USP25 Deficiency Exacerbates Acute Pancreatitis via Up-Regulating TBK1–NF-κB Signaling in Macrophages

Xin Liu,1 Wu Luo,2 Jiahao Chen,1 Chenghong Hu,1 Rumbidzai N. Mutsinze,1 Xu Wang,1 Yanmei Zhang,3 Lijiang Huang,4 Wei Zuo,4 Guang Liang,1,3 and Yi Wang1,4

1Chemical Biology Research Center, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China; 2Medical Research Center, First Affiliated Hospital, Wenzhou Medical University, Wenzhou, Zhejiang, China; 3School of Pharmaceutical Sciences, Hangzhou Medical College, Hangzhou, Zhejiang, China; and 4Department of Gastroenterology, Affiliated Xiangshan Hospital of Wenzhou Medical University, Xiangshan, Zhejiang, China

SUMMARY
Deficiency of macrophage ubiquitin-specific protease 25 enhances the activation of the TANK-binding kinase 1/nuclear factor-κB pathway, causing pancreatic and lung injury in different acute pancreatitis mouse models. Approaches to increase ubiquitin-specific protease 25 expression and function in macrophages may provide an anti-inflammatory therapy for acute pancreatitis.

BACKGROUND & AIMS: Severe acute pancreatitis can easily lead to systemic inflammatory response syndrome and death. Macrophages are known to be involved in the pathophysiology of acute pancreatitis (AP), and macrophage activation correlates with disease severity. In this study, we examined the role of ubiquitin-specific protease 25, a deubiquitinating enzyme and known regulator of macrophages, in the pathogenesis of AP.

METHODS: We used L-arginine, cerulein, and choline-deficient ethionine-supplemented diet–induced models of AP in Usp25−/− mice and wild-type mice. We also generated bone marrow Usp25−/− chimeric mice and initiated L-arginine–mediated AP. Primary acinar cells and bone marrow–derived macrophages were isolated from wild-type and Usp25−/− mice to dissect molecular mechanisms.

RESULTS: Our results show that Usp25 deficiency exacerbates pancreatic and lung injury, neutrophil and macrophage infiltration, and systemic inflammatory responses in L-arginine, cerulein, and choline-deficient ethionine-supplemented diet–induced models of AP. Bone marrow Usp25−/− chimeric mice challenged with L-arginine show that Usp25 deficiency in macrophages exaggerates AP by up-regulating the TANK-binding kinase 1 (TBK1)–nuclear factor-κB (NF-κB) signaling pathway. Similarly, in vitro data confirm that Usp25 deficiency enhances the TBK1–NF-κB pathway, leading to increased expression of inflammatory cytokines in bone marrow–derived macrophages.

CONCLUSIONS: Usp25 deficiency in macrophages enhances TBK1–NF-κB signaling, and the induction of inflammatory chemokines and type I interferon-related genes exacerbates pancreatic and lung injury in AP. (Cell Mol Gastroenterol Hepatol 2022;14:1103–1122; https://doi.org/10.1016/j.jcmgh.2022.07.013)

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Acute pancreatitis (AP) is a digestive system disease that comes on suddenly and requires hospitalization.1 Obstruction by gallstones is a common cause of AP.2 Alcohol consumption and smoking also can increase the
risk of AP. Clinically, most patients present with mild AP, which usually is self-limiting, and patients recover quickly. However, approximately 20% of the patients progress to severe AP, which carries a high mortality rate and requires intensive treatment. Severe AP easily can lead to systemic inflammatory response syndrome and multiple organ dysfunction syndrome. Among the complications associated with severe AP, acute lung injury is one of the most serious diseases. Unfortunately, the pathogenesis of severe AP-associated multiple organ dysfunction syndrome is not fully understood, but may involve pancreatic necrosis, bacteremia, intestinal barrier failure, and activation of inflammatory cascades and diffuse alveolar damage.

Clinical studies have shown that several inflammatory cytokines predict and mark AP disease severity. Corroborating experimental studies have highlighted the critical role of innate immune activation, neutrophils, and macrophages in AP. These studies suggest that mechanisms suppressing inflammatory responses should be explored for AP. In this context, the ubiquitin-specific protease 25 (USP25) warrants investigation. USP25 is a deubiquitinating enzyme that prevents proteasomal degradation of substrates by hydrolyzing ubiquitin moieties conjugated to substrates. USP25 has been associated with antiviral immunity, Alzheimer’s disease, cancers, diabetes, and other metabolic diseases. Specifically, expression of USP25 decreases lipopolysaccharide-induced inflammatory cytokine production in macrophages, and negatively regulates virus-induced type I interferon signaling in Human embryonic kidney (HEK)-293T cells. Based on this important role of USP25 in the suppression of inflammatory responses, we explored whether USP25 is involved in AP pathogenesis.

To investigate the role of USP25 in AP, AP was induced in Usp25 knockout (KO) mice through L-arginine, cerulein, or choline-deficient diet supplemented with dextrose and Levoisomer-ethionine (CDE). Compared with wild-type (WT) mice, Usp25 KO mice show aggravated AP, associated with increased inflammatory responses in the pancreas and lungs. Furthermore, USP25 expression in macrophages was shown to play a vital role in L-arginine–induced severe AP using a bone marrow transplantation chimeric mouse model. In vitro studies showed that Usp25−/− macrophages increase cytokine release after incubation with acinar cell supernatant (ACS) through activating the TANK-binding kinase 1 (TBK1) and nuclear factor-κB (NF-κB) signaling pathways. Our studies have shown a novel role of USP25 in AP pathogenesis.

