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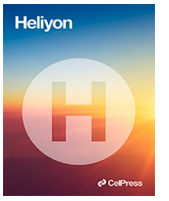

Fasudil and SR1001 synergistically protect against sepsis-associated pancreatic injury by inhibiting RhoA/ROCK pathway and Th17/IL-17 response

Pingping Liu [a], Zhenghui Xiao [a,*], Xiulan Lu [a], Xinping Zhang [a], Jiaotian Huang [a], Cheng Li [b]

[a] *Department of Emergency, Key Laboratory of Pediatric Emergency Medicine of Hunan Province, Hunan Children's Hospital, Changsha, 410007, Hunan, PR China*

[b] *Department of Respiratory and Critical Care Medicine, Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University, Changsha, 410005, Hunan, PR China*

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ABSTRACT

Sepsis is defined as a dysregulated host response to infection that can result in organ dysfunction and high mortality, which needs more effective treatment urgently. Pancreas is one of the most vulnerable organs in sepsis, resulting in sepsis-associated pancreatic injury, which is a fatal complication of sepsis. The aim of this study was to investigate the effect of combination of fasudil and SR1001 on sepsis-associated pancreatic injury and to explore the underlying mechanisms. The model of sepsis-associated pancreatic injury was induced by cecal ligation and puncture. Pancreatic injury was evaluated by HE staining, histopathological scores and amylase activity. The frequency of Th17 cells was analyzed by flow cytometry. Serum IL-17 level was determined by ELISA. Protein levels of RORγt, p-STAT3, GEF-H1, RhoA and ROCK1 were determined by Western blot. The apoptosis of pancreatic cells was examined by TUNEL analysis and Hoechst33342/PI staining. Compared to the sham group, the model group showed significant pathological injury including edema, hyperemia, vacuolization and necrosis. After treatment with fasudil, model mice showed an obvious reduction of Th17 cells and IL-17. SR1001 significantly reduced the expressions of GEF-H1, RhoA and ROCK1 in the model mice. The combination treatment with fasudil and SR1001 significantly inhibited the differentiation of Th17 cells, expressions of IL-17, GEF-H1, RhoA and ROCK1, which were more effective than each monotreatment. In addition, our data revealed a remarkable decrease of apoptosis in pancreatic acinar cells culturing with fasudil or SR1001, which was further inhibited by their combination culture. Lipopolysaccharide remarkably upregulated the differentiation of Th17 cells in vitro, which could be significantly downregulated by fasudil or SR1001, and further downregulated by their combination treatment. Taken together, the combination of fasudil with SR1001 has a synergistic effect on protecting against sepsis-associated pancreatic injury in C57BL/6 mice.

* Corresponding author.
E-mail address: xiaozh888@126.com (Z. Xiao).

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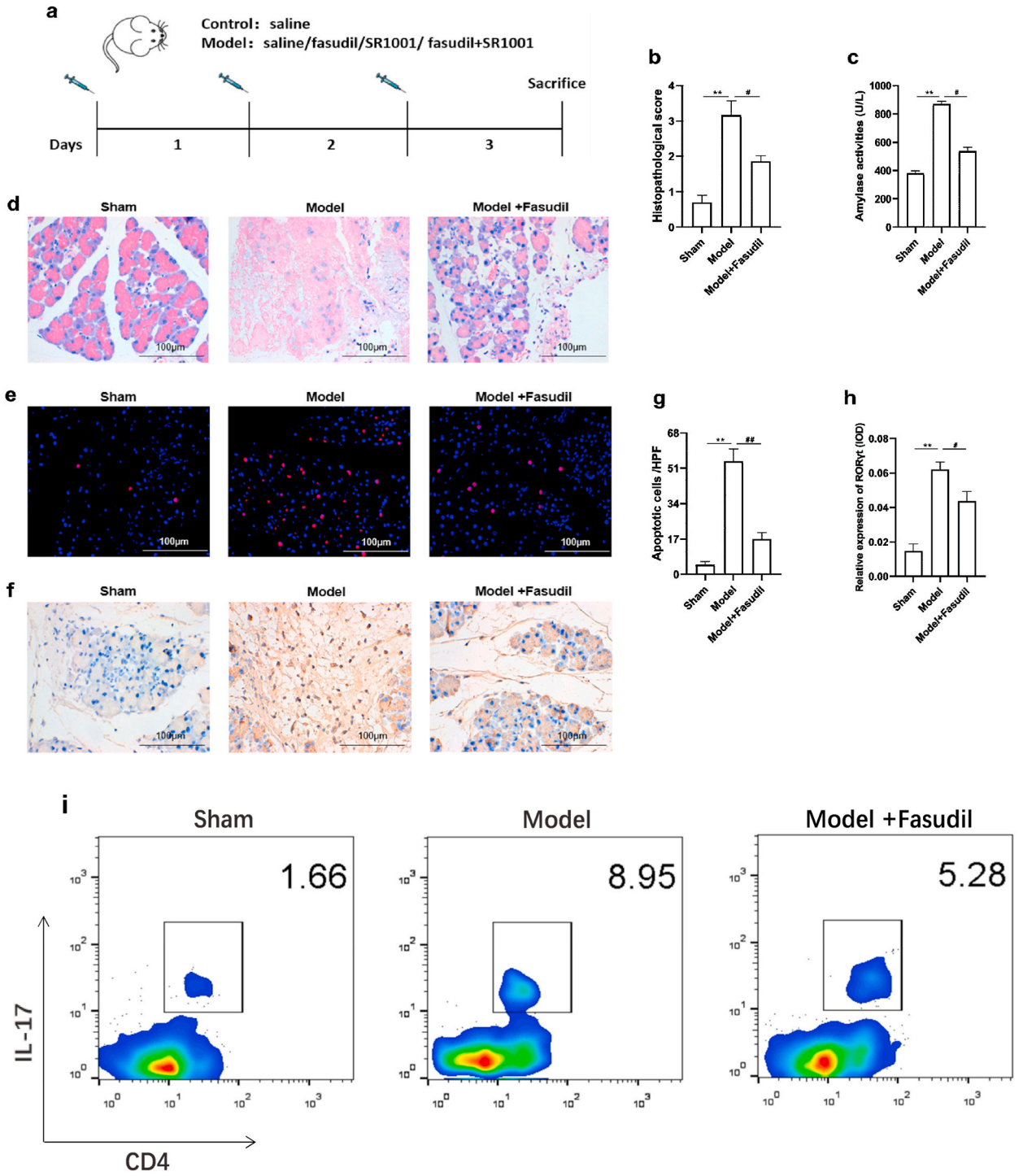

Fig. 1. Fasudil alleviated pancreatic injury and inhibited the differentiation of Th17 cells in CLP-induced sepsis model. (**a**) Experimental protocol of the study. (**b**) The histopathological scores of pancreatic injury. (**c**) Amylase activity in the serum was determined. (**d**) Representative HE pathological staining of pancreatic tissues section (orginal magnification × 400). (**e**) Apoptotic cells were examined by TUNEL analysis. (**f**) Immunohistochemistry analysis of RORγt in the pancreatic tissues. (**g**) Apoptotic cells were quantified by histogram (high power fields at × 400 magnification). (**h**) Relative expression level of RORγt was analyzed by histogram. (**i**) Percentage of Th17 cells in the spleen. (**j**) Percentage of Th17 cells was analyzed by histogram. (**k**) Serum IL-17 was analyzed by ELISA. (**l**) Expression of p-STAT3 in the spleen analyzed by Western blot. (**m**) (**n**) Expressions of p-STAT3 and RORγt in the pancreatic tissues analyzed by Western blot. (**o**) Quantitative analysis of p-STAT3 in the spleen. (**p**) (**q**) Quantitative analysis of p-STAT3 and RORγt in the pancreatic tissues. Values were means ± SD, n = no. of animals. $^{**}P < 0.01$, $\#P < 0.05$.

