Original Article Follistatin-like 1 ameliorates severe acute pancreatitis associated lung injury via inhibiting the activation of NLRP3 inflammasome and NF-κB pathway

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Abstract: Objective: Severe acute pancreatitis (SAP) is one of the most common abdominal conditions of digestive system that usually causes acute lung injury through systemic inflammation. Follistatin-like 1 (FSTL-1) has been reported to have anti-inflammatory and anti-apoptotic effects in a variety of diseases. The aim of this study was to investigate the effects of FSTL-1 on SAP-associated lung injury (SAPALI) and the underlying mechanism. Methods: SAP model was induced by intraperitoneal injection of the L-arginine in C57BL/6 mice. The haematoxylin and eosin (H&E) staining was applied to determine the severity of lung and pancreatic injury. ELISA kits were used to determine serum amylase and inflammatory cytokines levels. TUNEL staining was carried out to measure cell apoptosis. Western blotting was applied to analyze the related proteins of NLRP3 inflammasome and NF-κB pathways. Results: FSTL-1 was significantly increased in the lung of SAP mice. Knockout of FSTL-1 ameliorated pancreatic injury, lung injury, inflammation and apoptosis in mice with SAP. Moreover, the protein levels of NLRP3, ASC, Caspase-1, p-p65 and p-lkBα were obviously reduced in the FSTL-1 KO+SAP group in comparison with SAP group, suggesting that inhibition of FSTL-1 repressed the activation of the NLRP3 inflammasome and NF-κB pathway. Conclusion: This study helps us understand the mechanism of FSTL-1 in SAPALI and might provide a potential new strategy for the treatment of SAPALI.

Keywords: Severe acute pancreatitis, SAP-associated lung injury, Follistatin-like 1, inflammation, apoptosis

Introduction

Severe acute pancreatitis (SAP) is a common acute abdominal disease associated with systemic inflammation [1, 2]. SAP-associated lung injury (SAPALI) is the earliest, most common and serious systemic complication of SAP, and its pathogenesis is complex [3]. In recent years, studies on the mechanism of SAPALI have mainly focused on the activation pathways of inflammatory cells and mediators [4, 5]. Substantial evidence has demonstrated that decreased level of inflammatory cytokines has a therapeutic effect on SAPALI [6, 7]. However, the mechanism of lung injury caused by inflammatory cytokines in the pathogenesis of SAP remains unclear.

Follistatin-like 1 (FSTL-1) is a glycoprotein secreted by mesenchymal derived cells and expresses in osteoblasts, chondrocytes, adipocytes, fibroblasts and bone marrow mesenchymal stem cells. It has many biological functions such as regulating cell apoptosis, skeletal muscle growth and fibrosis, and tumor metastasis [8-10]. Previous studies have shown that FSTL-1 is significantly elevated in various inflammatory diseases, including osteoarthritis, rheumatoid arthritis, polymyositis, and dermatomyositis [11, 12]. For instance, silence of FSTL-1 inhibited inflammation through suppressing the TNF- α and Smad signaling [13]. Li et al. has clarified that over-expression of FSTL-1 inhibited chondrogenesis via enhancing inflammatory signaling [14]. However, the correlation between FSTL-1 and SAPALI, especially with regard to inflammation and apoptosis, has not yet been identified.

NLRP3 inflammasome is a kind of intracellular multiprotein complex distributed mainly in the interstitial and cell membrane and is known to

be involved in various host immune and inflammatory responses. This inflammasome is composed of NLRP3, ASC and Caspase-1, which are the most potent cytokines in initiating inflammation [15]. NLRP3 inflammasome recognizes a range of exogenous and endogenous stimuli that transform Caspase-1 into an activated form, and the active Caspase-1 further stimulates the cleavage, maturation and activation of the precursors of the cytokines IL-1 β and IL-18, which are well-known most potent cytokines in initiating inflammation [16-18]. A previous study has shown that NLRP3 inflammasome plays a critical role in placental inflammation and pregnancy complications [19]. Another report demonstrated that inhibition of NLRP3mediated inflammation suppressed the growth of cancer cells [20]. Furthermore, Chaly et al. proposed that FSTL-1 promoted NLRP3 inflammasome activation and facilitated IL-1B and IL-1 secretion through monocytes/macrophages [21]. Besides, it has been reported that FSTL-1 promotes the activation of NF-kB signaling pathway in osteoarthritis [22].