### Results

**Usp25−/− Mice Show Aggravated L-Arginine–Induced Severe AP and Associated Lung Injury**

We first used the L-arginine model of severe AP. WT and Usp25−/− mice received 2 × 4 g/kg L-arginine and were killed 72 hours after the first injection, the point of peak pancreatic injury. Gross morphology of the harvested pancreas (Figure 1A) and pancreas-to-body weight ratios (Figure 1B) indicated edema after L-arginine injections, which was more severe in the Usp25−/− mice compared with WT mice. Pancreas injury was evaluated further by measuring the levels of serum amylase, lactate dehydrogenase (LDH), and lipase. These biochemical parameters were increased significantly after L-arginine administration (Figure 1B). Furthermore, the increase was greater in Usp25−/− mice compared with WT mice. Histopathologic assessment of pancreas showed increased pancreatic edema, inflammatory infiltration, and necrosis in Usp25−/− mice challenged with L-arginine compared with WT mice (Figure 1C and D).

We next examined lung tissues in mice challenged with L-arginine because acute lung injury is one of the common complications of severe AP. Compared with the WT mice, the Usp25−/− mice showed severe structural alterations as evident through histopathologic analysis (Figure 2A). Staining of lung tissues showed increased macrophage F4/80 antigen immunoreactivity in Usp25−/− mice compared with WT mice (Figure 2B and C). Furthermore, myeloperoxidase (MPO) activity was significantly higher in L-arginine–challenged mice, and in Usp25−/− compared with WT mice (Figure 2D). Increased F4/80 immunoreactivity and MPO activity followed the same pattern in pancreas (Figure 2B, D, F). In line with these results, pancreatitis-induced trypsinogen activation in the pancreas was significantly higher in Usp25−/− mice compared with WT mice (Figure 2G). These results indicated that Usp25 deficiency aggravates pancreatic injury and inflammatory cell infiltration in the L-arginine–induced model of severe AP.

**Usp25 Deletion in Bone Marrow–Derived Macrophages Aggravates L-Arginine–Induced Severe AP**

Based on our observation of increased macrophage F4/80 immunoreactivity in the pancreas and lung tissues of Usp25−/− mice challenged with L-arginine, as well as the known critical role of macrophages in AP, we explored the contribution of macrophage USP25 in AP. WT mice were irradiated and reconstituted with bone marrow cells derived from either WT donor mice or Usp25−/− mice. Similar to Usp25−/− mice challenged with L-arginine, WT mice, after receiving Usp25−/− bone marrow cells (KO→WT), showed severe pancreatic injury compared with WT mice that received WT bone marrow cells (WT→WT). This was
evident in gross tissue examination (Figure 3A), and pancreatic-to-body weight ratios, and levels of serum amylase, LDH, and lipase (Figure 3B). Histopathology confirmed that KO→WT mice had higher injury scores of edema, inflammatory infiltration, and necrosis (Figure 3C and D). Furthermore, F4/80 immunoreactivity in pancreas and lung (Figure 3E–G), MPO activity (Figure 3H and I), and pancreatic trypsin activity (Figure 3J) all were higher in KO→WT mice compared with WT→WT mice. These data indicate that USP25 expression in macrophages plays a critical role in L-arginine–induced severe AP.

**TBK1–NF-κB Activation in Macrophages by Acinar-Derived Factors Requires USP25 Expression**

To further explore the role of macrophage USP25 in AP, we exposed bone marrow–derived macrophages (BMDMs) from WT and Usp25−/− mice to supernatants prepared from primary acinar cells (Figure 4A). Trypan blue staining showed that primary acinar preparation is viable (data not shown). Based on this observation, we collected ACS from WT cells only at 0 hours (immediately after culture) and 24
hours. Supernatant then was applied to BMDMs to examine downstream activation.

A recent study showed that stimulator of interferon (IFN) genes (STING) and TBK1/NF-κB pathways promote inflammation in experimental AP. This prompted us to examine whether acinar-derived factors alter STING/TBK1/NF-κB in macrophages derived from WT and Usp25−/− mice. Phosphorylated forms of TBK1, phosphorylated interferon regulatory factor 3 (IRF3), and P65 protein (P65), as well as the total STING protein levels, were induced in WT BMDMs that were exposed to ACS for 24 hours (Figure 4B). Interestingly, we found that these changes were exaggerated significantly in BMDMs harvested from Usp25−/−, except for STING protein levels, which did not appear to increase (Figure 4B). Transcript levels of inflammatory factors downstream of TBK1/NF-κB including Ccl4, Ccl5, Cxcl10, and type 1 IFN-related genes Isg15 and Ifnb were increased significantly in WT BMDMs after ACS exposure (Figure 4C–G). As with the TBK1/NF-κB pathway itself, we noted that Usp25−/− BMDMs showed higher inductions of inflammatory factors when exposed to ACS compared with WT BMDMs. Moreover, IFNβ protein levels were found to be increased in Usp25−/− BMDMs compared with WT BMDMs (Figure 4H). These results may indicate that acinar cell death releases factors that activate TBK1/NF-κB in macrophages, and that USP25 may regulate TBK1/NF-κB through a STING-independent mechanism.

Because we used a Usp25−/− model with precise temporal regulation, there is a possibility that some of the effects noted may be owing to utilization of alternate signaling pathways. In an attempt to overcome this limitation, we restored Usp25 in BMDMs harvested from Usp25−/− mice, and measured the activation of the TBK1/NF-κB pathway after ACS exposure. Our results show that expression of Usp25 in Usp25−/− BMDMs largely reverses ACS-induced phosphorylation of TBK1, IRF3, and P65 (Figure 5A). Usp25 expression also prevented NF-kappa-B inhibitor alpha degradation, another measure of reduced NF-κB activity (Figure 5A). Furthermore, Usp25 expression reduced the level of Ccl4 and Ifnb induction in cells after ACS exposure (Figure 5C–G). However, no reductions were seen in Ccl5, Cxcl10, and Isg15 in cells transfected with Usp25 vector compared with control vector. These results suggest that exaggerated inflammatory responses seen in Usp25−/− BMDMs is owing to Usp25 deficiency.

