USP25 deficiency exacerbates acute pancreatitis via upregulating TBK1-NF-κB signaling in macrophages

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L-Arg Cerulein CDE...

Acini cells release damage DNA et

BMDM

Sting TBK1 NF-κB IRF3

USP25

Macrophage infiltration

Pancreas

Type I Interferons

Chemokines
USP25 deficiency exacerbates acute pancreatitis via upregulating TBK1-NF-κB signaling in macrophages

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Short title: USP25 deficiency exacerbates acute pancreatitis

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Conflicts of interest

The authors disclose no conflict.

Author Contributions

Yi Wang, Guang Liang, Lijiang Huang and Wei Zuo contributed to the literature search and study design. Yi Wang, Guang Liang and Xin Liu participated in the drafting of the article. Xin Liu, Wu Luo, Jiahao Chen, Chenghong Hu, Rumbidzai N. Mutsinze, and Yanmei Zhang carried out the experiments. Yi Wang, Guang Liang, Xin Liu, Wu Luo, Xu Wang and Yanmei Zhang participated in data collection and analysis. Yi Wang, Guang Liang, Lijiang Huang and Wei Zuo revised the manuscript.

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Deficiency of macrophage USP25 enhances the activation of TBK1/NF-κB pathway, causing pancreatic and lung injury in different acute pancreatitis mouse models. Approaches to increase USP25 expression and function in macrophages may provide an anti-inflammatory therapy for acute pancreatitis.

Abbreviations:
ACS: acinar cell supernatant; AP: acute pancreatitis; BMDMs: bone marrow-derived macrophages; BSA: bovine serum albumin; CCK: cholecystokinin; CD: chow diet; CDE: choline-deficient ethionine-supplemented diet; IHC: immunohistochemistry; IL: interleukin; KO: knockout; LDH: lactate dehydrogenase; MODS: multiple organ dysfunction syndrome; MPO: myeloperoxidase; NETs: neutrophil extracellular traps; NF-κB: nuclear factor-κB; OD: optical density; SBTI: soybean trypsin inhibitor; SIRS: systemic inflammatory response syndrome; STING: stimulator of interferon genes; TBK1: TANK-binding kinase 1; TLR: toll-like receptor; TRAF: tumor necrosis factor receptor–associated factor; USP25: ubiquitin-specific protease 25; WT: wild-type.
Abstract

**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 (USP25), a deubiquitinating enzyme and known regulator of macrophages, in the pathogenesis of AP.

**Methods:** We utilized L-arginine, cerulein, and choline-deficient ethionine-supplemented diet (CDE)-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 (BMDMs) 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 CDE-induced models of AP. Bone marrow Usp25−/− chimeric mice challenged with L-arginine shows that Usp25 deficiency in macrophages exaggerates AP by upregulating the TBK1-NF-κB signaling pathway. Similarly, *in vitro* data confirm that Usp25-deficiency enhances TBK1-NF-κB pathway, leading to increased expression of inflammatory cytokines in BMDMs.

**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.

**Keywords:** severe acute pancreatitis; deubiquitinating enzymes; systemic inflammatory response syndrome; bone marrow-derived macrophages.
Introduction

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 can also increase the risk of AP [3, 4]. Clinically, most patients present with mild AP, which is usually self-limiting, and patients recover quickly. However, about 20% of the patients progress to severe AP, which carries a high mortality rate and requires intensive treatment [5-7]. Severe AP can easily lead to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS). Among the complications associated with severe AP, acute lung injury is one of the most serious diseases [5, 8, 9]. Unfortunately, the pathogenesis of severe AP-associated MODS is not fully understood, but may involve pancreatic necrosis, bacteremia, intestinal barrier failure, and activation of inflammatory cascades and diffuse alveolar damage [10-13].

Clinical studies have revealed that several inflammatory cytokines predict and mark AP disease severity [14]. Corroborating experimental studies highlight the critical role of innate immune activation, neutrophils, and macrophages in AP [15-17]. These studies suggest that mechanisms suppressing inflammatory responses should be explored for AP. In this context, the ubiquitin-specific protease 25 (USP25) warrants investigation [18]. 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 [19]. Specifically, expression of USP25 decreases lipopolysaccharide-induced inflammatory cytokine production in macrophages, and negatively regulates virus-induced type I interferon signaling in HEK-293T cells [20-22]. 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 mice through L-arginine, cerulein, or choline-deficient diet supplemented with DL-ethionine (CDE). Compared to the wild-type mice, Usp25 knockout mice show aggravated AP, associated with increased inflammatory responses in the pancreas and lungs. Furthermore, USP25 expression in macrophages was demonstrated to play a vital role in L-arginine-induced severe AP using a bone marrow transplantation chimeric mouse model. In vitro studies show that Usp25-knockout macrophages increase cytokine release after incubation with acinar cell supernatant (ACS) through activating the TANK-binding kinase 1 (TBK1) and NF-κB signaling pathways. Our studies have revealed a novel role of USP25 in AP pathogenesis.
Results

Usp25<sup>-/-</sup> mice show aggravated L-arginine-induced severe acute pancreatitis and associated lung injury.

We first utilized the L-arginine model of severe AP [23]. Wild-type (WT) and Usp25<sup>-/-</sup> mice received 2 × 4 g/kg L-arginine and were sacrificed at 72 hr after the first injection, point of peak pancreatic injury. Gross morphology of the harvested pancreas (Fig. 1A) and pancreas-to-body weight ratios (Fig. 1B) indicated edema after L-arginine injections, which was more severe in the Usp25<sup>-/-</sup> mice compared to WT mice. Pancreas injury was further evaluated by measuring the levels of serum amylase, lactate dehydrogenase (LDH), and lipase. These biochemical parameters were significantly increased after L-arginine administration (Fig. 1B). Furthermore, the increase was greater in Usp25<sup>-/-</sup> mice compared to WT mice.