In this study, we aimed to investigate whether FSTL-1 modulated SAPALI through NLRP3 inflammasome and the NF-κB signaling pathway. The functional role of FSTL-1 in SAPALI model mediated by L-arginine, and the molecular mechanisms were further explored. We have shown evidence that knockout of FSTL-1 exerted an inhibitory effect on SAPALI progression via suppressing NLRP3 inflammasome and NF-κB pathway activation, suggesting that FSTL-1 might be a promising prognostic biomarker and therapeutic target for SAPALI.

Materials and methods

Animal

Seventy-two male C57BL/6 mice and FSTL-1 knockout (FSTL-1 KO) C57BL/6 mice (6-8 weeks, 16-20 g) were customized in Cyagen Biosciences Inc (Suzhou, China). The experimental mice were kept at 23°C with 12 h light-dark cycle, and the feeding conditions met the SPF standard. The experiment was conducted after more than 3 days of adaptation. This animal study was abided by the experimental ethics of Weifang People's Hospital. This animal experiments were endorsed by the animal research committee of Weifang People's Hospital (No. 2021-33).

Severe acute pancreatitis (SAP) model

The mice were randomly divided into four groups: Control group (4 g/kg normal saline), FSTL-1 KO group (FSTL-1 knockout+4 g/kg normal saline), SAP group (4 g/kg L-arginine), and FSTL-1 KO+SAP group (FSTL-1 knockout+4 g/kg L-arginine). The mice in control group and FSTL-1 KO group were injected with the same volume of normal saline. The SAP model was established as previously reported [23]. Briefly, the mice were intraperitoneally injected with L-arginine (diluted with normal saline at pH 7.0) twice with 1 h apart, at a concentration of 4 g/ kg body weight. The mice were anaesthetized with 1% pentobarbital sodium via intraperitoneal injection after 24 h. The blood specimens were collected and centrifuged at 1500 rpm for 15 min at 4°C to obtain serum. The lungs and pancreas were then removed after mice were sacrificed. One portion was fixed with 4% formaldehyde for histology analysis and the other was stored at -80°C for RT-PCR assays and western blotting.

Hematoxylin-eosin (HE) staining and histological assessment

After fixed with 4% formaldehyde, the lung and pancreas tissues were embedded in pre-dissolved paraffin, followed by resection and HE staining (hematoxylin and Eosin Stain Kit, CA, USA). Eight random fields were selected, and the images were observed by light microscopy (Olympus BX51, Tokyo, Japan).

Immunohistochemical test

Lung tissue sections were blocked with 30 $\text{uL H}_2\text{O}_2$ at room temperature for 30 min. Then, 40 uL of diluted primary anti-FSTL-1 antibody (Abcam, 1:200, ab232777) was added and incubated at 4°C overnight. After washing with TBST for 4-5 times, 20 uL biotinylated secondary antibody was added and incubated at room temperature for 2 h, followed by 20 uL of HRP-labeled avidin at room temperature for 30 min. DAB was used for color rendering. Finally, the lung tissue sections were examined under microscope, dehydrated and sealed.

Pancreatic damage was determined based on acinar cell necrosis, interstitial aggravation, interstitial edema, and vacuolization [24], with a scale of 0 to 3 (0 points, normal; 3 points, severe). Lung damage was determined based on alveolar wall thickening, infiltration of neutrophils in the vessel walls, bleeding, and alveolar congestion, with a scale of 0 to 4 (0 points, minimal damage, 4 points, maximal damage) [25].

Biochemical tests

The serum amylase and lipase activities were measured using commercial kits as per manufacturer's instructions (C016; A054-2; Jiancheng Bioengineering Institute). The values are expressed in U/L.

