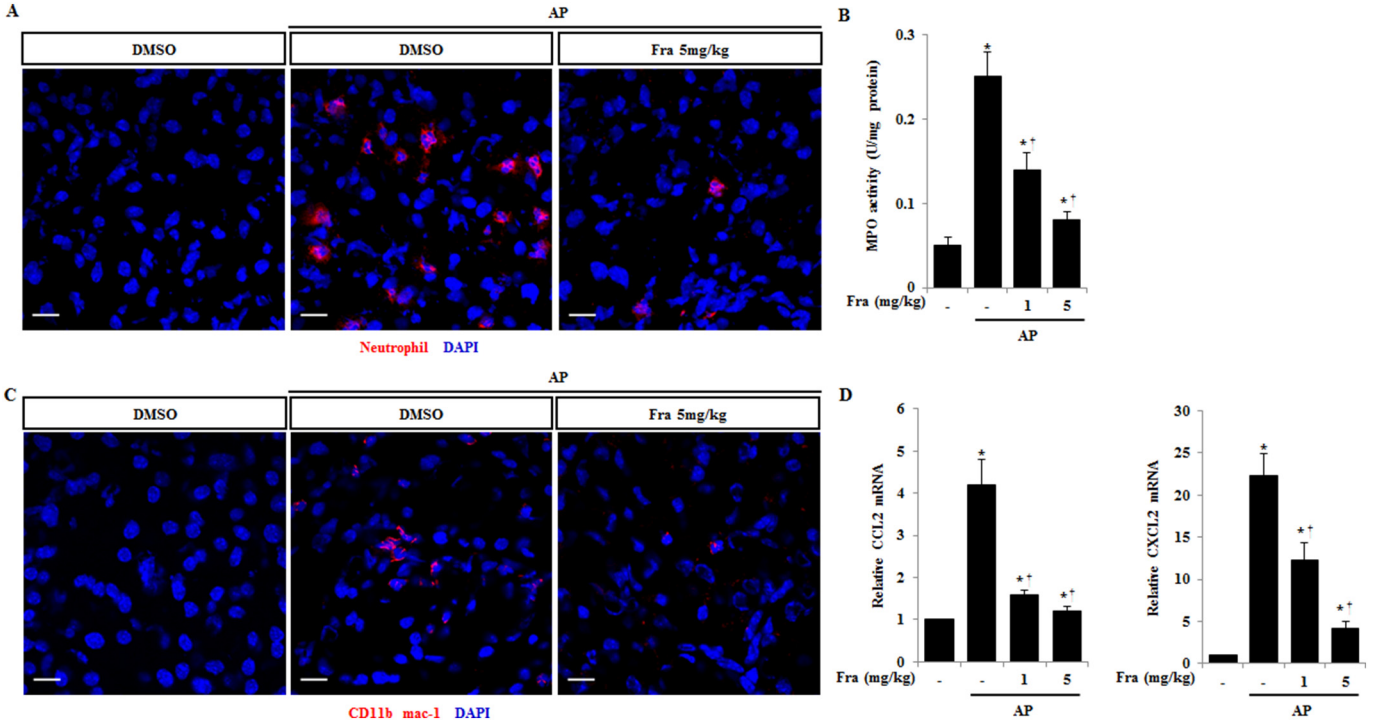

Fig. 3. Effects of fraxinellone (Fra) on neutrophil/macrophage infiltration during AP. Mice were pretreated with fraxinellone (1 or 5 mg/kg) or DMSO 1 h before the first cerulein (50 μg/kg) injection. Mice were sacrificed 6 h after the last cerulein injection. (A) Immunofluorescence staining of neutrophils (red) and DAPI staining in the pancreas. (B) Pancreatic MPO activity. (C) Immunofluorescence staining of $CD11b^+$ mac-1 for macrophages (red) and DAPI staining in the pancreas. (D) mRNA levels of CCL2 and CXCL2 in the pancreas. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. $^*P < 0.05$ versus DMSO treatment alone, $^\dagger P < 0.05$ versus cerulein treatment alone. Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

developed using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) according to the manufacturer's recommended protocol.