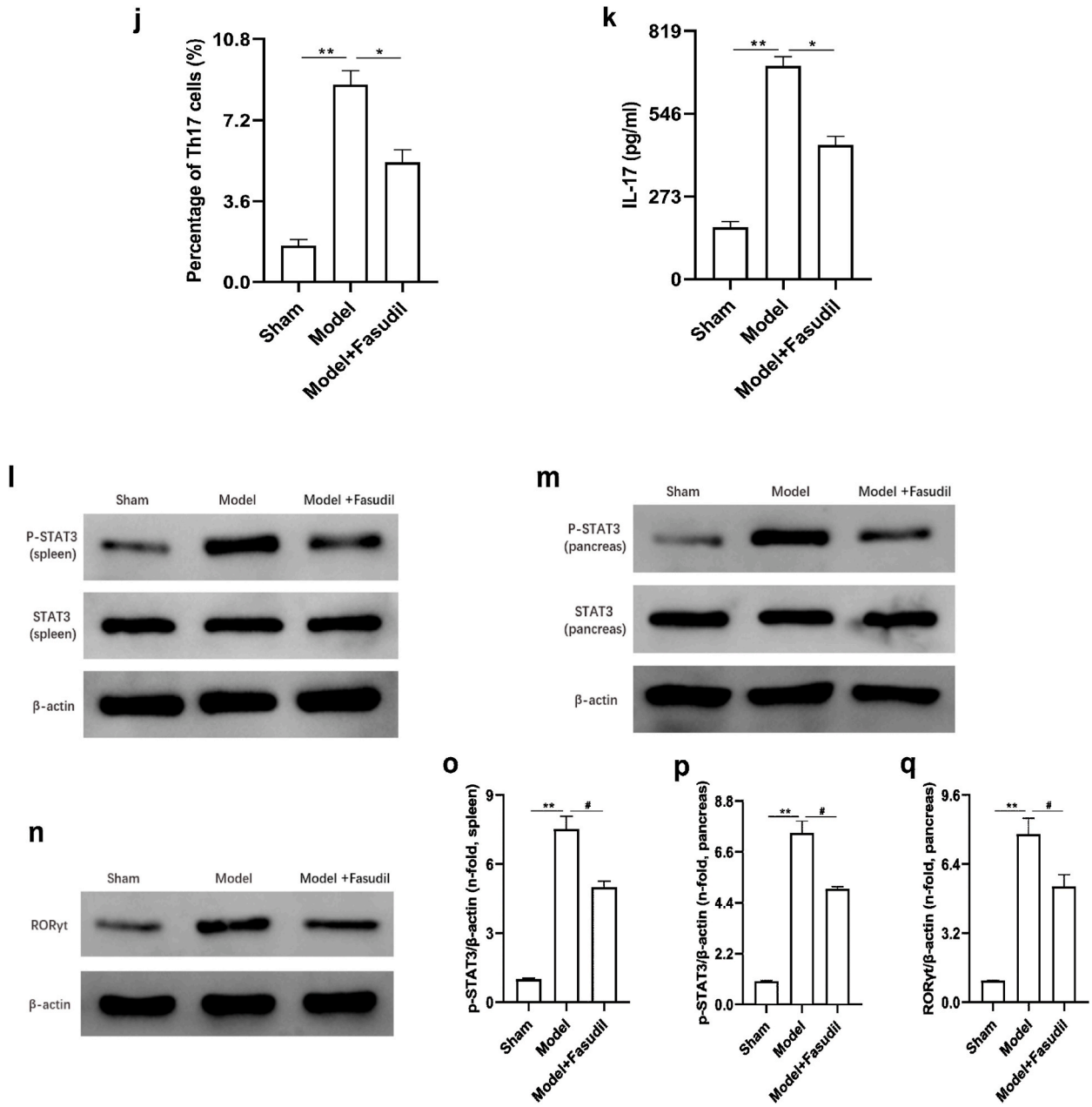

Fig. 1. (*continued*).

1. Introduction

Sepsis is a life-threatening clinical syndrome resulting from systemic inflammation to infection that can progress to multi-organ dysfunction syndrome with a high mortality rate [1]. Sepsis affects more than 48 million people annually, leading to the death of approximately 11 million people across the world. Currently there is no specific treatment for sepsis. Lethal outcome is depending under several factors, such as infectious agent, site of infections, severity of sepsis and organ dysfunction. No organ is exempt from sepsis-induced dysfunction. Sustained, uncontrolled inflammatory activity triggers a sequence of systemic mechanisms that tend to affect organ and hemodynamic stability. The pathogenesis of sepsis seems to be complex, involving metabolic dysfunction, epigenetic reprogramming, immune dysfunction, intestinal barrier failure, coagulation abnormalities, neuroendocrine disorders, and many other scientific issues [2]. Due to the inadequacy of diagnostic markers and treatment strategies, the treatment for sepsis may be a huge challenge.

Although the most common dysfunction in patients with sepsis is kidney, liver and lung [3–5], the pancreas is also vulnerable to

injury and dysfunction in patients and animal models of sepsis [6]. Pancreatic injury was found to be an important pathological change in sepsis, which is an important cause of mortality of patients with sepsis. Our group also indicated that pancreatic injury was common in critically ill children, which positively correlated with severity of the disease and the prognosis [7]. Therefore, the inhibition of pancreatic injury is the key to treat sepsis and improve the prognosis of sepsis.

It has been reported that Ras homolog gene family member A (RhoA)/Rho-kinase (ROCK) signaling pathway is likely to play a significant role in sepsis. The ROCK family, consisting of ROCK1 and ROCK2, plays a key role in a wide range of fundamental cellular functions. ROCK1 is mainly expressed in pancreas, kidney tissues and cardiomyocyte, and ROCK2 is mainly expressed in nervous tissues [8,9]. Previous studies have indicated that the expression of RhoA/ROCK pathway were upregulated in lung vascular leak and impair cardiac function of sepsis. Unfractionated heparin attenuated lipopolysaccharide (LPS)-induced lung vascular leak by inhibiting the activated RhoA/ROCK pathway. Cardioprotection in sepsis by RhoA/ROCK inhibition may be related to changes in actin cytoskeleton reorganization and mitochondrial homeostasis. Improvement of LPS-induced cardiac dysfunction by fasudil was attributed to inhibition of RhoA/ROCK-dependent Drp 1 phosphorylation and activation of autophagic processes [10,11]. In addition, our recent study showed that RhoA/ROCK pathway was involved in pancreatic injury of sepsis, which could be inhibited by baicalin [12]. These data demonstrated the importance of RhoA/ROCK pathway in organ dysfunction and provided the novel mechanism that were also likely to influence the progress and severity of the sepsis.

In sepsis, alterations in immune cells such as T lymphocytes, B lymphocytes and natural killer cells are largely responsible for the increased mortality and poor prognosis [13]. Th17 cells are important pro-inflammatory cell and the foremost players in inflammatory diseases. IL-17 is a proinflammatory cytokine and plays essential roles in adaptive and innate immune responses. A previous research found that Th17 cells proportion was elevated in septic patients, which was correlated with higher inflammation level, increased disease severity and worse prognosis in septic patients [14]. Moreover, Th17 cells in peripheral blood was significantly increased in septic patients with acute lung injury and that may be used as a potential biomarker for diagnosis of acute lung injury of sepsis [15]. Our study also suggested the significant increments of Th17 cells and its associated cytokines IL-17 in peripheral blood, spleen and pancreatic tissue in sepsis-associated pancreatic injury (SPI) model, and baicalin evidently reduced Th17/IL-17 and ameliorated pancreatic injury [12]. These reports supported that immune cell was important for sepsis and provided novel insight into the mechanism involved in the initiation and progress of sepsis.

A series of studies have shown the association between RhoA/ROCK pathway and Th17/IL-17. Rho-GTPases (which include Rho subfamilies) proteins are key regulators of T-lymphocyte development, differentiation, migration, and activation [16]. One of the early lines of evidence demonstrated that IL-17 activated RhoA/ROCK leading to endothelial dysfunction and hypertension [17]. These data may indicate the interplay of RhoA/ROCK pathway and Th17/IL-17.

Based on the important role of RhoA/ROCK pathway and Th17/IL-17 axis in sepsis, this study is designed to evaluate whether the combination of SR1001 (a novel effect of Th17 cells inhibitor) and fasudil (a RhoA/ROCK pathway inhibitor) could ameliorate SPI. Furthermore, we also assessed whether the combination treatment of SR1001 and fasudil was more effective than each monotreatment, and the underlying mechanisms of SR1001 and fasudil regulating Th17/IL-17 axis and RhoA/ROCK pathway. Thus, this study aimed to provide more evidence and a potential therapeutic strategy for SPI.