ELISA kits assay

Serum levels of IL-6, IL-1 β , and TNF- α were measured using corresponding ELISA kits, according to the company's prospectuses. The ELISA kits used in this experiment were as follows: mouse IL-6 ELISA Kit (RAB0308, Sigma-Aldrich, Shanghai, China), mouse IL- β ELISA Kit (RAB0274, Sigma-Aldrich, Shanghai, China), mouse TNF- α ELISA Kit (RAB0477, Sigma-Aldrich, Shanghai, China). Unknown samples and standard curves were prepared using the Master QT program (MiraiBio, Alameda, CA, USA).

Western blotting assay

The lysis buffer was applied to homogenize lung tissues. BCA protein assay kit (Beyotime Biotechnology, Nanjing, China) was applied for determining protein concentrations. Proteins were subjected to 10% SDS-PAGE, followed by transferred to PVDF membranes. Subsequently, the membranes were blocked with 5% skim milk, and then incubated with primary antibodies against FSTL-1 (1:1000, ab224496, abcam), cleaved-caspase-3 (1:2000, ab214-430, abcam), pro-caspase-3 (1:2000, ab324-99, abcam), cleaved-caspase-8 (1:1000, #85-92, Cell Signaling Technology), pro-caspase-8 (1:1000, ab108333, abcam), NLRP3 (1:1000, ab108333, abcam), ASC (1:1000, #67824, Cell Signaling Technology), caspase-1 (1:1000, #13833, Cell Signaling Technology), p-p65 (1:1000, #13346, Cell Signaling Technology), p65 (1:1000, #8242, Cell Signaling Technology), p-IκB-α (1:1000, #2859, Cell Signaling Technology), IκB-α (1:1000, #4812, Cell Signaling Technology), β-actin (1:1000, ab8227, abcam), followed by incubation with the secondary HRP antibodies (1:2000, ab205718, ab6789, abcam) for 1 h at 37°C. The ECL detection system was used to develop the membranes, and Image J software was applied to analyze image intensity.

Immunofluorescence analysis

Lung tissue sections were incubated with 3% Triton-X100 at room temperature for 30 min, followed by blocking with 5% serum. Then, the primary anti-CD11b antibody (abcam, ab8878, 1:500) and anti-Ly6G antibody (abcam, ab25-377, 1:500) were added and incubated at 4°C overnight. After washing with PBS for 3 times, the secondary antibody was added and incubated at room temperature for 1 h. Finally, the lung tissue sections were examined under microscope, dehydrated and sealed.

Terminal-deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) staining

Lung tissues were incubated with 0.3% Triton X-100 for 30 min at room temperature, followed by incubating with $0.3\% H_2O_2$. Then, the lung tissues were incubated with TUNEL detection solution (Beyotime, Beijing, China) in the dark at 37°C for 1 h, followed by the streptavidin-HRP working solution (Beyotime, Beijing, China) for 30 min. The Hoechst 33342 was used for staining nucleus and a fluorescence microscope (Fluoview FV1000, Tokyo, Japan) was applied for photographing.

Statistical analysis

Data were presented as the mean \pm SD. The significant differences between two groups or the means of groups were determined by unpaired 2-tailed t-test or one-way ANOVA by Tukey's post hoc tests. GraphPad Prism 8.0 was used for statistical analysis. The differences in histopathological score were determined by Mann-Whitney U test. P < 0.05 or less was considered to be statistically significant.