Figure 2. Increased inflammatory cell infiltration in Usp25-deficient mice after L-arginine (L-Arg) administration. (A) Representative images of H&E-stained sections of lungs. Scale bar: 100 μm. (B) Representative immunohistochemical staining of lung and pancreas for macrophage F4/80 antigen. Red arrows indicate positive staining. Scale bar: 100 μm. Quantification of F4/80-immunoreactive (staining positive) area per high-power field (HPF) in (C) lung and (D) pancreatic tissues of mice. MPO activity in (E) lung and (F) pancreas of experimental mice. (G) Trypsin activity in the pancreas of mice. (E–G) Data shown are means ± SD; n = 5–6. *P < .05, and **P < .01. SAP, severe acute pancreatitis.
Figure 3. Deficiency in macrophage-expressed Usp25 worsens L-arginine–induced pancreatitis. WT mice were irradiated and reconstituted with bone marrow from either WT (WT → WT) or Usp25−/− (KO → WT) mice. Mice then were subjected to a L-arginine–induced model of pancreatitis. (A) Gross morphology of the pancreas 72 hours after L-arginine administration. (B) Pancreas-to-body-weight ratios, and serum levels of amylase, LDH, and lipase in mice. (C) Histopathologic scoring of pancreas tissues. (D) Representative H&E-stained images of pancreas and lung tissues. Scale bar: 100 μm. (E) Representative immunohistochemical staining images showing F4/80 in the pancreas and lung tissues. Red arrows indicate positive staining. Scale bar: 100 μm. (F and G) Quantification of F4/80 staining area in (F) pancreas and (G) lung. (H and I) MPO activity in the (H) pancreas and (I) lung. (J) Trypsin activity in the pancreas. Data are shown as means ± SD; n = 5–6. *P < .05 and **P < .01. HPF, high-power field.
Involvement of STING in AP has been reported previously and studies have shown that STING activation by Dimethylxanthine acetic acid (DMXAA) worsens AP through activation of downstream pathways. We exposed WT and Usp25−/− BMDMs to DMXAA and examined the downstream pathways. Interestingly, Usp25−/− BMDMs showed increased phosphorylated TBK1, IRF3, and P65 proteins compared with WT BMDMs when exposed to DMXAA (Figure 5B). Similarly, DMXAA increased messenger RNA levels of Ccl4, Ccl5, Cxcl10, Isg15, and Ifnb in WT BMDMs, which were increased further in DMXAA-treated Usp25−/− BMDMs (Figure 5H–L). These data indicate that Usp25 deficiency in macrophages exacerbates inflammatory responses, potentially through enhanced TBK1/NF-κB signaling.

Usp25−/− Mice Show Exaggerated TBK1/NF-κB Activation in the L-Arginine–Induced Severe AP Model

Based on our culture studies showing that Usp25−/− BMDMs activate TBK1/NF-κB in response to ACS, we probed for TBK1/NF-κB activation and downstream cytokine
expression in the L-arginine–induced mouse model of AP, including the bone marrow chimeric mice. Pancreatic tissues showed that USP25 protein levels are significantly lower in KO/WT mice upon L-arginine administration compared with WT/WT mice (Figure 6A). Levels of STING also were found to be lower in the KO→WT mice. However, downstream factors, including Phosphorylated-TBK1, Phosphorylated-IRF3, and Phosphorylated-P65 all were significantly higher in KO→WT mice compared with WT→WT mice (Figure 6A). As expected from these results, Ccl4, Ccl5, Cxcl10,
Isg15 (Figure 6C) and serum IFNβ (Figure 6D) were significantly higher in the pancreatic tissues of KO→WT mice compared with WT→WT mice. Analysis of tissues from WT and Usp25−/− mice (without bone marrow reconstitution) showed the expected readouts: decreased STING levels, increased TBK1/NF-κB activity, and significantly induced levels of inflammatory factors (Figure 6B, E, and F). Furthermore, we found that the well-established NF-κB inhibitor BAY11-7082 decreased pancreatic injury in Usp25−/− mice in response to L-arginine (Figure 7A and B). A protective effect of BAY11-7082 also was observed in histopathologic examination of pancreatic tissues from Usp25−/− mice (Figure 7C and D). These data, at a minimum, support the involvement of NF-κB in exaggerated AP phenotype observed in Usp25−/− mice challenged with L-arginine.

Usp25−/− Mice Show Exacerbated Cerulein-Induced Pancreatitis and Associated Lung Injury

No experimental model of AP is perfect, and different models offer different advantages and disadvantages. One of the most widely used model of AP is induced by repetitive injections of cerulein.25 This treatment reliably generates mild, edematous pancreatitis in C57BL/6J mice when administered in 6–10 intraperitoneal injections.25 To bolster our finding that Usp25 deficiency worsens AP, we challenged WT and Usp25−/− mice with 8 cerulein injections given at hourly intervals. First, to examine the effects of Usp25 deletion soon after developing cerulein-induced pancreatitis, lungs and pancreas were harvested from mice at different time points, including 9, 13, and 17 hours after the first cerulein injection (data not shown). Our data
show that cerulein challenge caused pancreatic injury that was evident at the 9-hour time point. Even though both lung and pancreas tissues recovered at 13 and 17 hours, it was not a full recovery when compared with saline-injected Usp25−/− mice (data not shown). At a later time point, gross morphologic examination showed white and enlarged pancreas in cerulein-challenged Usp25−/− mice, indicating edema (Figure 8A). Histologic analysis and subsequent injury scoring showed that Usp25 deficiency increases edema and inflammatory cell infiltration after cerulein administration compared with mice with intact Usp25 (Figure 8B and C). The level of necrosis, however, was not statistically different between WT and Usp25−/− mice. Biochemical assays showed higher serum amylase, lipase, and LDH levels in Usp25−/− mice in response to cerulein administration compared with WT mice (Figure 8D).
Lung tissues harvested from mice after cerulein challenge showed more tissue damage in Usp25−/− mice compared with WT mice (Figure 8F). We then evaluated macrophage infiltration in pancreas and lung sections using F4/80 immunohistochemistry staining, as performed in the L-arginine model. A greater F4/80-stained lung and pancreas area was observed in Usp25−/− mice (Figure 8E and G). Similarly, MPO activity in the lung and pancreas (Figure 8H and I) was higher in Usp25−/− mice compared with WT mice after cerulein injections. Furthermore, the trypsin activity in the pancreas was higher in the Usp25−/− mice (Figure 8J). Collectively, these data confirm that Usp25 deficiency also aggravates cerulein-induced pancreatitis, as observed in L-arginine–induced pancreatitis.