Histopathological assessment of pancreas showed increased pancreatic edema, inflammatory infiltration, and necrosis in Usp25<sup>-/-</sup> mice challenged with L-arginine compared to WT mice (Fig. 1C and 1D).

We next examined lung tissues in mice challenged with L-arginine, as acute lung injury is one of the common complications of severe AP. Compared to the WT mice, the Usp25<sup>-/-</sup> mice exhibited severe structural alterations as evident through histopathological analysis (Fig. 2A). Staining of lung tissues revealed increased macrophage F4/80 antigen immunoreactivity in Usp25<sup>-/-</sup> mice compared to WT mice (Fig. 2B and 2C). Furthermore, myeloperoxidase (MPO) activity was significantly higher in L-arginine-challenged mice, and in Usp25<sup>-/-</sup> compared to WT (Fig. 2E). Increased F4/80 immunoreactivity and MPO activity followed the same pattern in pancreas (Fig. 2B, 2D and 2F). In line with these results, pancreatitis-induced trypsinogen activation in the pancreas was significantly higher in Usp25<sup>-/-</sup> mice compared to WT mice (Fig. 2G). These results indicate that Usp25 deficiency aggravates pancreatic injury and inflammatory cell infiltration in 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<sup>-/-</sup> mice challenged with L-arginine, as well as the known critical role of macrophages in AP [24], 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<sup>-/-</sup> mice. Like Usp25<sup>-/-</sup> mice-challenged with L-arginine, WT mice, after receiving Usp25<sup>-/-</sup> bone marrow cells (KO→WT), showed severe pancreatic injury as compared to WT mice that received WT bone marrow cells (WT→WT). This was evident in gross tissue examination (Fig. 3A), and pancreatic-to-body weight ratios, and levels of serum amylase, LDH, and lipase (Fig. 3B). Histopathology confirmed that KO→WT mice had higher injury scores of edema, inflammatory infiltration, and necrosis (Fig. 3C and 3D).

Furthermore, F4/80 immunoreactivity in pancreas and lung (Fig. 3E-3G), MPO activity (Fig. 3H and 3I), and pancreatic trypsin activity (Fig. 3J) were all higher in KO→WT mice compared to 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<sup>−/−</sup>* mice to supernatants prepared from primary acinar cells (Fig. 4A). Trypan blue staining showed that primary acinar preparation is viable (data not shown). Based on this observation, we collected acinar cell supernatant (ACS) from WT cells only at 0 hrs (immediately after culture) and 24 hrs, respectively. Supernatant was then applied to BMDMs to examine downstream activation.

A recent study showed that stimulator of interferon genes (STING) and TBK1/NF-κB pathways promote inflammation in experimental AP [12]. This prompted us to examine whether acinar-derived factors alter STING/TBK1/NF-κB in macrophages derived from WT and *Usp25<sup>−/−</sup>* mice. Phosphorylated forms of TBK1, IRF3, and P65, as well as the total STING protein levels were induced in WT BMDMs that were exposed to ACS for 24 hr (Fig. 4B). Interestingly, we found that these changes were significantly exaggerated in BMDMs harvested from *Usp25<sup>−/−</sup>* mice, except for STING protein levels which did not appear to increase (Fig. 4B). Transcript levels of inflammatory factors downstream of TBK1/NF-κB including *Ccl4*, *Ccl5*, *Cxcl10*, and type I interferon-related genes *Isg15* and *Ifnb* were significantly increased in WT BMDMs following ACS exposure (Fig. 4C-4G). As with the TBK1/NF-κB pathway itself, we noted that *Usp25<sup>−/−</sup>* BMDMs exhibit higher inductions of inflammatory factors when exposed to ACS compared to WT BMDMs. Moreover, IFNβ protein levels were found to be increased in *Usp25<sup>−/−</sup>* BMDMs compared to WT BMDMs (Fig. 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.