Results

FSTL-1 increased in SAP mice lung tissues

To investigate the association of FSTL-1 with SAPALI, FSTL-1 expression in the lung tissues was measured. Results from RT-PCR discovered that FSTL-1 was obviously elevated in SAP



Figure 1. FSTL-1 was increased in the lung tissues of SAP mice. A: FSTL-1 mRNA was tested in SAP mice by RT-PCR; B: The protein level of FSTL-1 was measured in SAP mice by western blotting; C: FSTL-1 level was measured by IHC in SAP mice (200× magnification, Scale bars = 50 μ m). n = 6 for each group. Compared with control, *P < 0.05, **P < 0.01, ***P < 0.001. FSTL-1: Follistatin-like 1; SAP: severe acute pancreatitis; IHC: immunohistochemical.

mice, relative to control mice (Figure 1A). Moreover, the protein level of FSTL-1 was increased as well in mice after L-arginine treatment, which was measured by western blotting (Figure 1B). Furthermore, IHC results showed that FSTL-1 was up-regulated in SAP mice versus to that in control group (Figure 1C). These data indicated that FSTL-1 might be involved in the development of SAPALI.

Silence of FSTL-1 attenuated the pancreatic injury and inflammation in SAP mice

Then, the effect of FSTL-1 on inflammation and pancreatic injury was explored in SAP mice. The severity of SAP was determined by histological scores of necrosis, inflammation, and tissue oedema. FSTL-1 expression was detected by western blotting and RT-PCR in FSTL-1 KO mice prior to SAP. Results displayed that FSTL-1 was significantly reduced in FSTL-1 KO mice compared with control group (**Figure 2A**). HE results discovered that there was no damage to pancreatic tissue in control and FSTL-1 KO control mice, while there was pancreatic tissue damage in SAP group. Notably, knockout of FSTL-1 significantly reduced the pancreatic injury in mice (**Figure 2B-F**). Moreover, ELISA kits assays revealed that the serum amylase and lipase levels in SAP group was significantly elevated in comparison with control group, and inhibition of FSTL-1 significantly reduced these changes (**Figure 2G** and **2H**). Furthermore, the levels of IL-1 β , IL-6 and TNF- α (**Figure 2I**) in the SAP group were increased, while these levels were decreased significantly in SAP+FSTL-1 KO group. These results indicated that silence of FSTL-1 has a protective effect on L-arginineinduced pancreatic injury in mice.

Silence of FSTL-1 reduced the lung damage and inflammation in SAPALI mice

Next, the effect of FSTL-1 on SAPALI was determined. Compared with the control group, HE staining showed that the pulmonary alveolar structure of mice in SAP group was severely damaged, with a large number of neutrophil



Figure 2. Silence of FSTL-1 attenuated inflammation and pancreatic injury in SAP mice. A: The mRNA and protein level of FSTL-1 in SAP mice; B: HE staining of the pancreas tissues (200× magnification, Scale bars = 50 µm); C: Overall histopathological score; D: Acinar cell necrosis; E: Inflammatory cell infiltration; F: Tissue edema; G-I: ELISA kits analysis of the levels of amylase, lipase, TNF- α , IL-6, and IL-1 β . n = 6 for each group. Compared with control, **P < 0.01, ***P<0.001; compared with FSTL-1 KO, *P < 0.05, #*P < 0.01. FSTL-1: Follistatin-like 1; SAP, severe acute pancreatitis; SAP: severe acute pancreatitis; HE: haematoxylin and eosin; TNF- α : tumor necrosis factor- α ; IL-6: Interleukin-6; IL-1 β . Interleukin-1 β .

infiltrates and pulmonary interalveolar capillary hyperemia. Inhibition of FSTL-1 attenuated the injury induced by L-arginine treatment (**Figure 3A**). Moreover, the lung histopathological score in SAP group was higher than that of control group. FSTL-1 KO decreased the scores induced by SAP (**Figure 3B**). The results from ELISA revealed that the levels of TNF- α , IL-6, and IL-1 β in lung tissues were remarkably up-regulated in SAP group versus to those in control group which were reduced after FSTL-1 knockout (Figure 3C-E). In addition, the immunofluorescence staining was applied to measure macrophages (CD11b) and neutrophils (Ly6G) in lung tissue sections. As expected, SAP induction led to an increase of CD11b and Ly6G numbers, but FSTL-1 KO attenuated the increased number of cells in the lung tissues (Figure 3F and