2.6. Histological analysis

For histological examination and scoring, the pancreas tissues were fixed in 4% paraformaldehyde solution, embedded in paraffin using standard methods, cut into 4-μm sections, stained with hematoxylin-eosin (H&E), and then examined under a light microscope. The pancreas samples from each treatment group were examined and semi-quantitatively assessed for the levels of edema and inflammation. Levels of edema, inflammation, necrosis, and vacuolization were scored on a scale of 0 to 3 (0 being normal and 3 being severe) [16,17].

2.6.1. IHC analysis

The expressions of IL-1β, $CD11b^+$ mac-1 (for macrophages), and neutrophils in pancreas tissues were evaluated with immunofluorescence. The frozen sectioned tissues (9-μm thickness) were stained with primary antibodies at 4 °C overnight, followed by the treatment with Alexa Fluor 488 or 594-labeled secondary antibodies at room temperature for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Stained sections were visualized using a confocal laser microscope (Olympus, Japan).

2.7. Determination of pancreatic edema

The ratio of pancreatic weight (P.W.) to body weight (B.W.) was evaluated as an estimate of the degree of pancreatic edema. At the time of sacrifice, the pancreas and body of mice were weighed. P.W. was divided by B.W. and then multiplied by 1000 to obtain the value of the ratio as a natural number.

2.8. Biochemical analysis

Fraxinellone was intraperitoneally administered to mice ($n = 6$ per group). Twenty-four hours after administration, serum was obtained and separated using a centrifuge maintained at 5000 rpm for 5 min. Fresh serum was used for the analysis of urine sodium (UN), creatinine (CREA), glutamic-oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and alkaline phosphatase (ALP) levels using biochemical kits (Span Diagnostics, Surat, France).

2.8.1. Quantification of serum amylase and lipase levels

Amylase and lipase activities were determined by an assay kit of Bio Assay Systems (CA, USA).

2.8.2. Myeloperoxidase (MPO) activity

Sequestration of neutrophils within the pancreas and lung was evaluated by measuring tissue MPO activity. Briefly, tissue samples were weighed, homogenized with 20 mM of phosphate buffer (pH 7.4), and centrifuged at 10,000 ×*g* for 10 min at 4 °C. The pellets were re-suspended in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM phosphate buffer (pH 6.0). The samples were centrifuged at 10,000 ×*g* for 5 min at 4 °C, and the MPO activity was determined using 1.6 mM TMB, 80 mM sodium phosphate buffer (pH 5.4), and sodium phosphate buffer. This mixture was incubated at 37 °C for 110 s and the reaction was terminated with the addition of 2 mol/L of sulfuric acid (H_2SO_4). The absorbance was measured at 450 nm wavelength and MPO activity was expressed as unit activity per milligram of protein.

2.9. Statistical analysis

Results are expressed as means ± standard error of means (SEM). The significance was evaluated using two-way analysis of variance