2. Materials and methods

2.1. Mice and treatment

A total of 88 male C57BL/6 mice weighing 23–26 g (8–10 weeks of age) were purchased from Hunan Silaikejingda Laboratory Animal Co. Ltd., Changsha, China. All animal studies were performed in accordance with the policy of Chinese Association of Laboratory Animal Science. All procedures were approved by the Ethics Committee of Hunan Children's Hospital (NO.KYSQ2021-194). This animal study consists of three parts as follows (Fig. 1a). Twenty-four mice were randomly divided into three groups (n = 8): sham group, model group, and model + SR1001 group. Another 24 mice were randomly divided into three groups (n = 8): sham group, model group, and model + fasudil group. The model of sepsis-associated pancreatic injury induced by cecal ligation and puncture (CLP) was established according to our previous report [12]. Following CLP surgery, mice in model group were intraperitoneally injected 2 ml/kg normal saline daily. Mice in model + fasudil group were intraperitoneally injected 30 mg/kg fasudil (Manufacturer: Sigma-Aldrich, batch No.: CDS021620, diluted with normal saline by 2 ml/kg) daily [18,19]. Mice in model + SR1001 group were intraperitoneally injected 25 mg/kg SR1001 (Manufacturer: Abcam, batch No.: ab141462, diluted with normal saline by 2 ml/kg) daily. Mice in sham group were received the same dose of normal saline as model group daily. Fasudil and SR1001 doses were similar to those used in previous literature [20–22]. The remaining forty mice were randomly assigned into other five groups (n = 8): sham group, model group, model + SR1001 group, model + fasudil group, and model + SR1001+fasudil group. The mice models were established as mentioned above. Mice in sham group and model group were intraperitoneally injected 2 ml/kg normal saline daily. Mice in model + fasudil group and model + SR1001 group were intraperitoneally injected 30 mg/kg fasudil and 25 mg/kg SR1001 daily, respectively. Mice in model + SR1001+fasudil group were received 30 mg/kg fasudil and 25 mg/kg SR1001 daily. This animal experiment lasted for three days. Three days later, all the mice were anesthetized using sodium pentobarbital and killed for blood samples, spleen and pancreatic tissues.

2.2. Histopathological analysis

The pancreatic tissues were fixed with formalin for 24 h, which were embedded in paraffin and sectioned at about 5-μm for each

group. Slides were stained with hematoxylin and eosin (H&E) according to manufacturer's protocol. At least 10 randomly selected microscopic fields were evaluated in each tissue by two experienced investigators who were blinded as to the study. Pancreatic histological injury was assessed according to the method described previously [6].

2.3. Amylase activity measurement

All blood samples were collected. The severity of pancreatic injury was evaluated by serum amylase activity, which was measured by using a commercial kit (Sigma-Aldrich) [6]. Levels of amylase were determined in accordance with the manufacturer's protocols.

2.4. Flow cytometric analysis

The flow cytometric analysis was performed as the previous report [23]. Briefly, individual cell suspensions of splenocytes of experimental mice and CD4+T cells cultured in vitro were prepared. These individual cells were pre-cultured with PMA (50 ng/ml, Sigma-Aldrich), ionomycin (1 mg/ml, Sigma-Aldrich) and Brefeldin A (0.5 mg/ml, Sigma-Aldrich) for 5 h. Afterward, these cells were stained with FITC-*anti*-CD4 (eBioscience, USA), APC-*anti*-CD3 (eBioscience, USA) and PE-*anti*-IL-17 (eBioscience, USA) antibodies. The T cells labeled $CD3^{+}CD4+IL\text{-}17+$ represent Th17 cell, which were analyzed by flow cytometer (FC500, Beckman, USA) and FlowJo 7.6.1 software (Tree Star, USA).

2.5. ELISA

The concentration of IL-17 in serum was measured by using commercially murine enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer's protocols (R&D, USA). All assays were analyzed in triplicate.

2.6. Western blot analysis

Proteins were extracted from spleen [for detection of phosphorylated signal transducer and activator of transcription 3 (p-STAT3)], pancreatic tissues [for detection of guanine nucleotide exchange factor-H1 (GEF-H1), RhoA, ROCK1, p-STAT3, retinoic acid receptor-related orphan receptor γt (RORγt)], CD4+T cells cultured in vitro (for detection of RhoA) and pancreatic acinar AR42J cells (for detection of RhoA) by using a protein extraction buffer kit according to manufacturer's protocol. BCA protein assay kit was used to evaluate the protein concentration. Western blot was performed as described previously using primary antibodies for p-STAT3 (ab76315, Abcam, 1:500) antibody, RORγt (ab207082, Abcam, 1:500) antibody, RhoA (ab187027, Abcam, 1:500) antibody, GEF-H1 (ab155785, Abcam, 1:1000) antibody, ROCK1 (ab134181, Abcam, 1:500) antibody. A chemiluminescence substrate kit (Millipore, WBLUR0100) was used for chemiluminescent signals detection. Membranes were imaged and quantified by densitometry with image software ChemiDoc XRS + system (Bio-Rad Co,. USA).

2.7. Immunohistochemistry

The paraffin-embedded pancreatic tissues were sectioned about 5 μm for immunohistochemistry. Briefly, paraffin sections were dewaxed and incubated with 3% H_2O_2 to block endogenous peroxidase activity. After the sections were incubated with 10% goat serum, they were added with primary antibodies at 4 °C overnight: RhoA (ab187026, Abcam, 1:200) antibody, RORγt (ab207082, Abcam, 1:200) antibody. Subsequently, the sections were incubated with secondary antibody at 1:2000 dilution. DAB was used as chromogenic substrate to incubate for immunohistochemical staining, and then the slides were dehydrated, sealed and viewed by an Olympus microscope (Olympus AG, Zurich, Switzerland). The positive cells were counted at high-power field (× 400 magnification).

2.8. Isolation and culture of $CD4^{+}$ T cells

Splenocytes were harvested and distributed into 96-well plates as described previously [24]. CD4+T cells were isolated using immunomagnetic beads (MACS beads, USA) and examined by the flow cytometry for purity. The purified CD4+T cells were added IL-6 (20 ng/ml), TGF-β (1 ng/ml), anti–IFN–γ (10 μg/ml) and anti-IL-4 (5 mg/ml) for inducing Th17 differentiation. This $CD4^{+}$ T cells study consists of five groups as follows. (1) CD4+T cells group, (2) CD4+T cells + LPS group in which $CD4^{+}$ T cells were cultured in the presence of LPS (10 μg/ml), (3) CD4+T cells + LPS + fasudil group in which $CD4^{+}$ T cells were cultured in the presence of LPS (10 μg/ml) and fasudil (15 μg/ml), (4) CD4+T cells + LPS + SR1001 group in which $CD4^{+}$ T cells were cultured in the presence of LPS (10 μg/ml) and SR1001 (15 μg/ml). (5) CD4+T cells + LPS + fasudil + SR1001 group in which $CD4^{+}$ T cells were cultured in the presence of LPS (10 μg/ml), fasudil (15 μg/ml) and SR1001 (15 μg/ml). Each well was inoculated 10^{6}/ml of cells and fostered for 24 h.

2.9. Experiment protocols of pancreatic acinar AR42J cells

Pancreatic acinar AR42J cells (Procell, Wuhan, China) were maintained in Ham's F12 medium and plated in 6-well cluster dishes at a density of 1×10^{5} cells/ml and incubated in a humidified incubator with 5% CO2 and 95% atmospheric air at 37 °C. This AR42J cells study consists of six groups as follows. (1) AR42J cells group, (2) AR42J cells + $CD4^{+}$T cells (Th17 polarization, 10^{6}/ml), (3) AR42J cells + $CD4^{+}$T cells (Th17 polarization, 10^{6}/ml) + LPS (10 μg/ml), (4) AR42J cells + $CD4^{+}$T cells (Th17 polarization, 10^{6}/ml) + LPS

(10 μg/ml) + SR1001 (15 μg/ml) group, (5) AR42J cells + $CD4^+$T cells (Th17 polarization, 10^6/ml) + LPS (10 μg/ml) + fasudil (15 μg/ml) group, (6) AR42J cells + $CD4^+$T cells (Th17 polarization, 10^6/ml) + LPS (10 μg/ml) + SR1001 (15 μg/ml) + fasudil (15 μg/ml) group. AR42J cells in each group were fostered for 24 h.