**Usp25−/− Mice Also Show Exaggerated Diet-Induced Pancreatitis**

We next used a diet-induced model of pancreatitis to confirm the role of Usp25. A CDE has been shown to produce necrotizing pancreatitis with hemorrhage in young female mice.12,26 For this model, 4-week-old female WT and Usp25−/− mice were given a CDE or standard rodent chow (chow diet [CD]; control) diet for 72 hours. We found obvious signs of edema in WT and Usp25−/− mice fed a CDE (Figure 9A). Quantitative assessment of pancreatic injury showed that Usp25−/− deficiency worsens CDE-induced pancreatitis in mice (Figure 9B and C). Lung histopathology and MPO activity in lung and pancreatic tissues of mice also showed exaggerated responses in Usp25−/− mice compared with WT mice (Figure 9D–F). In addition, pancreatic trypsin activity levels were significantly higher in Usp25−/− mice after CDE feeding (Figure 9G).

A slightly modified CDE model was used to assess survival in mice. Usp25−/− and WT mice were fasted for 12 hours and fed a CDE for 4 days, followed by normal diet for another 4 days. Usp25−/− mice fed a CDE appeared to have a poorer prognosis compared with WT mice (Figure 9H). However, the results did not reach statistical significance (P = .1215).

**The Role of Usp25 in AP Is Not Independent of the Associations With TLR4 and Tumor Necrosis Factor–Receptor–Associated Factor**

USP25 is a deubiquitinating enzyme. Activity of this protein has been implicated in human diseases, including cancers and various inflammatory conditions.27,28,29 The molecular mechanisms by which USP25 participates may vary depending on the disease. Some of the reported mechanisms include regulation of suppressor of cytokine signaling 3-Phosphorylated signal transducer and activator of transcription 3, Wnt pathway/β-catenin, and NF-κB and c-Jun-N-terminal kinase (JNK) signaling pathways.27,28,30,29 A recent report showed that USP25 inhibited Toll-like receptor 4 (TLR4)-triggered proinflammatory signaling and promoted type I interferon signaling through deubiquitination of tumor necrosis factor receptor–associated factor (TRAF)3.31 These researchers also found that USP25 associates with TRAF3 and TRAF6 after viral infection and protects against proteasome-dependent or -independent degradation of TRAF3 and TRAF6.32 To explore whether this mechanism is at play in AP, we probed for the levels of TLR4, TRAF3, and TRAF6 in BMDMs harvested from WT and Usp25−/− mice after ACS exposure. ACS exposure did not change the expression of TLR4 or TRAF3 in either WT or Usp25−/− BMDMs (Figure 10A). The levels of TRAF6, however, were suppressed in both WT and Usp25−/− BMDMs in response to ACS. Expression of Usp25 in Usp25−/− BMDMs failed to restore TRAF6 levels after ACS treatment (Figure 10B). Analysis of pancreatic tissues of WT and Usp25−/−, and chimeric KO → WT and WT → WT mice did not show any changes in TLR4, TRAF3, or TRAF6 expression after L-arginine challenge (Figure 10C and D). These results indicate that the involvement of USP25 in AP may be independent of the known associations with TLR4 and TRAF proteins.

**The Role of USP25 in AP Is Not Caused by the Modulation of Interleukin 17**

Zhong et al32 identified USP25 as a negative regulator of interleukin (IL)17-mediated signaling and showed that Usp25−/− mice show a greater sensitivity to IL17-dependent inflammation and autoimmunity. Because IL17 has been shown to participate in AP pathogenesis,29,33 we wondered if the anti-inflammatory effects of USP25 were mediated through the modulation of the IL17 pathway. Surprisingly, when compared with the WT BMDMs, transcript levels of Il17a and downstream Ccl7, Ccl20, Cxcl5, Tnf, and Cxcl1 were not increased significantly in ACS- or IL17A-exposed Usp25−/− BMDMs (Figure 11A and B). In addition, there was no significant change in mitogen activated kinase-like protein pathway activation, as assessed by phosphorylated protein levels, between WT and Usp25−/− BMDMs upon IL17A stimulation (Figure 11C). We also evaluated the levels of NF-κB signaling proteins in WT and Usp25−/− BMDMs after IL17A exposure. Interestingly, IL17A did not induce NF-κB activation in either WT or Usp25−/− BMDMs when tested up to 45 minutes (data not shown). These new data suggest that the effects of USP25 seen in our experimental platform are not caused by the modulation of IL17.