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

Involvement of STING in AP has been reported previously and studies have shown that STING activation by DMXAA worsens AP through activation of downstream pathways [12]. We exposed WT and *Usp25<sup>−/−</sup>* BMDMs to DMXAA and examined the downstream pathways.
Interestingly, Usp25−/− BMDMs showed increased phosphorylated TBK1, IRF3 and P65 proteins compared to the WT BMDMs when exposed to DMXAA (Fig. 5B). Similarly, DMXAA increased mRNA levels of Ccl4, Ccl5, Cxcl10, Isg15 and Ifnb in WT BMDMs, which were further elevated in DMXAA-treated Usp25−/− BMDMs (Fig. 5H-5L). 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 to WT→WT mice (Fig. 6A). Levels of STING were also found to be lower in the KO→WT mouse. However, downstream factors, including p-TBK1, p-IRF3 and p-P65 were all significantly higher in KO→WT mice compared to WT→WT mice (Fig. 6A). As expected from these results, Ccl4, Ccl5, Cxcl10, and Isg15 (Fig. 6C) and serum IFN-β (Fig. 6D) were significantly higher in the pancreatic tissues of KO→WT mice compared to 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 (Fig. 6B, 6E-6F). Furthermore, we found that a well-established NF-κB inhibitor BAY11-7082 decreased pancreatic injury in Usp25−/− mice in response to L-arginine (Fig. 7A-7B). Protective effect of BAY11-7082 was also observed in histopathological examination of pancreatic tissues from Usp25−/− mice (Fig. 7C-7D). 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 six to ten intraperitoneal injections [25]. To bolster our finding that Usp25 deficiency worsens AP, we challenged WT and Usp25−/− mice with eight cerulein injections given at hourly intervals. First, in order to examine the effects of USP25 deletion soon after developing cerulein-induced pancreatitis, lungs and pancreas were harvested from mice at different timepoints, including 9-hr, 13-hr, and 17-hr after the first cerulein injection (data not shown). Our data show that cerulein challenge caused pancreatic injury that was evident at the 9 h timepoint. Even though both lung and pancreas tissues recovered at 13 and 17 hrs, it is not a full recovery when compared to saline injected Usp25−/− mice (data not shown). In a later timepoint, gross morphological examination showed white and enlarged pancreas in cerulein-challenged Usp25−/− mice, indicating edema (Fig. 8A). Histological
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255 analysis and subsequent injury scoring revealed that Usp25 deficiency increases edema and
256 inflammatory cell infiltration following cerulein administration compared to mice with intact
257 Usp25 (Fig. 8B and 8C). Level of necrosis, however, was not statistically different between
258 WT and Usp25\(^{-/-}\) mice. Biochemical assays revealed higher serum amylase, lipase, and LDH
259 in Usp25\(^{-/-}\) mice in response to cerulein administration compared to WT mice (Fig. 8D).
260
261 Lung tissues harvested from mice after cerulein challenge showed more tissue damage in
262 Usp25\(^{-/-}\) mice as compared to WT mice (Fig. 8F). We then evaluated macrophage infiltration
263 in pancreas and lung sections using F4/80 immunohistochemistry staining, as performed in
264 the L-arginine model. Greater F4/80-stained lung and pancreas area was observed in Usp25\(^{-/-}\)
265 mice (Fig. 8E and 8G). Similarly, MPO activity in the lung and pancreas (Fig. 8H and 8I)
266 was higher in Usp25\(^{-/-}\) mice compared to WT mice following cerulein injections.
267 Furthermore, the trypsin activity in the pancreas was higher in the Usp25\(^{-/-}\) mice (Fig. 8J).
268 Collectively, these data confirm that Usp25 deficiency also aggravates cerulein-induced
269 pancreatitis, as observed in L-arginine-induced pancreatitis.
270
271 \textit{Usp25\(^{-/-}\) mice also show exaggerated diet-induced pancreatitis.}
272 We next utilized a diet-induced model of pancreatitis to confirm the role of USP25. Choline-
273 deficient diet supplemented with 0.5% DL-ethionine (CDE) has been shown to produce
274 necrotizing pancreatitis with hemorrhage in young female mice [12, 26]. For this model,
275 four-week-old female WT and Usp25\(^{-/-}\) mice were given CDE diet or standard rodent chow
276 (CD; control) diet for 72 hours. We found obvious signs of edema in WT and Usp25\(^{-/-}\) mice
277 fed a CDE (Fig. 9A). Quantitative assessment of pancreatic injury showed that Usp25\(^{-/-}\)
278 deficiency worsens CDE-induced pancreatitis in mice (Fig. 9B and 9C). Lung
279 histopathology and MPO activity in lung and pancreatic tissues of mice also showed
280 exaggerated responses in Usp25\(^{-/-}\) mice compared to WT mice (Fig. 9D-9F). In addition,
281 pancreatic trypsin activity levels were significantly higher in Usp25\(^{-/-}\) mice following CDE
282 feeding (Fig. 9G).
283
284 A slightly modified CDE-model was used to assess survival in mice. Usp25\(^{-/-}\) and WT mice
285 were fasted for 12 hrs and fed a CDE diet for 4 days, followed by normal diet for another 4
286 days. Usp25\(^{-/-}\) mice fed a CDE appeared to have poorer prognosis compared to WT mice
287 (Fig. 9H). However, the results did not reach statistical significance (p=0.1215).
288
289 \textbf{The role of USP25 in AP is independent of the associations with TLR4 and TRAF.}
290 USP25 is a deubiquitinating enzyme. Activity of this protein has been implicated in human
291 diseases, including cancers and various inflammatory conditions [27-29]. The molecular
292 mechanisms by which USP25 participates may vary depending on the disease. Some of the
293 reported mechanisms include regulation of SOCS3-pSTAT3, Wnt/β-catenin, and NF-κB and
294 JNK signaling pathways [27-30]. A recent report showed that USP25 inhibited toll-like
295 receptor 4 (TLR4)-triggered proinflammatory signaling and promoted type I interferon
296 signaling through deubiquitination of TRAF3 [31]. These researchers also found that USP25
297 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 following ACS exposure. ACS exposure did not change the expression of TLR4 or TRAF3 in either WT or Usp25−/− BMDMs (Fig. 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 following ACS treatment (Fig. 10B). Analysis of pancreatic tissues of WT and Usp25−/−, and chimeric KO→WT and WT→WT mice did not reveal any changes in TLR4, TRAF3, or TRAF6 expression following L-arginine challenge (Fig. 10C-10D). 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 due to the modulation of IL17.