2.10. Hoechst33342/PI staining of cell apoptosis

Cell apoptosis assay of AR42J cells was performed as described previously [25]. In short, dispersed AR42J cells were fixed with 4% formaldehyde solution for 15 min and stained with Hoechst33342 and propidium iodide for 15 min under indoor temperature. After rinsing PBS, the cells were examined analyzed it utilizing a fluorescence microscope. The red fluorescence-positive cells were calculated as the percentage of apoptotic cells.

2.11. Statistical analysis

All statistical analyses and data processing were accomplished using SPSS 26.0 software. The measurement data were expressed as mean ± standard deviation. Comparisons between three or more groups were evaluated by One-way analysis of variance (ANOVA)

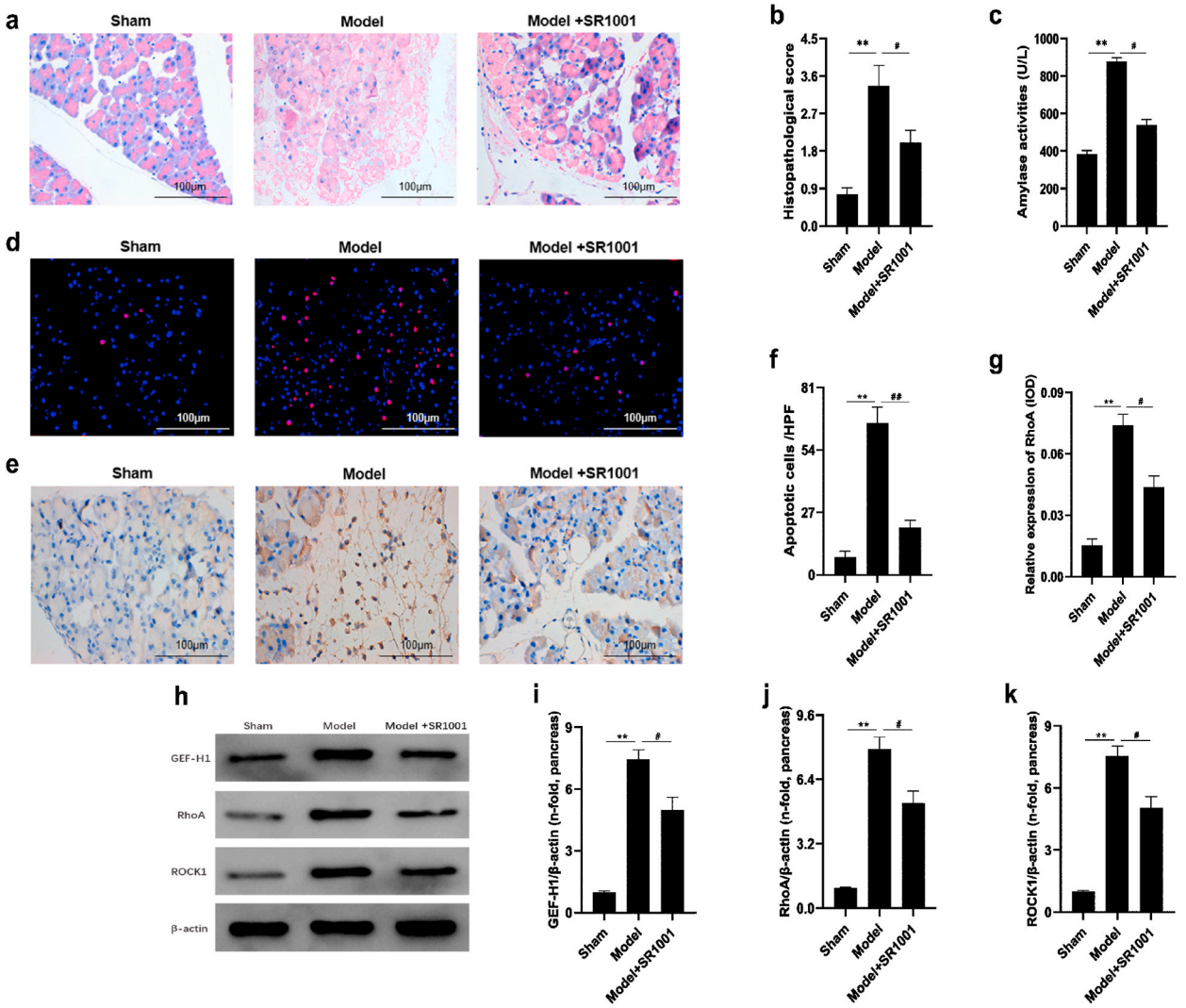

Fig. 2. SR1001 alleviated pancreatic injury and inhibited the differentiation of Th17 cells in CLP-induced sepsis model. (**a**) Representative HE pathological staining of pancreatic tissues section (orginal magnification × 400). (**b**) The histopathological scores of pancreatic injury. (**c**) Amylase activity in the serum was determined. (**d**) Apoptotic cells were examined by TUNEL analysis. (**e**) Immunohistochemistry analysis of RhoA in the pancreatic tissues. (**f**) Apoptotic cells were quantified by histogram (high power fields at × 400 magnification). (**g**) Relative expression level of RhoA was analyzed by histogram. (**h**) Expressions of GEF-H1, RhoA and ROCK1 in the pancreatic tissues analyzed by Western blot. (**i**) (**j**) (**k**) Quantitative analysis of GEF-H1, RhoA and ROCK1 in the pancreatic tissues. Values were means ± SD, n = no. of animals. $^{**}P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$.