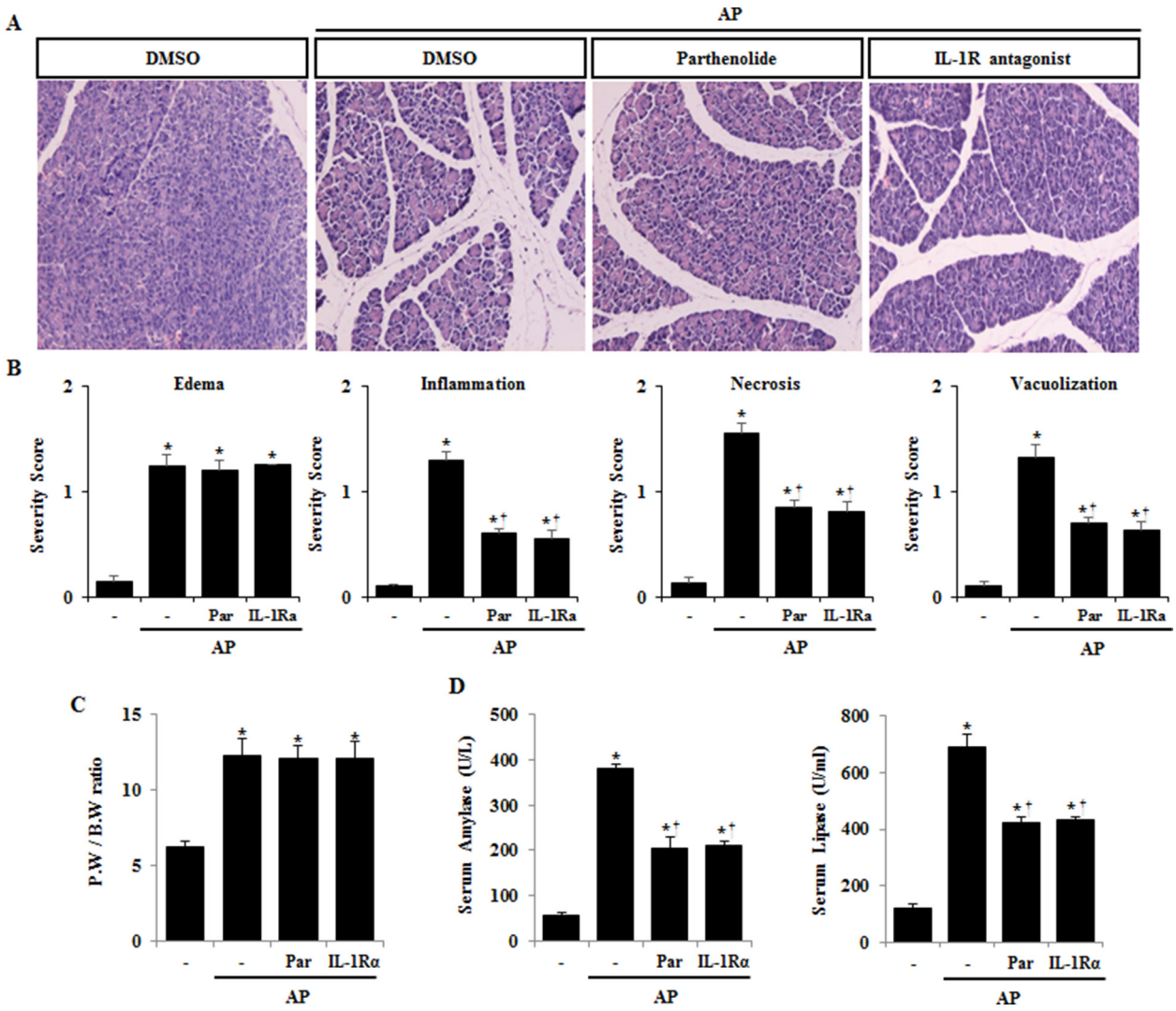

Fig. 4. Effect of inflammasome inhibition on pancreatic injury during cerulein-induced AP. Mice were pretreated with parthenolide (25 mg/kg) or DMSO 1 h before the first cerulein (50 μg/kg) injection. Mice were sacrificed 6 h after the last cerulein injection. (A and B) Representative H&E-stained sections of the pancreas (100× magnification) and histological score for edema, inflammation, necrosis and vacuolization. (C) P.W./B.W. ratio. (D) Serum amylase and lipase activities. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. *$P < 0.05$ versus DMSO treatment alone, †$P < 0.05$ versus cerulein treatment alone.

(ANOVA). Values of $P < 0.05$ were considered statistically significant. The results were similar in three independent experiments.

3. Results

3.1. Effects of fraxinellone on biochemical parameters in mice

To study the biological activity of fraxinellone, we examined its possible toxicity under normal conditions in mice. Mice were intraperitoneally administered with either fraxinellone or DMSO (for control) and serum samples were harvested after 24 h for the examination of biochemical factors that could influence the metabolism of mice. In comparison with DMSO treatment, the administration of fraxinellone at 50 mg/kg caused toxicity (Table 1), as observed with the levels of aspartate transaminase (AST), alanine aminotransferase (ALT), and creatinine. However, the administration of fraxinellone at concentrations < 10 mg/kg had no effect on the levels of these biochemical parameters (Table 1). Therefore, we used fraxinellone at concentrations below 10 mg/kg in the subsequent experiments to investigate its beneficial effects on AP.