Figure 3. Silence of FSTL-1 attenuated inflammation and lung damage in SAPALI mice. A: HE staining of the lung tissues (200× magnification, Scale bars = 50 µm). B: Scoring of the alveolar septal capillary density in lung tissues. C-E: The level of TNF- α , IL-6 and IL-1 β were analyzed by ELISA kits; F, G: The numbers of the Ly6G and CD11b detected by Immunofluorescence (200× magnification, Scale bars = 50 µm). n = 6 for each group. Compared with control, *P < 0.05, **P < 0.01, ***P < 0.001; compared with FSTL-1 KO, *P < 0.05. FSTL-1: Follistatin-like 1; SAP, severe acute pancreatitis; SAPALI: SAP-associated lung injury; HE: haematoxylin and eosin; TNF- α : tumor necrosis factor- α ; IL-6: Interleukin-6; IL-1 β .



Figure 4. Silence of FSTL-1 repressed cell apoptosis in SAPALI lung tissues. A: TUNEL staining of the apoptotic cells in the lung tissues (200× magnification, Scale bars = 50 μ m; B: Western blotting analysis of the cleaved-caspase-3 and cleaved-caspase-8 levels in SAPALI lung tissues. n = 6 for each group. Compared with control, *P < 0.05, **P < 0.01, ***P < 0.001; compared with FSTL-1 KO, *P < 0.05, **P < 0.01. FSTL-1: Follistatin-like 1; SAP, severe acute pancreatitis; SAPALI: SAP-associated lung injury.

3G). These data suggested that silence of FSTL-1 ameliorated SAPALI and inflammation.

Silence of FSTL-1 suppressed apoptosis in the lungs of SAPALI mice

Next, TUNEL staining was performed to investigate the effect of FSTL-1 on SAPALI-induced apoptosis of lung cells. Results showed that the apoptotic cells were significantly increased by L-arginine treatment, relative to that in the control group, but the apoptotic cells were suppressed by inhibition of FSTL-1 (**Figure 4A**). Moreover, western blotting assay discovered that the cleaved-caspase-3 and cleaved-caspase-8 levels were dramatically increased in SAP group as compared to control group which was reversed by FSTL-1 inhibition (**Figure 4B**). Thus, silence of FSTL-1 might reduce SAPALI through anti-apoptotic effects.

Silence of FSTL-1 inhibited the activation of NLRP3 inflammasome and NF-KB pathway in SAPALI mice

Previous studies have reported that NLRP3 inflammasome and NF-KB signaling pathway play important roles in lung injury. Western



Figure 5. Silence of FSTL-1 inhibited NLRP3 inflammasome and NF-**kB path**way activation in SAPALI mice. A: Western blotting assay was applied to analyze the levels of Caspase-1, NLRP3, and ASC in SAPALI lung tissues; B: p-p65 and p-lkB α levels were detected in SAPALI lung tissues. n = 6 for each group. Compared with control, *P < 0.05, **P < 0.01; compared with FSTL-1 KO, #P < 0.05. SAP, severe acute pancreatitis; FSTL-1: Follistatin-like 1; SA-PALI: SAP-associated lung injury.



Figure 6. Knockout of FSTL-1 repressed SAPALI via suppressing the activation of NF-kB pathway, thereby inhibiting the inflammation and apoptosis in mice after SAP. FSTL-1: Follistatin-like 1; SAP, severe acute pancreatitis; SAPALI: SAP-associated lung injury.

blotting assay was applied to analyze the levels of Caspase-1, ASC, and NLRP3, and the total and phosphorylated NF-kB and IkB expressions in lung tissues to access the mechanism of FSTL-1 in SAPassociated tissue damage. Results displayed that the levels of Caspase-1, ASC, NL-RP3, p-p65 and p-lkBα were markedly elevated in the SAP group in comparison with the control group. Moreover, compared with the SAP group, these protein levels were attenuated in FSTL-1 KO+SAP group (Figure 5A and 5B). These results suggested that inhibition of FSTL-1 repressed SAPALI via suppressing the activation of NF-kB pathway, thereby inhibiting the inflammation and apoptosis in mice after SAP (Figure 6).