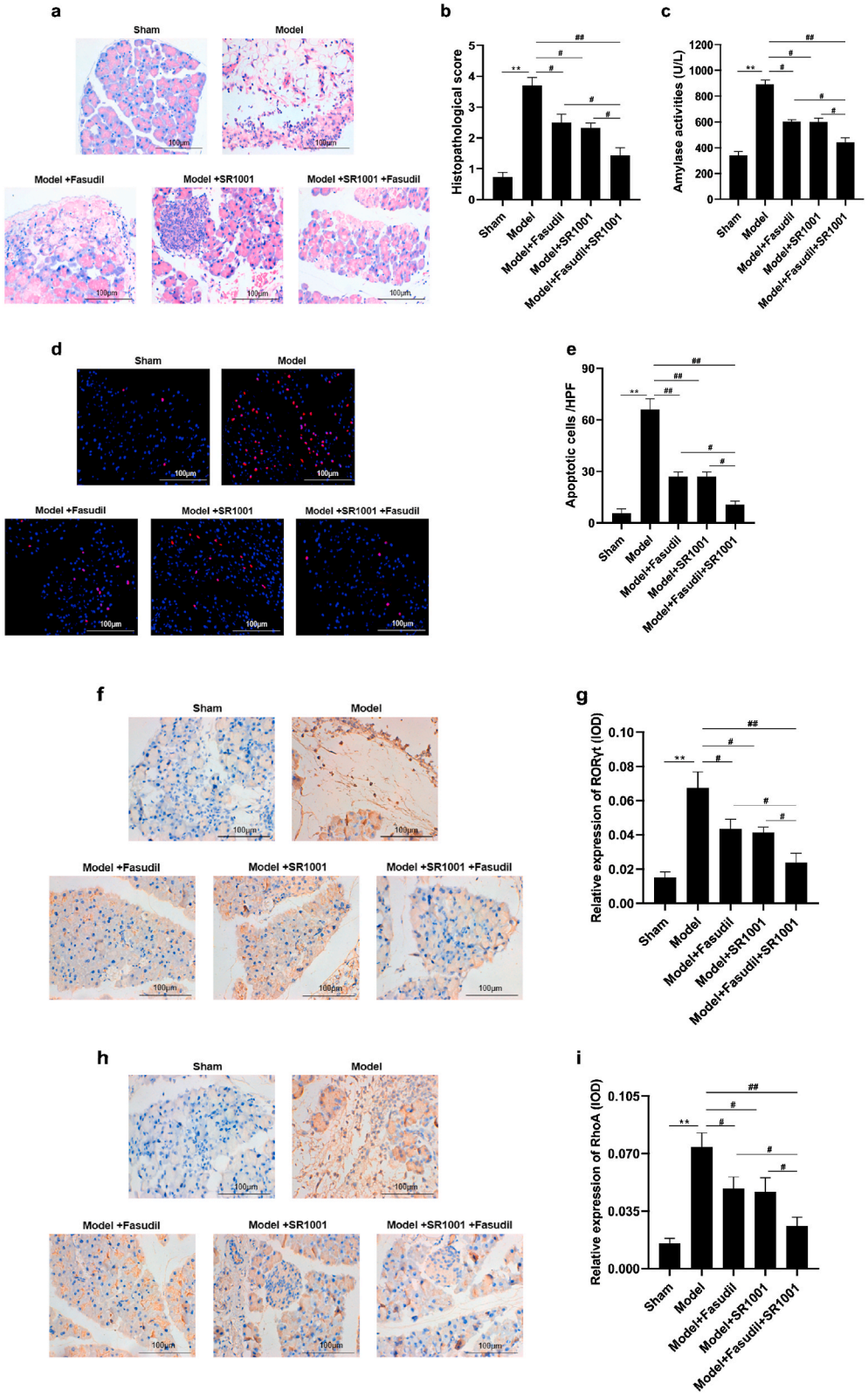

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Fig. 3. Combination of Fasudil and SR1001 alleviated pancreatic injury in CLP-induced sepsis model. (**a**) Representative HE pathological staining of pancreatic tissues section (orginal magnification × 400). (**b**) The histopathological scores of pancreatic injury. (**c**) Amylase activity in the serum was determined. (**d**) Apoptotic cells were examined by TUNEL analysis. (**e**) Apoptotic cells were quantified by histogram (high power fields at × 400 magnification). (**f**) Immunohistochemistry analysis of RORγt in the pancreatic tissues. (**g**) Relative expression level of RORγt was analyzed by histogram. (**h**) Immunohistochemistry analysis of RhoA in the pancreatic tissues. (**i**) Relative expression level of RhoA was analyzed by histogram. Values were means ± SD, n = no. of animals. $^{**}P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$.

and the Student's t-test was selected for two group comparisons. All results were repeated three times. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Fasudil alleviated pancreatic injury and inhibited the differentiation of Th17 cells

To evaluate the effect of fasudil on pancreatic injury in mice induced by CLP, HE staining was performed and we found the significant pathological injury in the model group including edema, hyperemia, vacuolization and necrosis compared to the sham group, while fasudil could obviously alleviate the pancreatic injury. The apoptosis of pancreatic cell was examined by the Tunel method. Quantitative analysis demonstrated that the number of apoptotic cells was higher in the model group compared to the sham group, and fasudil reduced the apoptosis of pancreatic cells (Fig. 1b, d, e, g). In addition, amylase activity was dramatically increased in the model

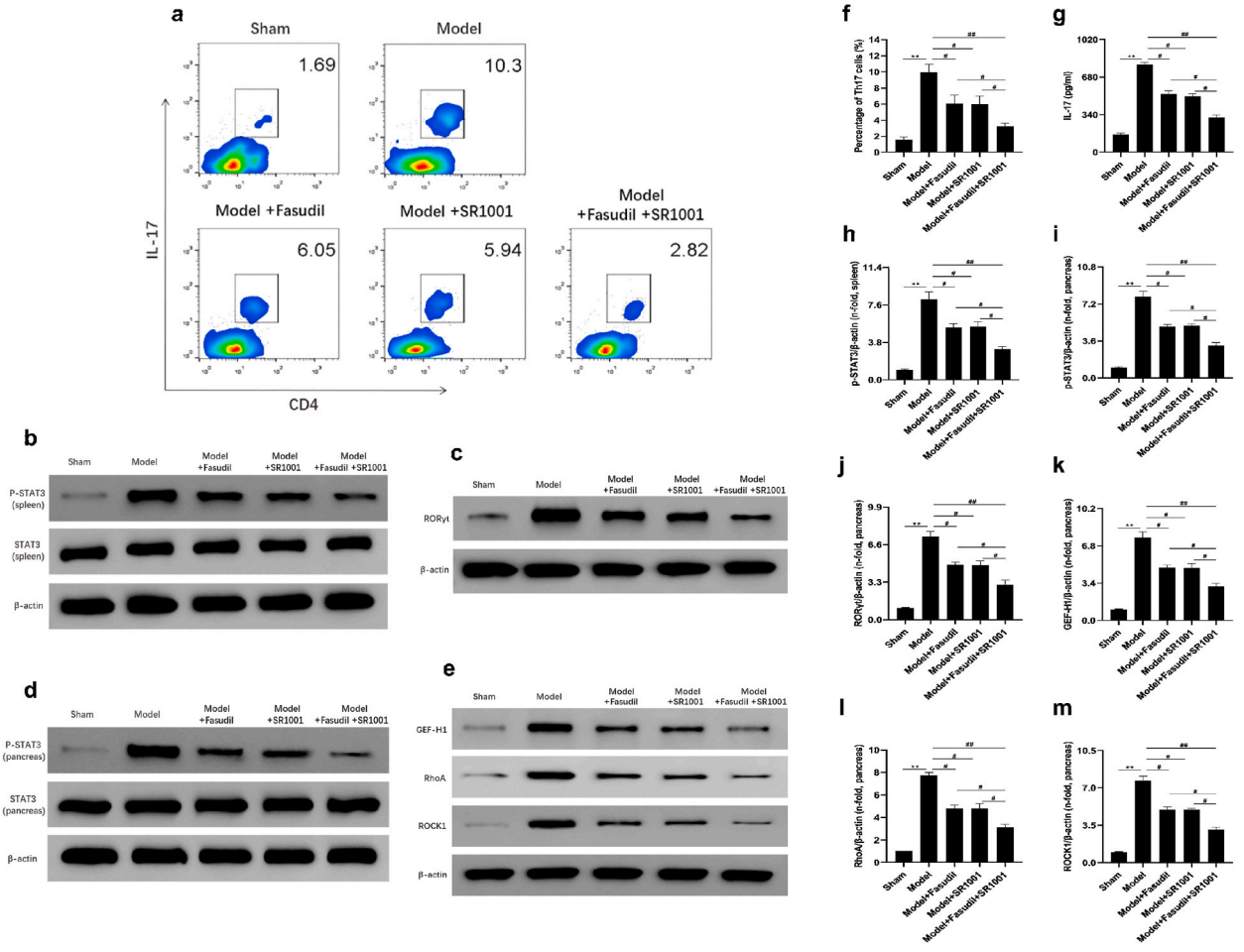

Fig. 4. Combination of Fasudil and SR1001 inhibited the differentiation of Th17 cells and expressions of RORγt and RhoA. (**a**) Percentage of Th17 cells in the spleen. (**b**) Expression of p-STAT3 in the spleen analyzed by Western blot. (**c**) Expression of RORγt in the spleen analyzed by Western blot. (**d**) (**e**) Expressions of p-STAT3, GEF-H1, RhoA and ROCK1 in the pancreatic tissues analyzed by Western blot. (**f**) Percentage of Th17 cells was analyzed by histogram. (**g**) Serum IL-17 was analyzed by ELISA. (**h**) Quantitative analysis of p-STAT3 in the spleen. (**i**) (**j**) (**k**) (**l**) (**m**) Quantitative analysis of p-STAT3, RORγt, GEF-H1, RhoA and ROCK1 in the pancreatic tissues. Values were means ± SD, n = no. of animals. $^{**}P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$.

group, which could be inhibited by fasudil (Fig. 1c). Then, we detected the percentage of Th17 cells in spleen by flow cytometry and expression of IL-17 in serum by ELISA. The results showed that percentage of Th17 cells and expression of IL-17 increased significantly compared to those of control group, while fasudil treatment decreased the percentage of Th17 cells and expression of IL-17 in model mice (Fig. 1i, j, k). STAT3 is a key transcription factor of Th17 cells and RORγt is the crucial control gene in development and function of Th17 cells. Consistent with the change of Th17 cells, the expressions of p-STAT3 and RORγt were significantly increased in the model group, and fasudil inhibited the expressions of p-STAT3 and RORγt (Fig. 1f, h, l-q). These results suggested that fasudil alleviated pancreatic injury and inhibited the differentiation of Th17 cells in mice induced by CLP.