3.2. Effect of fraxinellone on NLRP3 inflammasome during AP

The NLRP3 inflammasome is an intracellular complex triggering inflammatory responses during AP [9]. Therefore, we examined the effects of fraxinellone on NLRP3 inflammasome complexes (NLRP3, PY-CARD, and caspase-1) by treating mice with an intraperitoneal injection of fraxinellone for 1 h, followed by the induction of AP with cerulein. Parthenolide was used as an inhibitory drug to inhibit inflammasome activation (sFig. 1). In accordance with previous report [18], the mRNA expressions of NLRP3, PY-CARD, and caspase-1 increased, and caspase-1 was cleaved in mice with AP (Fig. 1A–D). In addition, IL-1β and IL-18 levels increased as a consequence of inflammasome activation (Fig. 1E–H). However, treatment with fraxinellone resulted in the inhibition of NLRP3 inflammasome activation and IL-1β and IL-18 production, as observed with parthenolide treatment (Fig. 1). Treatment of the experimental model with 10 mg/kg fraxinellone resulted in an inhibitory activity similar to that observed with 5 mg/kg fraxinellone (data not shown). Thus, we used 5 mg/kg of fraxinellone as the highest concentration to inhibit inflammasome activation in AP.

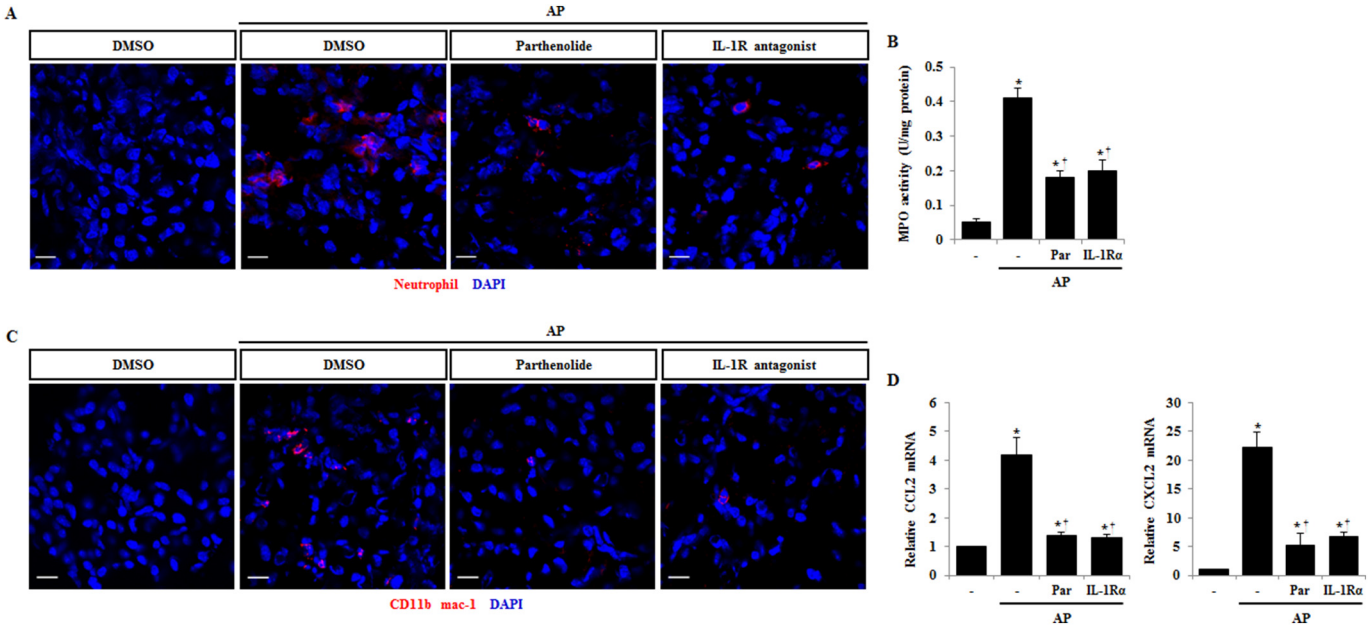

Fig. 5. Effect of inflammasome inhibition on neutrophil/macrophage infiltration during AP. Mice were pretreated with parthenolide (25 mg/kg) or DMSO 1 h before the first cerulein (50 μg/kg) injection. Mice were sacrificed 6 h after the last cerulein injection. (A) Immunofluorescence staining of neutrophils (red) and DAPI staining in the pancreas. (B) Pancreatic MPO activity. (C) Immunofluorescence staining of $CD11b^{+}$ mac-1 for macrophages (red) and DAPI staining in the pancreas. (D) mRNA levels of CCL2 and CXCL2 in the pancreas. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. $^{*}P < 0.05$ versus DMSO treatment alone, $^{\dagger}P < 0.05$ versus cerulein treatment alone. Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