Zhong and colleagues identified USP25 as a negative regulator of IL-17-mediated signaling and showed that Usp25−/− mice exhibit a greater sensitivity to IL17-dependent inflammation and autoimmunity [33]. Since IL-17 has been demonstrated to participate in AP pathogenesis [34, 35], we wondered if the anti-inflammatory effects of USP25 were mediated through the modulation of the IL-17 pathway. Surprisingly, when compared to the WT BMDMs, transcript levels of Il17a and downstream Ccl7, Ccl20, Cxcl5, Tnf, and Cxcl11 were not significantly increased in ACS- or IL17A-exposed Usp25−/− BMDMs (Fig. 11A and 11B). In addition, there was no significant change in MAPK pathway activation, as assessed by phosphorylated protein levels, between WT and Usp25−/− BMDMs upon IL-17A stimulation (Fig. 11C). We also evaluated the levels of NF-κB signaling proteins in WT and Usp25−/− BMDMs following IL-17A exposure. Interestingly, IL-17A did not induce NF-κB activation in either WT or Usp25−/− BMDMs, when tested up to 45 min (data not shown). These new data suggest that the effects of USP25 seen in our experimental platform are not due to the modulation of IL-17.
Discussion

Our study discovered a novel role of USP25 in AP. Comprehensive cell culture and mouse modeling studies demonstrated that Usp25 deficiency worsens AP. Specifically, our studies show that Usp25−/− mice exhibit exaggerated pancreatic injury induced by L-arginine, cerulein, or CDE diet feeding over wildtype mice. Without Usp25, increased inflammatory cell infiltration, inflammatory cytokine levels, and necrosis is evident. This worsening of AP presentation was likely mediated by the lack of Usp25 in macrophages. We tested this idea by reconstituting the bone marrow of wildtype 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 demonstrated that cells lacking Usp25 have 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 pathological 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 [7, 11, 36]. 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 [37, 38]. Neutrophil infiltration occurs in the early stages of AP, and neutrophil extracellular traps can cause duct blockage, activate pro-inflammatory signaling and active trypsinogen prematurely [39-41]. Macrophages also play key roles in local and systemic inflammation responses at the onset of AP [42-44]. Classically polarized M1 macrophages dominate in the pro-inflammatory phase of AP, while M2-like macrophages dominate the repair/regenerative phase [43]. Upon acinar cell necrosis, released pro-inflammatory mediators and chemokines, as well as damage-associated molecular patterns activate TLRs and inflammasome complexes in macrophages that may exacerbate pancreatic injury [45-47]. Recently, it has been reported that inhibition of CCL2-induced macrophage migration and blockade of cytokine signaling 3-dependent activation of macrophages can prevent the progression of AP and distant organ failure [48]. Furthermore, a single-cell mass cytometry analysis has revealed that a dynamic shift in pancreatic CD206+ macrophage population is observable during AP and recovery [49]. 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 [20]. Moreover, Usp25 knockdown has been shown to generate pro-inflammatory effects in Kupffer cells [50]. 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 due to the modulation of IL-17. We are also intrigued by the underlying mechanisms and why Usp25−/− has no effect on IL17-induced inflammatory factors. First, it is possible that IL-17
produced through NF-κB activation may exhibit pro-inflammatory effects at a later time point. There are recent studies that show that IL17 modulates macrophage phenotype in an NF-κB-dependent manner following 48 hours of exposure [51]. Other studies, utilizing a similar condition media experimental design, have shown that inflammatory factors may be induced by IL17 in macrophages after 24 hours [52]. Secondly, inflammatory factor induced by IL17, which may be independent of USP25, may also involve non-canonical factors such as GSK3 and CEBP. 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 pro-inflammatory cytokines. STING forms a complex with TBK1, and this complex phosphorylates IRF3 and NF-κB. Our studies revealed that L-arginine-induced AP increases the expression of STING, indicating that at least some of NF-κB activation may be attributed to STING upregulation. However, Usp25−/− mice do not show this increase in STING but still upregulate 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 [53]. It has further been reported that USP19 promotes TBK1 degradation through chaperone-mediated autophagy [54]. SARS-CoV-2 M protein also interacts with TBK1 and induces TBK1 degradation by K48-linked ubiquitination [55]. 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 reveals a novel role of macrophage-expressed USP25 in AP. Deficiency in macrophage USP25 enhances the activation of 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 diet, 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 number: wydw2021-142). C57BL/6 mice were obtained from Gempharmatech Co. LTD (Nanjing, China). USP25−/− mice on 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±2.0℃ and under a 12/12 h light/dark cycle. Mice were fed standard chow diet (CD). We generated three models of AP in wildtype and Usp25−/− mice: L-arginine, cerulein, and choline-deficient diet supplemented with DL-ethionine (CDE).

L-arginine-induced pancreatitis: Male mice at 8-weeks of age were fasted for 14 hours. Pancreatitis was induced by two intraperitoneal injections of L-arginine at a dose of 4 g/kg (Sigma, A5131) at 1 hour interval [56]. Control mice received the same volume of saline by i.p. injections. Mice were euthanized 72 h after the first injection. Serum, pancreas, and lung samples were collected for analyses. Pancreas weight to body weight ratios were recorded to assess edema.