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**Figure 8.** (See previous page). Usp25−/− mice show aggravated cerulein (Cer)-induced AP. (A) Gross morphology of pancreas after cerulein-induced pancreatitis. (B) Representative H&E-stained images of pancreatic tissues. Scale bar: 100 μm. (C) Levels of pancreatic edema, inflammatory cell infiltration, necrosis, and total injury score in pancreas of cerulein-induced pancreatitis in mice. (D) Pancreas-to-body-weight ratios, and serum levels of amylase, LDH, and lipase in WT and Usp25−/− mice. (E) Pancreas and lung were stained for macrophage marker F4/80. Quantitative measurement of F4/80 immunoreactivity is shown. (F) Representative H&E-stained images of lung tissue. Scale bar: 100 μm. (G) F4/80 staining in the pancreas and lung tissues of WT and Usp25−/− mice after cerulein-induced AP. Arrows indicate positive staining. Scale bar: 100 μm. MPO activity in the (H) lung and (I) pancreas of mice. (J) Trypsin activity in pancreas of mice. (C–E and H–J) Data are shown as means ± SD; n = 5–6. *P < .05 and **P < .01.
Figure 9. Exaggerated responses to CDE-induced pancreatitis in Usp25<sup>−/−</sup> mice. (A) Gross morphology of pancreatic tissues in WT and Usp25<sup>−/−</sup> mice after 72-hour CDE feeding. Control diet included standard rodent chow diet (CD). (B) Serum amylase, LDH, lipase, and IFNγ levels in CD- or CDE-fed WT and Usp25<sup>−/−</sup> mice. (C) Representative H&E-stained images of pancreas in mice. Scale bar: 100 μm. (D) Representative H&E-stained images of lung tissues in mice. (E and F) MPO activity in the (E) pancreas and (F) lung of CD- or CDE-fed WT and Usp25<sup>−/−</sup> mice. (G) Trypsin activity in the pancreas from CD or CDE-fed WT and Usp25<sup>−/−</sup> mice. (H) Survival rate in WT and Usp25<sup>−/−</sup> mice fed a CDE or control CD diet. (B and E–G) Data are shown as means ± SD; n = 5–6. *P < .05 and **P < .01. (H) n = 8.
Discussion

Our study discovered a novel role of USP25 in AP. Comprehensive cell culture and mouse modeling studies showed that Usp25 deficiency worsens AP. Specifically, our studies show that Usp25-/- mice show exaggerated pancreatic injury induced by L-arginine, cerulein, or CDE feeding over WT mice. Without Usp25, increased inflammatory cell infiltration, inflammatory cytokine levels, and necrosis is evident. This worsening of AP presentation likely was mediated by the lack of Usp25 in macrophages. We tested this idea by reconstituting the bone marrow of WT mice with Usp25-/- derived marrow cells and show that these chimeric mice mimic global Usp25-deficient mice. We then used primary macrophages derived from mice and showed that cells lacking Usp25 have an enhanced TBK1/NF-κB response when challenged with acinar cell factors or L-arginine.

There are many causes of AP. However, all leads trigger the same pathologic pathways and cellular dysfunction that culminate in acinar cell damage and local and systemic inflammation. Damaged acinar cells recruit various immune cells such as neutrophils, monocytes, and macrophages by releasing cytokines, chemokines, and expressing adhesion molecules. When immune cells migrate to these sites, the interaction between necrotic pancreatic tissue and immune cells, such as neutrophils and macrophages, further promotes local and systemic inflammatory responses, ultimately leading to organ injury. Neutrophil infiltration occurs in the early stages of AP, and neutrophil extracellular traps can cause duct blockage, activate proinflammatory signaling, and activate trypsinogen prematurely. Macrophages also play key roles in local and systemic inflammation responses at the onset of AP. Classically polarized M1 macrophages dominate in the proinflammatory phase of AP, while M2-like macrophages dominate the repair/regenerative phase. Upon acinar cell necrosis, released proinflammatory mediators and chemokines, as well as damage-associated molecular patterns, activate TLRs and inflammasome complexes in macrophages that may exacerbate pancreatic injury. Recently, it was reported that inhibition of C-C motif chemokine ligand 2-induced macrophage migration and blockade of}

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**Figure 10.** The effect of Usp25 deletion in macrophages on the expression of TRAF and TLR4. BMDMs were prepared from WT and Usp25-/- mice. Acinar cells were isolated from WT mice and cultured for 0 or 24 hours. ACS collected from 0 or 24 hours was applied to BMDMs. (A) BMDMs were exposed to ACS for 60 minutes. Lysates were collected and immunoblotted for the expression of TRAF3, TRAF6 and TLR4. (B) BMDMs from Usp25-/- mice were transfected with control or Usp25 expressing plasmid. Cells then were exposed to ACS for 60 minutes. Levels of TRAF and TLR4 proteins were detected by immunoblotting. (C) WT mice were irradiated and reconstituted with bone marrow cells derived from either WT (WT→WT) or Usp25-/- mice (KO→WT). Mice then were challenged with L-arginine. Pancreas was harvested and levels of TLR4 and TRAF were detected in tissue lysates. (D) WT and Usp25-/- mice were challenged with L-arginine. Lysates from pancreas were used for immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
cytokine signaling 3–dependent activation of macrophages can prevent the progression of AP and distant organ failure.46 Furthermore, a single-cell mass cytometry analysis has shown that a dynamic shift in pancreatic CD206+ macrophage population is observable during AP and recovery.47 Our study adds to the importance of macrophages in AP progression and shows that Usp25 deficiency in macrophages exacerbates L-arginine–induced AP and promoted macrophage infiltration into pancreas and lung. These findings are in line with previous studies showing that overexpression of USP25 reduces LPS-induced macrophage activation and inflammatory cytokines production.48 Moreover, Usp25 knockdown has been shown to generate proinflammatory effects in Kupffer cells.48 Taken together, these studies indicate that increasing Usp25 activity may counter inflammatory responses in AP.