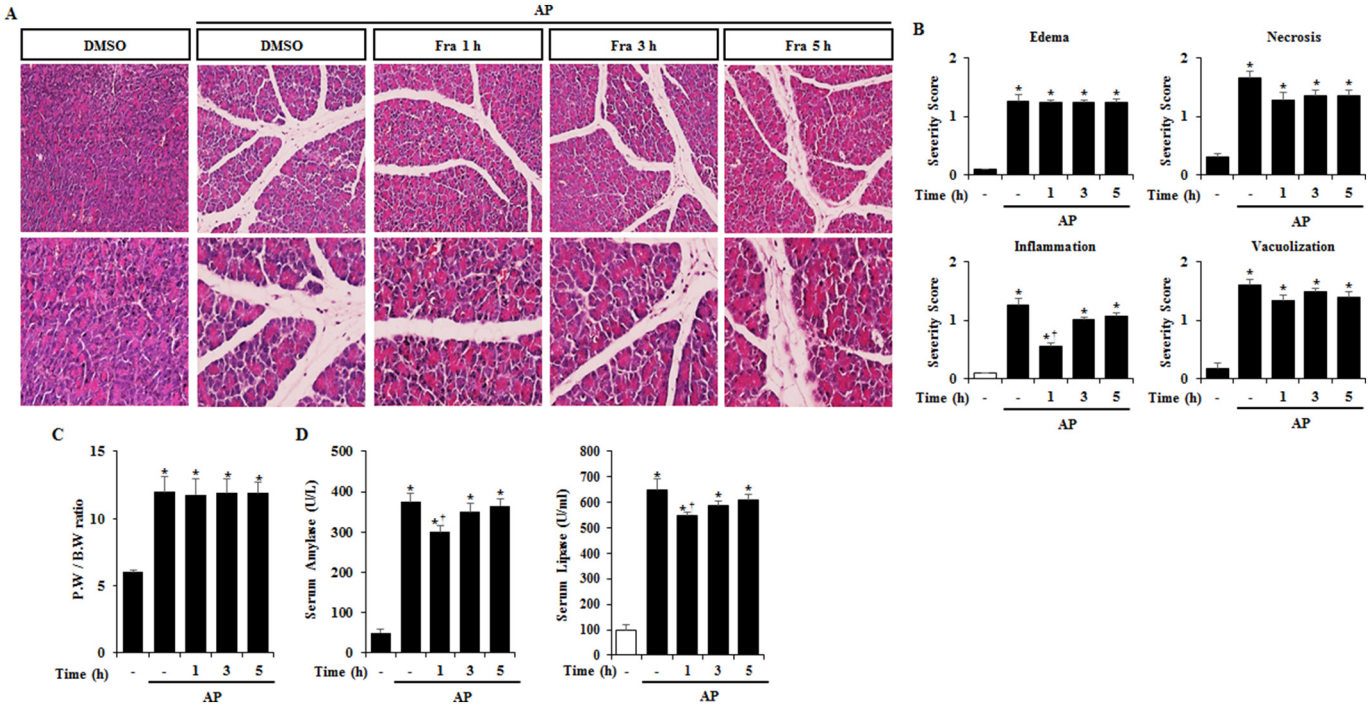

Fig. 6. Therapeutic effects of fraxinellone (Fra) on pancreatic injury during cerulein-induced AP. Mice were post-treated with fraxinellone (5 mg/kg) or DMSO 1, 3, or 5 h after the first cerulein (50 μg/kg) injection. Mice were sacrificed 6 h after the last cerulein injection. (A and B) Representative H&E-stained sections of the pancreas (upper panel, 100× and lower panel, 200× magnification) and histological score for edema, inflammation, necrosis and vacuolization. (C) P.W./B.W. ratio. (D) Serum amylase and lipase activities. Data are presented as means ± SEM, $n = 6$. Results are representative of three experiments. $^{*}P < 0.05$ versus DMSO treatment alone, $^{\dagger}P < 0.05$ versus cerulein treatment alone.

3.3. Prophylactic effect of fraxinellone on the severity of cerulein-induced AP