Cerulein-induced pancreatitis: Male mice at 8-weeks of age were fasted for 14 hours, and then pancreatitis was induced by eight intraperitoneal injections of cerulein administered at a dose of 50 µg/kg at a 1 h interval [57]. Control mice received saline at the same time. Mice were euthanized at 21 h 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 were then fed a choline-deficient diet (XTCD10, Xietong, Jiangsu, China) supplemented with 0.5% DL-ethionine (Aladdin, E117217) for 3 days [12]. Control mice were fed a standard rodent chow diet (CD). Tissues were harvested at the experimental endpoint. For some studies, we examined the survival of mice following CDE diet feeding. Mice were fasted for 12 hrs and fed a CDE diet for 4 days, followed by 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 were then administered NF-κB inhibitor at 5 or 10 mg/kg BAY 11-7082 (Medchem Express, Monmouth Junction, NJ, HY-13453) by intragastric infusion. Six hours later, mice received two intraperitoneal injections of L-arginine at a dose of 4 g/kg at 1 hour interval. Mice received 5 or 10 mg/kg BAY 11-7082 again six 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 were then subjected to amylase activity and lipase activity tests using commercially available assay kits (BioAssay Systems, Hayward, CA). Lactate dehydrogenase kit (BioAssay Systems, Hayward, CA) was used to measure the LDH activity. In addition, interferon-beta levels in mouse serum were determined using ELISA kits (R&D Systems, Minneapolis, MN).
Bone marrow transplantation
Bone marrow chimeric mice were generated as previously described [58]. Wildtype recipient mice were subjected to irradiation with a dose of 6 Gy. Bone marrow cells isolated from femur and tibia of either wildtype 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 L-arginine method described above.

Isolation of bone marrow-derived macrophages
Bone marrow-derived macrophages (BMDMs) were isolated from femur and tibia of wildtype and Usp25−/− mice. Briefly, bones were flushed with RPMI 1640 containing 100 units/mL penicillin and 100 µg/mL streptomycin. Then, samples were filtered using a 70 mm nylon mesh and collected in the 50 mL tube s. Red blood cell lysis was performed (Solarbio, R1010) and samples were spun at 1000 × g for 5 minutes. Cells were cultured in 20% L929 cell-culture medium. On day 3 and day 5, fresh DMEM medium containing 20% L929 cell-culture medium and 10% fetal bovine serum were added. Cells were used for experiments on day 7. Studies included exposure of BMDMs to: acinar cell supernatant to assess activation, STING agonist DMXAA for signaling pathway determination, and interleukin-17A to assess crosstalk between signaling axes. Following exposure of BMDMs to acinar cell supernatant, levels of interferon-beta were detected by ELISA.

For some studies, BMDMs isolated from Usp25−/− mice were transfected with control (GENECHEM, Shanghai, China, Cat# B21030500) or plasmid expressing Flag-tagged Usp25 (GENECHEM, Shanghai, China, Cat# NM_013918).

Isolation of pancreatic acinar cells
Pancreatic acinar cells were isolated from 8-10-week-old wildtype and Usp25−/− mice by a collagenase digestion method, essentially as previously described [59]. Briefly, pancreas tissue was cut into 1 mm³ 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, Sigma, A1933) and 100 µg/mL soybean trypsin inhibitor (SBTI, LS003571, Worthington, Lakewood NJ) at 37 °C for 20 min. 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 gently layered onto culture plates using a transfer pipette. Once cells had settled, the supernatant was replaced with fresh 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 two 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 bone marrow-derived macrophages. A 24-hour supernatant was also collected and tested on bone marrow-derived macrophages. A lactate dehydrogenase assay was performed on 0 hr and 24 hr supernatant samples. Cells were also examined for cell death by trypan blue exclusion test.
Myeloperoxidase assay

Myeloperoxidase (MPO) activity was measured in pancreatic and lung lysates using methods described previously [60, 61]. Tissues were homogenized in 0.1 M phosphate buffer (pH 7.4) containing protease inhibitors (Beyotime, P1051) with Tissuelyser (Jingxin, China). Samples were centrifuged at 16,000 × g for 15 min at 4 °C. The pellets were resuspended in 0.1 M phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, H6269), 10 mM ethylene diamine tetra-acetic acid, and protease inhibitors. The pellets were further subjected to three cycles of sonication, freezing and thawing. The extract was then centrifuged, and the supernatant was used for MPO assay. MPO levels were measured by a colorimetry method using the 3,3′,5,5′-tetramethylbenzidine with 0.03% H₂O₂. The reaction was stopped with 2N H₂SO₄. Optical density (OD) was read at 450 nm. Protein concentration of the supernatant were measured using the Micro BCA Protein Assay Kit (Thermo, 23235). MPO protein (Sigma, M6908) was used to establish the standard curve. MPO levels were expressed in mUnits/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 histopathological assessment, slides were stained with Hematoxylin and eosin (H&E). Injury scores were graded from 0 to 3 as described [62, 63]. Briefly, scores were generated for edema (0 = absent; 1 = diffuse expansion of interlobar; 2 = same as 1 + diffuse expansion of inter-acinar; 3 = same as 2 + diffuse expansion of intercellular), inflammatory infiltration (0 = absent; 1 = around pancreatic duct; 2 = intralobular or perivascular, <50% of the lobules; 3 = intralobular or perivascular, >50% of the lobules), and necrosis (0 = absent; 1 = periductal necrosis, <10% of cells; 2 = focal acinar cells necrosis, 30 to 50% of cells; 3 = diffuse acinar cells necrosis, >50% of cells).

For immunohistochemistry, dewaxed and rehydrated sections were subjected to antigen retrieval in 0.01M citrate buffer (pH 6.0) for 3 minutes at boiling temperature. Slides were then blocked with 3% H₂O₂ for 30 minutes at room temperature. Primary antibodies against macrophage antigen F4/80 (1:400) and myeloperoxidase (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, Japan).