Our data suggest that the effects of USP25 seen in our experimental platform are not owing to the modulation of IL17. We also are intrigued by the underlying mechanisms and why Usp25−/− has no effect on IL17-induced inflammatory factors. First, it is possible that IL17 produced through NF-κB activation may show proinflammatory effects at a later time point. There are recent studies that have shown that IL17 modulates macrophage phenotype in a NF-κB–dependent manner after 48 hours of exposure.49 Other studies, using a similar condition media experimental design, have shown that inflammatory factors may be induced by IL17 in macrophages after 24 hours.50 Second, inflammatory factor induced by IL17, which may be independent of USP25, also may involve noncanonical factors such as glycogen synthase kinase 3 and CCAAT enhancer binding protein alpha. Therefore, a comprehensive study is needed to elucidate the possible mechanisms in the future.

One puzzling finding in our study is related to STING. STING is an important protein that regulates the transcription of host defense genes such as type I IFNs and proinflammatory cytokines. STING forms a complex with TBK1, and this complex phosphorylates IRF3 and NF-κB. Our studies showed that L-arginine–induced AP increases the
expression of STING, indicating that at least some NF-κB activation may be attributed to STING up-regulation. However, Usp25/−/− mice do not show this increase in STING but still up-regulate TBK1/NF-κB. This raises the question of how USP25 may induce TBK1 phosphorylation and activation. Previous studies have shown that TBK1 stability could be regulated by ubiquitinating modification.51 It has been reported further that USP19 promotes TBK1 degradation through chaperone-mediated autophagy.52 Severe acute respiratory syndrome coronavirus 2 M protein also interacts with TBK1 and induces TBK1 degradation by K48-linked ubiquitination.53 However, Usp25 deficiency in our study increased TBK1 phosphorylation but failed to change the TBK1 protein levels, indicating that USP25 does not affect TBK1 protein stability or deubiquitination. Empirically, we found no direct association between USP25 and TBK1 using lysates from macrophages or pancreatic tissue from WT mice (data not shown).

Conclusions
Our study shows a novel role of macrophage-expressed USP25 in AP. Deficiency in macrophage USP25 enhances the activation of the TBK1/NF-κB pathway, resulting in elaboration of cytokines and type I interferon-related genes. Usp25 deficiency exacerbated pancreatic and lung injury induced by L-arginine, cerulein, and CDE, and increased neutrophil and macrophage infiltration and systemic inflammatory responses. Thus, approaches to increase USP25 expression and function in macrophages may provide an anti-inflammatory therapy for AP.

Methods
Animal Experiments
All animal studies were approved by the Institutional Animal Policy and Welfare Committee of Wenzhou Medical University (approval: wydw2021-142). C57BL/6 mice were obtained from Gempharmatech, Co, Ltd (Nanjing, China). Usp25/−/− mice on a C57BL/6 background were provided by Professor Jian Yuan (Tongji University, Shanghai, China). All mice were housed under specific-pathogen free conditions with 50% ± 5% humidity at 22°C ± 2°C and under a 12/12-hour light/dark cycle. Mice were fed a standard chow diet (CD). We generated 3 models of AP in WT and Usp25/−/− mice: L-arginine, cerulein, and CDE. L-arginine–induced pancreatitis. Male mice at 8 weeks of age were fasted for 14 hours. Pancreatitis was induced by 2 intraperitoneal injections of L-arginine at a dose of 4 g/kg (A5131; Sigma) at 1-hour intervals.54 Control mice received the same volume of saline by intraperitoneal injections. Mice were killed 72 hours after the first injection. Serum, pancreas, and lung samples were collected.

CDE model of pancreatitis. Female mice at 4 weeks of age were fasted for 12 hours. Mice then were fed a choline-deficient diet (XTCD10; Xietong, Jiangsu, China) supplemented with 0.5% dextroisomer and Levoisomer-ethionine (E117217; Aladdin, Shanghai, China) for 3 days.12 Control mice were fed a standard rodent chow diet (CD). Tissues were harvested at the experimental end point. For some studies, we examined the survival of mice after CDE feeding. Mice were fasted for 12 hours and fed a CDE for 4 days, followed by a normal diet for another 4 days.

To examine the role of NF-κB in AP models, male Usp25/−/− mice at 8 weeks of age were fasted for 14 hours. Mice then were administered NF-κB inhibitor at 5 or 10 mg/kg BAY 11-7082 (HY-13453; Medchem Express, Monmouth Junction, NJ) by intragastric infusion. Six hours later, mice received 2 intraperitoneal injections of L-arginine at a dose of 4 g/kg at 1-hour intervals. Mice received 5 or 10 mg/kg BAY 11-7082 again 6 hours after the last L-arginine injection. Tissues were harvested at 72 hours from the first L-arginine injection.

Blood samples from mice were used to prepare serum. Samples then were subjected to amylase activity and lipase activity tests using commercially available assay kits (BioAssay Systems, Hayward, CA). A lactate dehydrogenase kit (BioAssay Systems) was used to measure the LDH activity. In addition, IFNβ levels in mouse serum were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).

Bone Marrow Transplantation
Bone marrow chimeric mice were generated as previously described.9 WT recipient mice were subjected to irradiation with a dose of 6 Gy. Bone marrow cells isolated from the femur and tibia of either WT or Usp25/−/− mice were administered in donors at 5.0 × 106 by tail vein injection. Eight weeks later, WT→WT and KO→WT mice were subjected to AP modeling using the L-arginine method described earlier.