Discussion

Despite new theories and new treatments, the mortality rate

of SAP has remained between 30%-60% in the past 10 years [26]. SAPALI is the most common cause of early death of SAP, so it is of great significance to understand the pathogenesis of SPALI so as to reduce the mortality of severe pancreatitis [3]. Previous studies have shown that FSTL-1 has a variety of anti-inflammatory effects on intervertebral disc degeneration, osteoarthritis and pulmonary fibrosis, but the precise molecular mechanism of FSTL-1 remains unclear [13, 22, 27]. This study showed that knockout of FSTL-1 attenuated SAPrelated lung and pancreatic tissue damage and inhibited related inflammation and cell apoptosis. In addition, inhibition of FSTL-1 suppressed the activation of NLRP3 inflammasome and NF-KB pathway in SAP model in vivo. To best of our knowledge, this is the first time to report that FSTL-1 is an effective target for the treatment of SPALI.

FSTL-1, a secreted glycoprotein mainly derived from interstitium, has been reported in embryonic development, inflammatory disease, autoimmune disease, cardiovascular disease and cancers [28-30]. Recent studies found that the dysregulation of FSTL-1 is closely associated with the occurrence and development of inflammatory diseases. For instance, FSTL-1 knockdown inhibits ox LDL-induced inflammatory response through MAPK signaling [31]. Wang et al. discovered that FSTL-1 played a critical role in allergic airway inflammation via NLRP3/IL-1β activation [32]. Moreover, inhibition of FSTL-1 reduced inflammation through suppressing the TNF-α response and Smad pathway in intervertebral disc degeneration [13]. In this study, we showed that FSTL-1 was up-regulated in lung tissues of SAP model. It has previously been reported that FSTL-1 was highly expressed in S. pneumonia-induced pneumonia [33]. In addition, knockdown of FSTL-1 attenuated SAPrelated lung and pancreatic tissue damage, and reduced inflammation and cell apoptosis. These findings revealed that FSTL-1 knockout could ameliorate SAPALI, and FSTL-1 might be a potential therapeutic target for SAP.

NLRP3 inflammasome, an important component of innate immunity, plays a critical role in the body's immune response and disease development, including auto-inflammatory response, atherosclerosis, Alzheimer's, and type 2 diabetes [34-37]. A recent study has reported that NLRP3 inflammasome was involved in the development of SAP, and inhibition of NLRP3 could reduce the severity of pancreatitis [38]. NF-kB signaling pathway was activated in various inflammatory responses in the lungs [39]. The NF-kB signaling pathway includes receptor and receptor proximal signal transfer protein, IkB kinase complex, IkB protein and NF-kB dimer. Under stimulation. IkB kinase is activated, leading to phosphorylation and ubiquitination of IkB, and free phosphorylated NF-kB is transferred to the nucleus, thereby stimulating the expression of various types of cytokines and inflammation-related genes [40]. This study found that the protein levels of NLRP3 Complex and p-IkB and p-p65 in the lung tissues of FSTL-1 KO mice were significantly increased, relative to control group, suggesting that inhibition of FSTL-1 repressed the activation of NLRP3 inflammasome and NF-KB pathway.

This study has some limitations. Firstly, only the L-arginine-induced model of SAP was used in the study, and the results should be verified in

other SAP models in future experiments. Secondly, it is unclear how FSTL-1 regulates the activation of NLRP3 inflammasome by either direct interaction with the NLRPL3 inflammasome or indirect effects via other signals, which remains to be further studied in the future.

Conclusion

This study demonstrated that inhibition of FSTL-1 ameliorated SAP-induced ALI as well as pancreatic injury in SAP mice. FSTL-1 KO might exert these effects by inhibiting the activation of NLRP3 inflammasome and NF- κ B signaling, indicating that FSTL-1 may be a potential target for treating SAP and SAP-induced ALI.

Disclosure of conflict of interest

None.

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