Pancreatic trypsin activity assay

Pancreatic tissue was homogenized in ice cold buffer containing 5 mM 4-morpholineethanesulfonic acid (pH 6.5), 1 mM MgSO₄, and 250 mM sucrose. Samples were then mixed with assay buffer containing 50 mM Tris-HCl (pH=8.0), 150 mM NaCl, 1 mM CaCl₂ and 0.1 mg/mL BSA. Trypsin activity was determined by adding Boc-Gln-Ala-Arg-MCA-HCl [64] (Bachem, 4017019). Excitation/emission at 380 nm/440 nm was measured...
[65, 66]. Purified trypsin (Worthington, Freehold, NJ) was used to generate standard curve.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from mouse tissues and cultured BMDMs using Trizol (Thermo Fisher). A total of 1 μg RNA was used for reverse transcription with PrimeScript RT reagent with gDNA Eraser (Takara). Quantitative polymerase chain reaction was conducted using TB Green Premix Ex Taq II (Takara) and 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 (Beyotime, P0013B) containing protease and phosphatase inhibitor cocktail (Beyotime, P1051). Protein concentrations were measured using a quick start Bradford kit (Bio-Rad). Approximately 40 μg total proteins were loaded and electrophoresed in 10% SDS–polyacrylamide gels. Samples were then transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 5% skim milk for 1 hour, and then incubated overnight with primary antibodies. Horseradish conjugated secondary antibodies and ECL substrates were used for detection with ChemiDoc XRS+ system (Bio-Rad). Image J analysis software version 1.38e (NIH, Bethesda, MD) was used for densitometric quantification of blots.

Antibodies against STING (13647), phosphorylated (p−)-TBK1 (Ser172; 5483), TBK1 (3013), p-IRF-3 (Ser396; 4947), IRF-3 (4302), p-NF-κB p65 (Ser536; 3033), NF-κB p65 (8242), IκBα (4812), p-SAPK/JNK (Thr183/Tyr185; 4668), SAPK/JNK (9252), p-Erk1/2 (Thr202/Tyr204; 4370), Erk1/2 (4695), p-p38 (Thr180/Tyr182; 9211), p38 (8690), and GAPDH (5174) were obtained from Cell Signaling Technology (Pudong, Shanghai, China). Antibodies against TRAF6 (66498-1-Ig), TRAF3 (18099-1-AP), and FLAG Tag (20543-1-AP) were obtained from Peprotech (Cranbury, NJ, USA). TLR4 (sc-293072) antibody was obtained Santa Cruz and USP25 (ab187156) was from Abcam.

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, Minneapolis, MN).

Statistical Analysis

All data are expressed as Mean ± SD. Statistical analyses were performed using GraphPad Pro Prism 8.0 (GraphPad, San Diego, CA). Student’s t-test or one-way ANOVA followed by multiple comparisons test with Bonferroni correction was employed to analyze the differences between sets of data. P value < 0.05 was considered significant.
References


[12] Zhao QL, Wei Y, Pandol SJ, Li LY, Habtezion A. STING Signaling Promotes Inflammation in Experimental Acute Pancreatitis. Gastroenterology 2018;154(6):1822-+


USP25 deficiency exacerbates acute pancreatitis

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USP25 deficiency exacerbates acute pancreatitis. Liu et al.


Macrophage phenotypic switch orchestrates the inflammation and repair/regeneration following acute pancreatitis injury. EBioMedicine 2020;58:12.


translational research 2016;8(9):3645-55.


# Table 1. RT-qPCR primer sequences for mouse genes

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*Rna18s was used as housekeeping gene. Data was normalized by ΔΔCt method.
Figure Legend

Figure 1. Usp25 knockout mice show exacerbated L-arginine-induced pancreatitis.
(A) Gross morphology of the pancreas 72 hours after injection of saline or L-arginine (L-Arg) in wildtype (WT) and Usp25−/− mice. (B) Pancreas-to-body weight ratios, and serum levels of amylase, LDH and lipase in mice. (C) Representative images of H&E-stained pancreatic tissues from mice [Bar = 100 µm]. (D) Histopathological scoring of pancreas tissues. Data in panels B and D are shown as mean ± SD; n = 5-6; ns = not significant, *p<0.05, **p<0.01, and ***p<0.001.

Figure 2. Increased inflammatory cell infiltration in Usp25 deficient mice following L-arginine administration.
(A) Representative images of H&E-stained sections of lungs [Bar = 100 µm]. (B) Representative immunohistochemical staining of lung and pancreas for macrophage F4/80 antigen. Red arrows indicate positive staining [Bar = 100 µm]. Quantification of F4/80-immunoreactive (staining positive) area per high power field (HPF) in lung (C) and pancreatic tissues of mice (D). MPO activity in lung (E) and pancreas (F) of experimental mice. (G) Trypsin activity in the pancreas of mice. Data in panels E-G are shown as mean ± SD; n = 5-6; ns = not significant, *p<0.05, **p<0.01, and ***p<0.001.

Figure 3. Deficiency in macrophage-expressed Usp25 worsens L-arginine-induced pancreatitis.
Wildtype mice were irradiated and reconstituted with bone marrow from either WT (WT→WT) or Usp25−/− (KO→WT) mice. Mice were then subjected to 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) Histopathological scoring of pancreas tissues. (D) Representative H&E-stained images of pancreas and lung tissues [Bar = 100 µm]. (E) Representative immunohistochemical staining images showing F4/80 in the pancreas and lung tissues. Red arrows indicate positive staining [Bar = 100 µm]. (F and G) Quantification of F4/80 staining area in pancreas (F) and lung (G). (H and I) MPO activity in the pancreas (H) and lung (I). (J) Trypsin activity in the pancreas. Data shown as mean ± SD; n = 5-6; ns = not significant, *p<0.05, **p<0.01, and ***p<0.001.