Isolation of BMDMs
BMDMs were isolated from the femur and tibia of WT and Usp25/−/− mice. Briefly, bones were flushed with RPMI 1640 containing 100 U/mL penicillin and 100 μg/mL streptomycin. Then, samples were filtered using a 70-mm nylon mesh and collected in 50-mL tubes. Red blood cell lysis was performed (R1010; Solarbio, Beijing, China) and samples were spun at 1000 × g for 5 minutes. Cells were cultured in 20% L929 cell culture medium. On days 3 and 5, fresh Dulbecco’s modified Eagle medium (DMEM) containing 20% L929 cell culture medium and 10% fetal bovine serum was added. Cells were used for experiments on day 7. Studies included exposure of BMDMs to the following: acinar cell supernatant to assess activation, STING agonist DMXAA for signaling pathway determination, and IL17A to assess crosstalk between signaling axes. After exposure of
BMDMs to acinar cell supernatant, levels of IFNγ were detected by ELISA.

For some studies, BMDMs isolated from Usp25<sup>−/−</sup> mice were transfected with control (B21030500; Genechem, Shanghai, China) or plasmid expressing Flag-tagged Usp25 (NM_013918; Genechem).

**Isolation of Pancreatic Acinar Cells**

Pancreatic acinar cells were isolated from 8- to 10-week-old WT and Usp25<sup>−/−</sup> mice by a collagenase digestion method, essentially as previously described.<sup>56</sup> Briefly, pancreas tissue was cut into 1-mm<sup>3</sup> pieces and digested in DMEM supplemented with 1 mg/mL collagenase type 4 (LS004188; Worthington, Lakewood, NJ), 2.5 mg/mL bovine serum albumin (BSA) (A1933; Sigma), and 100 μg/mL soybean trypsin inhibitor (SBTI) (LS003571; Worthington) at 37°C for 20 minutes. The digestion was repeated once more with fresh media. Digested sample was passed through a 100-μm nylon mesh and rinsed with DMEM containing 10 mg/mL BSA and 100 μg/mL SBTI. Cells were resuspended in DMEM supplemented with 40 mg/mL BSA and 100 μg/mL SBTI. Cells were centrifuged at 50 × g and resuspended in the same formulation. After 2 more washes, cells were resuspended in DMEM supplemented with 1 mg/mL BSA and 100 μg/mL SBTI and cultured. After 30 minutes, supernatant was collected (time zero) and used to expose BMDMs. A 24-hour supernatant also was collected and used for MPO assay. MPO levels were measured by a colorimetry method using 3,3′,5,5′-tetramethylbenzidine with 0.03% H<sub>2</sub>O<sub>2</sub>. The reaction was stopped with 2 N H<sub>2</sub>SO<sub>4</sub>. Optical density was read at 450 nm. The protein concentration of the supernatant were measured using the Micro BCA Protein Assay Kit (23235; Thermo, Carlsbad, CA). MPO protein (M6908; Sigma) was used to establish the standard curve. MPO levels were expressed in mU/mg protein.

**MPO Assay**

MPO activity was measured in pancreatic and lung lysates using methods described previously.<sup>57,58</sup> Tissues were homogenized in 0.1 mol/L phosphate buffer (pH 7.4) containing protease inhibitors (P1051; Beyotime, Shanghai, China) with TissueLyser (Jingxin, Shanghai, China). Samples were centrifuged at 16,000 × g for 15 minutes at 4°C. The pellets were resuspended in 0.1 mol/L phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (H6269; Sigma), 10 mmol/L ethylene diamine tetra-acetic acid, and protease inhibitors. The pellets further were subjected to 3 cycles of sonification, freezing, and thawing. The extract then was centrifuged, and the supernatant was used for MPO assay. MPO levels were measured by a colorimetry method using 3,3′,5,5′-tetramethylbenzidine with 0.03% H<sub>2</sub>O<sub>2</sub>. The reaction was stopped with 2 N H<sub>2</sub>SO<sub>4</sub>. Optical density was read at 450 nm. The protein concentration of the supernatant were measured using the Micro BCA Protein Assay Kit (23235; Thermo, Carlsbad, CA). MPO protein (M6908; Sigma) was used to establish the standard curve. MPO levels were expressed in mU/mg protein.

**Histology and Immunohistochemistry**

Pancreas and lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Slices at 5-μm were prepared. Slides were dewaxed and rehydrated. For histopathologic assessment, slides were stained with H&E. Injury scores were graded from 0 to 3 as described.<sup>59,60</sup> Briefly, scores were generated for edema (0, absent; 1, diffuse expansion of interlobar; 2, same as 1 + diffuse expansion of interacinar; and 3, same as 2 + diffuse expansion of intercellular), inflammatory infiltration (0, absent; 1, around pancreatic duct; 2, intralobular or perivascular, <50% of the lobules; and 3, intralobular or perivascular, >50% of the lobules), and necrosis (0, absent; 1, periductal necrosis; 2, <10% of cells; 2, focal acinar cells necrosis, 30–50% of cells; and 3, diffuse acinar cells necrosis, >50% of cells).

For immunohistochemistry, dewaxed and rehydrated sections were subjected to antigen retrieval in 0.01 mol/L citrate buffer (pH 6.0) for 3 minutes at boiling temperatures. Slides then were blocked with 3% H<sub>2</sub>O<sub>2</sub> for 30 minutes at

| Gene  | Forward primer | Reverse primer |
|-------|----------------|----------------|
| Ccl4  | TTCCTGCTGTTCCTCTACACCT | CTGTCGTCCCTTGGTGCAG |
| Ccl5  | GCTGCTGTCGTTGTCCTTCTCC | TGGTGAGTCCACGAGCTCAG |
| Cxcl10 | AAGTGCCTGCGGCTCATTTTCTG | TCTCCTATGCGCCCTCATCCT |
| Lsg15 | GGTGTCTGCTGACTAACCTCC | TGGAGGAGTTAGAGCGTCCT |
| Ifn | CAGGCTCAGAAAGAGGAGCAGAC | GCGAGTCGTAACCTTCCTGT |
| Il17a | TTTTAACCTCCTGGGCAAAA | CTTTCCTCCCGGATCGAC |
| Tnf  | CTGAGGTCAATCTGCGCAATG | CTTCACAGAGCAATAGCTCAG |
| Cxcl1 | CACCCAAACGAGATCATAGC | TGGGGGACCACTTTGACATC |
| Cxcl5 | TTGGCCTGCTGCTGATACTG | CTGCCAGCTAGGCGACTG |
| Ccl20 | GCTCCTGCTACATACGAGC | CGACGTCGCTGCTGAGAG |
| Rna18s<sup>+</sup> | AGCTCCTGCTCCCCTGTACACA | CGATCCGAGGCGCCTCAG |