We hypothesized that the inhibition of inflammasome activation could attenuate the severity of AP. We evaluated the effects of fraxinellone on pancreatic injury during cerulein-induced AP. As shown in Fig. 2A, the pancreas from AP mice were characterized with interstitial edema, inflammatory cell infiltration, acinar cell necrosis and

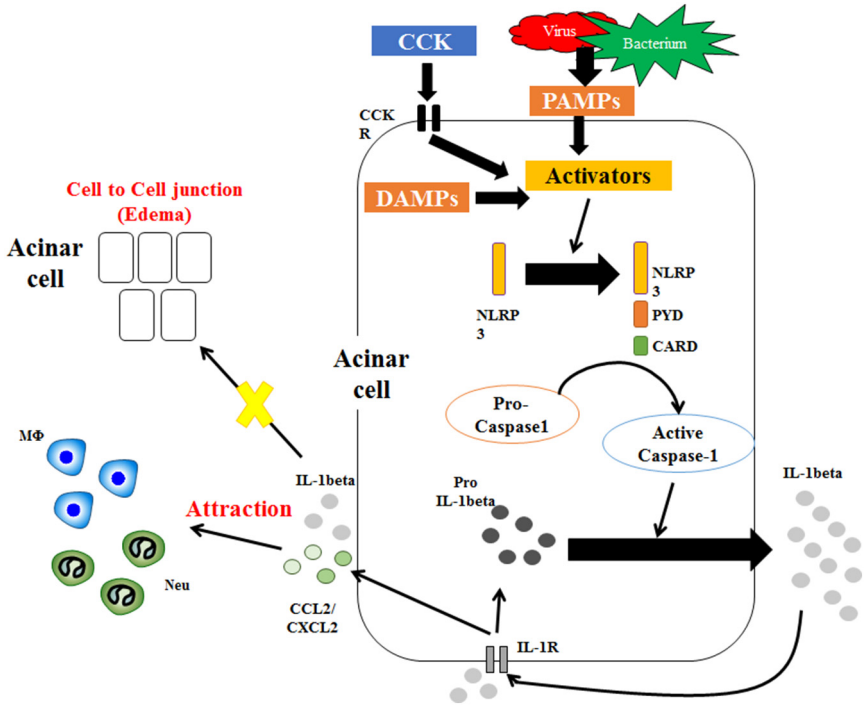

Fig. 7. Schematic representation about the inhibitory effects of fraxinellone against cerulein-induced acute pancreatitis.

vacuolization. However, inflammatory cell infiltration into pancreas, acinar cell necrosis and vacuolization, but not edema, was suppressed following treatment with fraxinellone (Fig. 2A and B). In accordance with histological data, fraxinellone failed to inhibit the P.W./B.W. ratio, the typical marker of pancreatic edema (Fig. 2B and C). In addition, fraxinellone could decrease the serum levels of amylase and lipase, which served as the markers of the severity of pancreatitis (Fig. 2D).