3.2. *Effect of SR1001 on pancreatic injury and expression of RhoA/ROCK1 pathway*

To investigate the effect of SR1001 on CLP-induced model mice, SR1001 was injected into mice and pancreatic injury was assessed. It was shown that pancreatic injury induced by CLP could be prevented by SR1001 treatment. Further analysis indicated that SR1001 inhibited the apoptosis of pancreatic cells and reduced the amylase activity (Fig. 2a–d, f). To explore the effect of SR1001 on RhoA, expression of RhoA was detected by immunohistochemistry and Western blot. The results showed that expression of RhoA was increased in the model group, while could be reversed by SR1001 treatment. GEF-H1 is a RhoA specific guanine nucleotide exchange factor, which leading to RhoA activation. ROCK1 is a downstream effector of RhoA. We also found that the expressions of GEF-H1 and ROCK1 were significantly increased in the model group, which could be inhibited by SR1001 treatment (Fig. 2e, g, h-k). These results suggested that the protective role of SR1001 on pancreatic injury induced by CLP might be mediated by the RhoA/ROCK1 pathway.

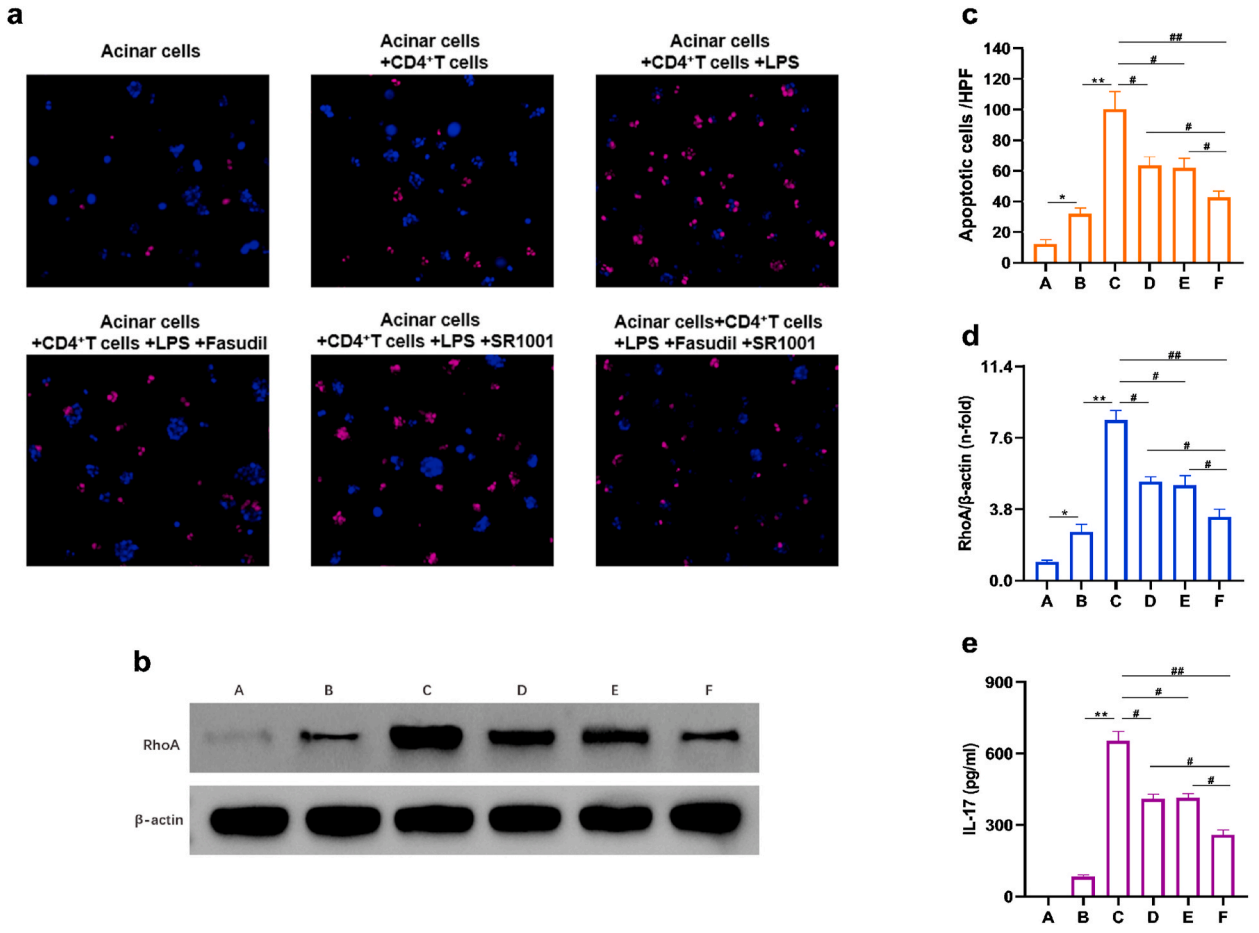

Fig. 5. Combination of Fasudil and SR1001 inhibited the apoptosis of pancreatic acinar cells and expression of RhoA in vitro. (**a**) Apoptotic cells were examined by Hoechst33342/PI staining. (**b**) Expression of RhoA analyzed by Western blot. (**c**) Apoptotic cells were quantified by histogram (high power fields at × 400 magnification). (**d**) Quantitative analysis of RhoA. (**e**) Expression of IL-17 was analyzed by ELISA. Values were means ± SD, n = 6. $*P < 0.05$, $**P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$.

3.3. Effect of combination with fasudil and SR1001 on pancreatic injury, expression of RhoA/ROCK1 pathway and differentiation of Th17 cells

A histological examination showed that significant pathological injury exhibited in the model group compared to sham group. In contrast, the treatment with fasudil or SR1001 significantly inhibited the pancreatic injury, which was further inhibited by their combination treatment. Also, the treatment with fasudil or SR1001 inhibited the apoptosis of pancreatic cells (Fig. 3a, b, d, e) and reduced the amylase activity (Fig. 3c), which was further inhibited by their combination treatment. Furthermore, the combination treatment markedly inhibited the Th17 cell differentiation and expression of IL-17 compared with the mono-treatment with fasudil or SR1001 (Fig. 4a, f, g). Next, we analyzed the levels of p-STAT3, RORγt, GEF-H1, RhoA and ROCK1 protein expressions in different groups. Western blot analysis showed that CLP-induced increment of p-STAT3 (Fig. 4b, d), RORγt (Fig. 3f and g, Fig. 4c, j), GEF-H1 (Fig. 4e, k), RhoA (Fig. 3h and i, Fig. 4e, l) and ROCK1 (Fig. 4e, m) protein expressions were blocked by fasudil or SR1001, and further blocked by their combination treatment. Results from immunohistochemistry indicated that expressions of RORγt and RhoA were promoted by CLP while inhibited by fasudil or SR1001, which was further inhibited by their combination treatment. These results indicated that the combination with fasudil and SR1001 showed the synergistic effects on pancreatic injury.

3.4. Effect of combination with fasudil and SR1001 on apoptosis of pancreatic acinar cells in vitro

In coculture, $CD4^+$ T cells (Th17 polarization) promoted the apoptosis in pancreatic acinar cells, which was further promoted after culturing with LPS in vitro (Fig. 5a, c). The results revealed a remarkable decrease of apoptosis in pancreatic acinar cells culturing with fasudil or SR1001, which was further inhibited by their combination culture. Further data showed that expressions of RhoA and IL-17 were promoted by coculture with $CD4^+$ T cells (Th17 polarization) and LPS, the combination culture markedly inhibited the expressions of RhoA and IL-17 compared with the mono-culture with fasudil or SR1001 (Fig. 5b, d, e). These results indicated that the synergistic effects of fasudil and SR1001 on reducing the apoptosis of pancreatic acinar cells in vitro.