Figure 4. Bone marrow-derived macrophages from Usp25−/− mice show enhanced inflammatory responses.
(A) Schematic illustration of the experimental model. Acinar cells were isolated from mice and cultured for 0 or 24 h. Acinar cell supernatant (ACS) was collected and applied to BMDMs harvested from WT and Usp25−/− mice. BMDM responses were then measured. (B) BMDMs from WT and Usp25−/− mice were exposed to ACS for 60 min. Cell lysates were probed for downstream signaling proteins by immunoblotting. (C-G) mRNA levels of inflammatory factors in BMDMs from WT and Usp25−/− mice that were exposed to ACS for 9h. (H) BMDMs were exposed to ACS for 18h, and IFNβ levels in culture medium were determined. Data in panels C-H are shown as mean ± SD; n = 3 independent experiments; ns
Figure 5. Expression of USP25 in Usp25<sup>−/−</sup> BMDMs reverse acinar cell supernatant-induced inflammatory responses in cells and STING agonist activates downstream signaling in both wildtype and Usp25 deficient macrophages.

Usp25<sup>−/−</sup> BMDMs were transfected with control plasmid or plasmid expressing Flag-tagged Usp25 (GENECHEM, Shanghai, China, Cat# NM_013918). Acinar cells were isolated from WT mice and cultured for 0 or 24 h. Acinar cell supernatant (ACS) collected from 0 or 24 h was applied to control- and Usp25-plasmid transfected BMDMs. (A) ACS exposure of BMDMs was carried out for 60 minutes. Lysates were then probed for activation of downstream inflammatory response proteins. Antibody against Flag was used to detect USP25 expression. (B) Bone marrow-derived macrophages (BMDMs) were prepared from wildtype (WT) and Usp25<sup>−/−</sup> mice. Cells were treated with 40 µg/mL STING agonist DMXAA or vehicle for 1 h, respectively. Lysates were then probed for activation of downstream inflammatory response proteins. Total proteins and GAPDH were used as control. (C-G) ACS exposure was called out for 9 h. mRNA levels of inflammatory factors were then measured by qPCR. (H-L) BMDMs were treated with DMXAA for 9 h. mRNA levels of inflammatory factors were then measured. Data in C-L are shown as mean ± SD; n = 3; ns = not significant, *p<0.05, **p<0.01.

Figure 6. Bone marrow-derived macrophages deficient in Usp25 show enhanced inflammatory responses to L-arginine.

(A) Bone marrow of WT mice was irradiated and reconstituted with bone marrow cells from either WT mice (WT→WT) and Usp25<sup>−/−</sup> mice (KO→WT). Mice were then challenged with L-arginine to induce pancreatitis. Pancreatic lysates were probed for inflammatory pathway activation by immunoblotting. (B) WT and Usp25<sup>−/−</sup> mice were injected with saline or L-arginine. Pancreatic lysates were used for immunoblotting. (C) mRNA levels of Ccl4, Ccl5, Cxcl10 and Isg15 in the pancreas of mice. (D) Serum IFNβ levels were measured by ELISA. (E) mRNA levels of Ccl4, Ccl5, Cxcl10 and Isg15 in the pancreas of WT and Usp25<sup>−/−</sup> mice. (F) Serum IFNβ levels in mice. Data in panels C-F are shown as mean ± SD; n = 5-6; ns = not significant, *p<0.05, **p<0.01, and ***p<0.001.

Figure 7. NF-κB inhibitor suppresses L-arginine-induced pancreatitis in Usp25<sup>−/−</sup> mice.

(A) Schematic illustration of the model. Usp25<sup>−/−</sup> mice received BAY 11-7082, a NF-κB inhibitor, at 5 mg/kg or 10 mg/kg. Control mice received vehicle alone. Six hours later, mice received 2 injections of L-arginine with one hour interval in between. Finally, mice received another dose of BAY 11-7082, six hours after the last L-arginine injection. Pancreas and lungs were harvested from mice and examined for tissue injury. (B) Pancreas-to-body weight ratios, and levels of serum amylase, lactate dehydrogenase (LDH), and lipase in mice. (C) Representative H&E-stained images of pancreas and lung tissues in mice at experimental endpoint [Bar = 100 µm]. (D) Histopathological assessment of edema, inflammatory cell infiltration, necrosis, and total pancreatic injury in mice. Data in B and D are shown as mean ± SD; n = 4-5; ns = not significant, *p<0.05, and **p<0.01.
Figure 8. Usp25−/− mice exhibit aggravated cerulein-induced acute pancreatitis.

(A) Gross morphology of pancreas after cerulein-induced pancreatitis. (B) Representative H&E-stained images of pancreatic tissues [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) Exaggerated responses to CDE feeding. Control diet included standard rodent chow diet (CD). (B) Serum amylase, LDH, lipase, and IFNβ levels in CD- or CDE-fed WT and Usp25−/− mice. (C) Representative H&E-stained images of pancreas in mice [Bar = 100 μm]. (D) Representative H&E-stained images of lung tissues in mice [Bar = 100 μm]. (E and F) MPO activity in the pancreas (E) and lung (F) of CD- or CDE-fed WT and Usp25−/− mice. (G) Trypsin activity in the pancreas from CD or CDE-fed WT and Usp25−/− mice. (H) Survival rate in WT and Usp25−/− mice fed a CDE or control CD diet. Arrows indicate positive staining [Bar = 100 μm]. MPO activity in the lung (H) and pancreas (I) of mice. (J) Trypsin activity in pancreas of mice. Data in panels C-E and H-G are shown as mean ± SD; n = 5-6; ns = not significant, *p<0.05, **p<0.01, and ***p<0.001.