To prove the general inhibitory effects of fraxinellone on AP, we additionally examined the inhibitory effect of fraxinellone on both pancreatic duct ligation (PDL) and L-arginine-induced severe AP model. As shown in sFigs. 2 and 3, treatment of fraxinellone inhibited pancreatic injury, pancreatic edema, inflammatory cell infiltration, acinar cell necrosis and vacuolization against PDL and L-arginine-induced severe AP which could suggest fraxinellone has general inhibitory activities on AP.

3.4. Effect of fraxinellone on the infiltration of inflammatory cells during AP

To evaluate the detailed inhibitory effects of fraxinellone on inflammatory cell infiltration, we investigated the infiltration of representative inflammatory cells, including macrophages and neutrophils, into the pancreas. Macrophages and neutrophils are known to migrate into the inflamed pancreas during AP [17,19–21]. Therefore, we evaluated the levels of neutrophils and macrophages as infiltrated inflammatory cells during AP. We found that neutrophils (for anti-neutrophil and MPO activity) and macrophages (for $CD11b^+$ mac-1) infiltrated into the inflamed pancreas (Fig. 3A–C). However, treatment with fraxinellone inhibited the infiltration of neutrophils and macrophages into the pancreas in a dose-dependent manner (Fig. 3A–C).

To examine the chemokine involved in the regulation of neutrophils and macrophages, we analyzed the levels of several well-known chemokines such as CXCL1, 2, 3, and 5 and CCL2. Of these, CCL2 (monocyte chemoattractant protein-1) and CXCL2 (macrophage inflammatory protein-2) were inhibited by fraxinellone during AP (Fig. 3D).

3.5. Effect of inflammasomes during cerulein-induced AP

Based on the inhibitory effects of fraxinellone on inflammatory cell infiltration (Fig. 3), we thought that inflammasomes may be involved in the regulation of inflammatory cell infiltration, but not edema, during cerulein-induced AP. To examine the role of inflammasomes on infiltrated cells during AP, we used parthenolide (a well-known inflammasome inhibitor) and IL-1 receptor antagonist mice to inhibit IL-1 signaling. As shown in Fig. 4A–C, pharmacological (parthenolide) and genetic (IL-1 receptor antagonist) inhibition of inflammasome activation inhibited the infiltration of inflammatory cells into pancreas, acinar cell necrosis and vacuolization, but no effect was observed on edema. In addition, the serum levels of amylase and lipase were impaired following inhibition of inflammasome activation (Fig. 4D). The inhibition of inflammasome activation significantly reduced the infiltration of neutrophils (indicated by immunofluorescence staining and MPO activity) and macrophages as well as levels of CCL2 and CXCL2 (Fig. 5).

3.6. Therapeutic effect of fraxinellone on the severity of cerulein-induced AP

To examine the potential therapeutic value of fraxinellone against AP, we administered fraxinellone at 1, 3, and 5 h after the first cerulein injection. After treatment with fraxinellone for 1 h, but not 3 and 5 h, the infiltration of inflammatory cells, acinar cell necrosis and vacuolization, were significantly inhibited; no effect was observed on edema (Fig. 6A–C). In addition, the elevated levels of serum amylase and lipase were impaired after treatment with fraxinellone at 1 h but not after 3 h and 5 h of fraxinellone treatment (Fig. 6D).

4. Discussion

Acute pancreatitis is a rapid pancreatic inflammatory response, which may lead to a systemic inflammatory syndrome and multi-organ failure [22]. AP is triggered by initial acinar cell death with intrapancreatic trypsinogen activation, subsequently leading to an inflammatory response [23]. Hence, there is an unmet need for the

development of potent new agents. In this study, we demonstrate the excellent use of fraxinellone in pancreatic inflammation. Our data highlight the inhibitory effect of fraxinellone on the activation of inflammasome cascades such as NLRP3, PY-CARD, caspase-1 and IL-1β. Pre- and post-treatment of mice with fraxinellone significantly attenuated the severity of AP. In addition, fraxinellone treatment inhibited the infiltration of macrophages and neutrophils and reduced the elevated levels of CCL2 and CXCL2. These findings, for the first time, indicate the inhibitory activities of fraxinellone on immune cell infiltration through inflammasome deactivation during AP.