3.5. Effect of combination with fasudil and SR1001 on differentiation of Th17 cells in vitro

To determine the effect of combination with fasudil and SR1001 on the differentiation of Th17 cells in vitro. The coculture systems

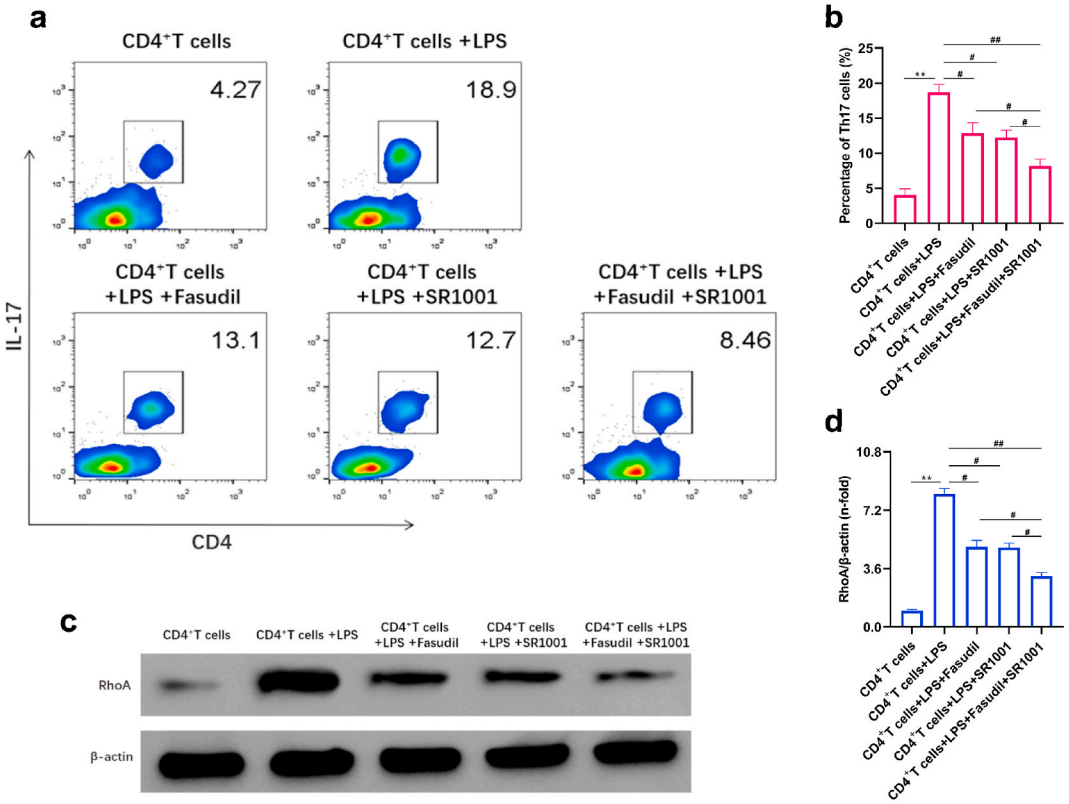

Fig. 6. Combination of Fasudil and SR1001 inhibited the differentiation of Th17 cells and expression of RhoA in vitro. (**a**) Percentage of Th17 cells in vitro. (**b**) Percentage of Th17 cells was analyzed by histogram. (**c**) Expression of RhoA analyzed by Western blot. (**d**) Quantitative analysis of RhoA. Values were means ± SD, n = 6. $^{**}P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$.

of $CD4^+$ T cells and LPS treated with fasudil and/or SR1001 in vitro were constructed. In the co-culture system, LPS remarkably upregulated the differentiation of Th17 cells, which could be significantly downregulated by fasudil or SR1001, and further downregulated by their combination treatment (Fig. 6a and b). Further analysis indicated that the expression of RhoA in vitro was promoted by LPS while inhibited by fasudil or SR1001, which was further inhibited by their combination treatment (Fig. 6c and d). These data further demonstrated that the synergistic effects of fasudil and SR1001 on differentiation of Th17 cells in vitro.

4. Discussion

This study provided strong evidence that combination of fasudil with SR1001 has a synergistic effect on ameliorating SPI. This concept is based on the following findings: 1) Fasudil alleviated pancreatic injury and inhibited the differentiation of Th17 cells in mice induced by CLP. 2) The protective role of SR1001 on pancreatic injury induced by CLP might be mediated by the RhoA/ROCK1 pathway. 3) The combination with fasudil and SR1001 showed the synergistic effects on pancreatic injury in vivo and in vitro.

SPI: Sepsis is a devastating clinical condition and leading cause of death in critically ill patients [26]. Pancreatic injury is responsible for higher mortality and poorer outcomes in septic patients [27]. In our study, there were remarkable pancreatic injury induced by sepsis, including edema, hyperemia, vacuolization and necrosis, which were observed in the pancreatic tissue by using histopathological analysis. Moreover, The level of serum amylase is an important biomarker used to detect pancreatic injury. Our recent clinical study also showed that serum amylase level could serve as independent biomarker to predict pancreatic injury in critically ill children [28]. In this study, amylase activity was dramatically increased in the model group, which could be inhibited in the treatment group. However, the pathogenesis of pancreatic injury in sepsis remains to be further elucidated.

RhoA/ROCK in SPI: RhoA is one of the most studied members of the Rho-GTPases family, which activates a downstream effector ROCK. A recent study by Li and colleagues demonstrated that RhoA/ROCK was a crucial pathway to induce pulmonary endothelial cell dysfunction in sepsis, which inhibited by hydrogen treatment [29]. In other published data, RhoA/ROCK pathway also participated in sepsis-induced acute lung, kidney injury and intestinal injury of animal models [30,31]. In this current research, we also found the expression of RhoA/ROCK1 was significantly increased in the model group, while inhibition of RhoA/ROCK1 pathway could reverse pancreatic injury. Thus, these data may indicate a potential therapeutic target for treating sepsis. GEF-H1 is a unique guanine nucleotide exchange factor that activates RhoA/ROCK pathway [32]. Our findings indicated that GEF-H1 was promoted in SPI, which suggested that the expression of RhoA/ROCK1 was regulated through GEF-H1.

Th17/IL-17 in SPI: Sepsis is characterized with imbalanced inflammatory reaction and T lymphocyte dysregulation [13,33]. $CD4^+$ T cell dysfunction plays a crucial role in humoral and cellular immune responses in sepsis [34]. It has also been demonstrated that abnormal Th17 cell is involved in sepsis-associated acute kidney injury, lung capillary leak, intestinal injury and lung injury [35–38]. Interleukin-17 (IL-17) is secreted by the Th17 subset of $CD4^+$ T cell, which exerts potent pro-inflammatory effects [39,40]. Here, we presented evidence that Th17/IL-17 significantly increased compared to those of control group, which suggested that Th17/IL-17 contributed to SPI. The retinoic acid-related orphan nuclear receptor (ROR) belongs to a family of ligand regulated transcription factors, which is composed of RORα, RORβ and RORγ. The expression of RORγt has been detected in Th17 cells, and it has been shown to play a critical role in differentiation of Th17 cells. It is well known that signal transducer and activator of transcription 3 (STAT3) is a key transcription factor to regulate RORγt expression and involved in a variety of cellular biologic functions [41,42]. In the present study, consistent with the changes of Th17/IL-17, the expressions of p-STAT3 and RORγt were significantly increased in the model group, which be inhibited in the treatment group. Thus, our study demonstrated that Th17 responses in SPI was regulated by phosphorylation of STAT3.

Fasudil in SPI: Numerous studies demonstrated RhoA/ROCK pathway-mediated regulation of Th17 cell differentiation and function. For example, Zhang et al. found RhoA regulated airway inflammation of asthma by controlling Th17 cell differentiation [43]. Additionally, inhibition of RhoA/ROCK pathway by fasudil ameliorated hepatic fibrosis and downregulated Th17 cells responses [44]. Oxymatrine restrained the differentiation of Th17 cells and exhibited the protective effects in acute intestinal inflammation in mice model via inhibiting the RhoA/ROCK pathway [45]. Another study also indicated that RhoA deficiency impaired Th17 cell differentiation and effector cytokine IL-17 secretion, which alleviated house dust mite-induced allergic airway inflammation [46]. As fasudil is selective ROCK inhibitor [47], we further investigated the effect of RhoA/ROCK pathway on Th17 cell response in model. Our data revealed that fasudil suppressed Th17 cell response to ameliorate SPI by inhibiting the expression of RhoA-ROCK1 pathway. These findings indicated that the RhoA/ROCK pathway was a key regulator of Th17 cell differentiation.