<sup>+</sup>Rna18s was used as the housekeeping gene. Data were normalized by ∆Δ cyle threshold method.
room temperature. Primary antibodies against macrophage antigen F4/80 (1:400) and MPO (1:25) were applied for 2 hours at room temperature. Horseradish peroxidase–linked secondary antibodies and diaminobenzidine (brown color) were used for detection. Images were taken using bright-field illumination on an epifluorescence microscope equipped with digital camera (Nikon, Tokyo, Japan).

**Pancreatic Trypsin Activity Assay**

Pancreatic tissue was homogenized in ice-cold buffer containing 5 mmol/L 4-morpholineethanesulfonic acid (pH 6.5), 1 mmol/L MgSO4, and 250 mmol/L sucrose. Samples were then mixed with assay buffer containing 50 mmol/L Tris-HCl (pH = 8.0), 150 mmol/L NaCl, 1 mmol/L CaCl2, and 0.1 mg/mL BSA. Trypsin activity was determined by adding Boc-Glu-Ala-Arg-MCA–HCl 1–3 (4017019; Bachem, Bubendorf, Switzerland). Excitation/emission at 380 nm/440 nm was measured.62,63 Puriﬁed trypsin (Warthington) was used to generate a standard curve.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated from mouse tissues and cultured BMDMs using TRizol (Thermo Fisher, Carlsbad, CA). A total of 1 µg RNA was used for reverse-transcription with PrimeScript reverse transcription reagent with genomic DNA Eraser (Takara, Beijing, China). Quantitative polymerase chain reaction was conducted using TB Green Premix Ex Taq II (Takara) and the CFX96 Real-Time System (Bio-Rad, Hercules, CA). Primer sequences are listed in Table 1.

**Immunoblotting Assay**

Lysates were prepared from cultured BMDMs and mouse tissues in RIPA buffer (P0013B; Beyotime) containing protease and phosphatase inhibitor cocktail (P1051; Beyotime). Protein concentrations were measured using a quick start Bradford kit (Bio-Rad). Approximately 40 µg total proteins were loaded and electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gels. Samples then were transferred to polyvinylidene diﬂuoride membranes (Bio-Rad). Membranes were blocked with 5% skim milk for 1 hour, and then incubated overnight with primary antibodies. Horseradish-conjugated secondary antibodies and enhanced chemiluminescence substrates were used for detection with the ChemiDoc XRS+ system (Bio-Rad). ImageJ analysis software version 1.38e (National Institutes of Health, Bethesda, MD) was used for densitometric quantiﬁcation of blots.

Antibodies against STING (13647), phosphorylated (p)-TBK1 (Ser172; 54833), TBK1 (3013), p-IRF-3 (Ser396; 4947), IRF-3 (4302), p-NF-κB p65 (Ser536; 3033), NF-κB p65 (8242), NF-kappa-B inhibitor alpha (4812), p-stress-activated protein kinase/JNK (Thr183/Tyr185; 4668), SAPK/JNK (9252), p-Erk1/2 (Thr202/Tyr204; 4370), Erk1/2 (4695), p-p38 (Thr180/Tyr182; 9211), p38 (8690), and glyceraldehyde-3-phosphate dehydrogenase (5174) were obtained from Cell Signaling Technology (Pudong, Shanghai, China). Antibodies against TRAF6 (66498-1-lg), TRAF3 (18099-1–AP), and FLAG Tag (20543-1–AP) were obtained from Peprotech (Cranbury, NJ). TLR4 (sc-293072) antibody was obtained Santa Cruz, CA, and USP25 (ab187156) was from Abcam (Cambridge, MA).

**Enzyme-Linked Immunosorbent Assay**

IFNβ levels in sera and acini cell culture supernatant were determined with an ELISA kit according the manufacturer’s protocol (R&D Systems).

**Statistical Analysis**

All data are expressed as means ± SD. Statistical analyses were performed using GraphPad Pro Prism 8.0 (GraphPad, San Diego, CA). The Student t test or 1-way analysis of variance followed by the multiple comparisons test with Bonferroni correction was used to analyze the differences between sets of data. A P value less than .05 was considered significant.

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CRediT Authorship Contributions
Xin Liu (Data curation: Equal; Formal analysis: Lead; Investigation: Lead; Writing – original draft: Equal; Writing – review & editing: Equal)
Wu Luo (Formal analysis: Equal; Funding acquisition: Supporting; Investigation: Equal; Methodology: Supporting)
Jiahao Chen (Investigation: Supporting)
Chenghong Hu (Investigation: Supporting)
Rumbidzai N. Mutsinze (Investigation: Supporting)
Xu Wang (Formal analysis: Supporting)
Yanmei Zhang (Investigation: Supporting)
Lijiang Huang (Conceptualization: Supporting; Writing – review & editing: Supporting)

Wei Zuo (Conceptualization: Supporting; Writing – review & editing: Supporting)
Guang Liang (Conceptualization: Equal; Formal analysis: Equal; Funding acquisition: Equal; Investigation: Equal; Writing – original draft: Equal)
Yi Wang (Conceptualization: Equal; Data curation: Equal; Formal analysis: Equal; Funding acquisition: Lead; Supervision: Lead; Writing – original draft: Equal; Writing – review & editing: Lead)

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