In general, the injury to pancreatic acinar cells by external stimuli results in the synthesis and release of several pro-inflammatory cytokines [24–26]. Therefore, evaluation of the cytokine levels is important to predict the outcome of severe AP. We examined whether fraxinellone could inhibit the production of well-known pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α during cerulein-induced AP. However, only cerulein-induced IL-1β, not IL-6, and TNF-α production was inhibited by fraxinellone (Fig. 1 and sFigs. 4 and 5). IL-1β is known to be a major mediator of inflammation and injury responses in AP. Deficiency of IL-1R or injection of IL-1R antagonist attenuates the severity of AP [10,11] Constitutive overexpression of IL-1β in pancreatic acinar cells leads to pancreatic atrophy and fibrosis, suggesting that the expression of pancreatic IL-1β is sufficient to induce pancreatic damage [27]. Furthermore, IL-1β is a well-known final product from inflammasome activation [28]. Thus, we first determined whether fraxinellone inhibits inflammasome activation during AP. As expected, fraxinellone treatment inhibited the activation of NLRP3 inflammasome and production of inflammasome effectors such as IL-1β and IL-18 (Fig. 1). Although the inhibitory activity of fraxinellone on inflammasome activation has been reported in a colitis model [15], this is the first study to report the inhibitory activity of fraxinellone on inflammasomes during AP.

The activation of NLRP3 inflammasome could contribute to the development of AP. It has been reported that NLRP3 and caspase-1 genes are required for the development of pancreatic inflammation in AP [9]. Moreover, the involvement of the NLRP3 inflammasome in obesity-associated severe AP is well-documented [29]. Thus, the inhibition of inflammasome activation may serve as a potential therapeutic strategy for AP. In accordance with previous reports, the pancreatic inflammasome inhibitor fraxinellone attenuated the severity of cerulein-induced mild AP, and PDL or L-arginine induced severe AP (Fig. 2, sFigs. 2 and 3). While cerulein-induced pancreatic edema was not improved by fraxinellone, PDL and L-arginine induced pancreatic edema was attenuated by fraxinellone treatment. Although it is difficult to determine the reason underlying the lack of any effect on edema on cerulein-induced AP model, we suggest that IL-6 and TNF-α signaling may be involved in the maintenance of the gap junction between pancreatic acinar cells against cerulein. Because while the inhibition of all three cytokines such as IL-1β, IL-6, and TNF-α resulted in decrease of pancreatic edema (sFigs. 2 and 3), the inhibition of IL-1β only did not make improvement of pancreatic edema during AP even though the stimulator of AP is different (Fig. 2, sFigs. 4 and 5). Further study is warranted to evaluate the regulatory mechanism underlying pancreatic edema.

The most unique aspect of the present report is the elucidation of the role of inflammasomes during AP. Although many reports have focused on studying inflammasomes during AP, their detailed role during AP is still unclear. We suppose that inflammasomes may trigger the inflammatory cell infiltration because fraxinellone, the inflammasome inhibitor, dramatically inhibited the infiltration of neutrophil/macrophages and suppressed the expression of chemokines (Fig. 3). We further inhibited the activation of inflammasomes using IL-1R transgenic mice and parthenolide, a well-known inflammasome inhibitor. As expected, the blockade of IL-1 signaling inhibited the activation of NLRP3 inflammasome (sFig. 1). As observed with fraxinellone treatment, pharmacologic and genetic inhibition of inflammasomes attenuated the severity of AP characterized by pancreatic damages as well as increased levels of serum amylase and lipase but had no effect on pancreatic edema (Fig. 4). In addition, the pharmacologic and genetic inhibition prevented the infiltration of neutrophils and macrophages into pancreas, as observed with fraxinellone treatment (Fig. 5). Thus, our findings suggest that inflammasomes inhibit inflammatory cell infiltration during AP.

In summary, we have shown that fraxinellone is capable of ameliorating pancreatic injury and inhibiting the infiltration of inflammatory cells such as neutrophils and macrophages into the pancreas through the suppression of inflammasome signaling during AP (Fig. 7). Therefore, the inhibition of inflammasome signaling may serve as a promising strategy to prevent/or cure AP.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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