Figure 9. Exaggerated Reponses to CDE-induced pancreatitis in Usp25−/− mice.

(A) Gross morphology of pancreatic tissues in WT and Usp25−/− mice after a 72-hour CDE diet feeding. Control diet included standard rodent chow diet (CD). (B) Serum amylase, LDH, lipase, and IFNβ levels in CD- or CDE-fed WT and Usp25−/− mice. (C) Representative H&E-stained images of pancreas in mice [Bar = 100 μm]. (D) Representative H&E-stained images of lung tissues in mice [Bar = 100 μm]. (E and F) MPO activity in the pancreas (E) and lung (F) of CD- or CDE-fed WT and Usp25−/− mice. (G) Trypsin activity in the pancreas from CD or CDE-fed WT and Usp25−/− mice. (H) Survival rate in WT and Usp25−/− mice fed a CDE or control CD diet. In panels B and E-G, data are shown as mean ± SD; n = 5-6; ns = not significant, *p<0.05, **p<0.01, and ***p<0.001. In panel H, n = 8.

Figure 10. The effect of Usp25 deletion in macrophages on the expression of TRAF and TLR4.

Bone marrow-derived macrophages (BMDMs) were prepared from wildtype (WT) and Usp25−/− mice. Acinar cells were isolated from WT mice and cultured for 0 or 24 h. Acinar cell supernatant (ACS) collected from 0 or 24 h was applied to BMDMs. (A) BMDMs were exposed to ACS for 60 minutes. Lysates were probed TRAF3, TRAF6, and TLR4 by immunoblotting. (B) BMDMs from Usp25−/− mice were transfected with control or Usp25 expressing plasmid. Cells were then exposed to ACS for 60 min. 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) donor mice or Usp25−/− mice (KO→WT). Mice were then 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.

Figure 11. Enhanced inflammatory responses following Usp25 deletion in macrophages are independent of IL-17 signaling.

(A) Bone marrow-derived macrophages (BMDMs) were prepared from wildtype (WT) and Usp25−/− mice. Acinar cells were isolated from WT mice and cultured for 0 or 24 h. Acinar cells were isolated from WT and Usp25−/− mice.
cell supernatant (ACS) collected from 0 or 24 h was applied to BMDMs for 9 h. mRNA levels of Il17a, Ccl7, Ccl20 and Cxcl5 were measured. (B) BMDMs from WT or Usp25−/− mice were treated with IL-17A at 50 ng/mL for 4 or 8 h. mRNA levels of Tnf, Cxcl1, Ccl7 and Ccl20 were measured. (C) BMDMs from WT or Usp25−/− mice were treated with IL-17A at 100 ng/mL for up to 45 min. Levels of USP25 and phosphorylated mitogen-activated protein kinase (JNK, ERK, p38) were determined using Western blot. The total levels of MAPK proteins and GAPDH were used as control. In panels A and B, data are shown as mean ± SD; n = 3; ns = not significant, *p<0.05.
Figure 1

A

Saline  L-Arg

WT

Uap25

B

C

Saline  L-Arg

WT

Uap25

D
Figure 2

A

Saline  L-Arg

WT  Usp29

H&E

B

Lung  Pancreas

WT  L-Arg

Usp29  L-Arg

F4/80

C

D

L-Arg  L-Arg SAP

WT  Usp29

E

Long MPO

Saline  L-Arg  L-Arg

Saline  L-Arg  L-Arg

F

Permea MPO

Saline  L-Arg  L-Arg

Saline  L-Arg  L-Arg

G

Permea
Figure 3

A. L-Arg

B. DHR

C. Edema

D. Pancreas

E. Pancreas

F. Pancreas

G. Lung

H. Pancreas MPO

I. Lung MPO

J. Pancreas

WT = WT
KO = KO

Pancreas body weight (mg)

Pancreatic damage

Inflammatory infiltration

Necrosis

Total score

H&E

F4/80

Ferritin (ng/ml)

Lipase (U/L)

LDH (U/L)

Myeloperoxidase (U/g)

Pancreas

Lung

Field average score

Field average intensity
Figure 4

A

Wild type

Acinar cells culture for 0 or 24 h

ACS (Acini cells supernatant)

Western blot/PCR

WT or Usp25⁻⁻BMDM

B

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C

Ccl4

Relative expression

BMDM ACS 0 24h 0 24h

D

Ccl5

Relative expression

BMDM ACS 0 24h 0 24h

E

Cxcl10

Relative expression

BMDM ACS 0 24h 0 24h

F

Isg15

Relative expression

BMDM ACS 0 24h 0 24h

G

Iltn-β

Relative expression

BMDM ACS 0 24h 0 24h

H

IFN-β (pg/ml)

BMDM ACS 0 24h 0 24h
Figure 7

A

Two i.g of BAY 11-7082 or Ctrl solution

before and after L-arginine injection

Two i.p injection of L-arginine or saline

Tissue harvest

Time (hours)

-6 12 18 24 48 72

B

Pancreas

L-Arg

L-Arg-treating BAY

USP25

C

D
Figure 10

A

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