SR1001 in SPI: Interestingly, several previous studies have shown that IL-17 induced the activation of RhoA/ROCK pathway. For instance, IL-17 increased airway contractility of severe asthma via activating the RhoA/ROCK pathway [48]. Furthermore, IL-17 directly upregulated the expression of RhoA/ROCK, which resulted in an induction of the bronchial smooth muscle hyperresponsiveness of asthma [49]. In the study by Chen et al., IL-17 exhibited a proangiogenesis effect on human choroidal endothelial cells in vitro by upregulating activated RhoA protein [50]. To further investigate the effect of IL-17 on expression of RhoA/ROCK, the model was treated by intraperitoneal injection of SR1001, which could abrogate the development of Th17 cell and secretion of cytokine IL-17 [51]. Our findings confirmed for the first time that SR1001 treatment inhibited the expression of RhoA/ROCK and alleviated pancreatic injury. These results indicated that IL-17 was an important pro-inflammatory cytokine involved in SPI through inducing expression of RhoA/ROCK. This study further confirmed the interplay between RhoA/ROCK and Th17/IL-17 in SPI.

Combination treatment in SPI: Currently, the etiology of sepsis is not well understood and no effective therapeutic interventions is available for sepsis. If not promptly treated, sepsis rapidly progresses and develops septic shock or multiple organ failure [52]. Broad-spectrum antibiotics can help control the infection but cannot prevent the cytokine storm and organ damage. Therefore, novel therapeutic agents targeting new mechanisms in SPI are urgently needed. Fasudil, the specific RhoA/ROCK inhibitor, which has been

extensively explored in numerous studies of sepsis, including sepsis-induced cerebral and cognitive injury, acute lung injury, and acute renal injury [53–55]. It is also known that SR1001 specifically inhibits the development of Th17 cells and IL-17 expression [56,57]. Recent findings by Wang and colleagues indicated that SR1001 significantly alleviated acute pancreatitis of the mouse model by selectively decreased the Th17/IL-17 [58]. Similarly, our data also confirmed the therapeutic effect of SR1001 on SPI. Notably, the combination treatment with fasudil and SR1001 has a significant efficacy in SPI, which was more effective than each mono-treatment. Fasudil as a clinically approved RhoA/ROCK inhibitor has been investigated in some clinical studies. Fasudil may be a promising clinical therapeutic drug for the prevention and treatment of SPI. SR1001 as a novel class of compound has potential utility in the treatment of SPI. Our results will pave a novel direction for future clinic studies of combination treatment in SPI.

Limitations: Several limitations should be mentioned for the current study. First, the CLP-induced mice model might not fully represent sepsis in humans. Thus, the effectiveness of the combination treatment should be carefully evaluated in other animal models (such as lipopolysaccharide (LPS)-induced sepsis) with different etiologies. However, while LPS model is representative of Gram-negative bacteria, the method does not account for the effect of Gram-positive and polymicrobial sepsis, which lacks the infectious component found in human sepsis. Moreover, therapies developed based on the promising results obtained using CLP model could be difficultly translated to the clinic. Second, we used an animal model intervened with fasudil and SR1001, rather than a gene RhoA or IL-17 knockout model. Gene knockout is the complete deletion of genes, while inhibitors only inhibit gene expression. Third, male mice were used in our experiment. However, there were sex-specific differences in sepsis model. For example, sex differences of cardiac function and intestinal microbiota were found in sepsis model. Sex-specific differences in the outcome of infectious diseases and sepsis appear to favor females. Thus, the conditional gene knockout model and model of female mice may be established in the subsequent studies.

5. Conclusions

Up to now, there are no specific and effective drugs in clinical practice of sepsis. The efficacy of monotherapy is often disappointing in practice due to an inadequate anti-inflammatory response, combination therapy is a promising mean to improve therapeutic efficacy by exploiting synergistic interactions in different pathways. Taken together, we undertook this study, for the first time, that the combination of fasudil with SR1001 has a synergistic effect on ameliorating SPI in C57BL/6 mice. This study identifies the combination of fasudil with SR1001 as a treatment for SPI and provides a new therapeutic theory scheme for SPI patients.

Author contribution statement

Conceived and designed the experiments: Pingping Liu and Zhenghui Xiao. Performed the experiments: Pingping Liu, Jiaotian Huang and Cheng Li. Analyzed and interpreted the data: Pingping Liu and Zhenghui Xiao. Contributed reagents, materials, analysis tools or data: Xiulan Lu and Xinping Zhang. Wrote the paper: Pingping Liu and Cheng Li.

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Data availability statement

Data included in article/supp. material/referenced in article.
Supplementary content related to this article has been publish online at [URL].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20118.

References

[1] I. Rumienczyk, et al., Multi-organ transcriptome dynamics in a mouse model of cecal ligation and puncture-induced polymicrobial sepsis, J. Inflamm. Res. 14 (2021) 2377–2388.

[2] S.F. Assimakopoulos, et al., Fecal microbiota transplantation and hydrocortisone ameliorate intestinal barrier dysfunction and improve survival in a rat model of cecal ligation and puncture-induced sepsis, Shock 55 (5) (2021) 666–675.
[3] Z.L. Qiu, et al., Mortality and serum hepcidin are associated with persistent and transient acute kidney injury in septic patients, Clin. Nephrol. 95 (6) (2021) 303–311.
[4] L. Cao, et al., Xuebijing protects against septic acute liver injury based on regulation of GSK-3 beta pathway, Front. Pharmacol. 12 (2021), 627716.
[5] H. Ren, W. Mu, Q. Xu, miR-19a-3p inhibition alleviates sepsis-induced lung injury via enhancing USP13 expression, Acta Biochim. Pol. 68 (2) (2021) 201–206.
[6] Z. Liu, et al., Innate immune molecule surfactant protein D attenuates sepsis-induced acute pancreatic injury through modulating apoptosis and NF-kappaB-mediated inflammation, Sci. Rep. 5 (2015), 17798.
[7] National Research Coordination Group of the Critically Ill Children Secondary Pancreatic, I., [Relationship between changes of increased amylase or lipase levels and pancreas injury in critically ill children], Zhonghua Er Ke Za Zhi 54 (9) (2016) 658–664.
[8] J. Cai, et al., Effect of bone marrow mesenchymal stem cells on RhoA/ROCK signal pathway in severe acute pancreatitis, Am J Transl Res 11 (8) (2019) 4809–4816.
[9] Q. Zhang, et al., Signaling pathways and targeted therapy for myocardial infarction, Signal Transduct Target Ther 7 (1) (2022) 78.
[10] J. Han, et al., Unfractionated heparin attenuates lung vascular leak in a mouse model of sepsis: role of RhoA/Rho kinase pathway, Thromb. Res. 132 (1) (2013) e42–e47.
[11] S. Preau, et al., Endotoxemia engages the RhoA kinase pathway to impair cardiac function by altering cytoskeleton, mitochondrial fission, and autophagy, Antioxid Redox Signal 24 (10) (2016) 529–542.
[12] P. Liu, et al., Baicalin suppresses Th1 and Th17 responses and promotes Treg response to ameliorate sepsis-associated pancreatic injury via the RhoA-ROCK pathway, Int Immunopharmacol 86 (2020), 106685.
[13] W. He, et al., Immune cell number, phenotype, and function in the elderly with sepsis, Aging Dis 12 (1) (2021) 277–296.
[14] Y. Liu, X. Wang, L. Yu, Th17, rather than Th1 cell proportion, is closely correlated with elevated disease severity, higher inflammation level, and worse prognosis in sepsis patients, J. Clin. Lab. Anal. 35 (5) (2021), e23753.
[15] G. Li, et al., Increased Th17 and Th22 cell percentages predict acute lung injury in patients with sepsis, Lung 198 (4) (2020) 687–693.
[16] A. Saoudi, et al., Rho-GTPases as key regulators of T lymphocyte biology, Small GTPases 5 (2014).
[17] H. Nguyen, et al., Interleukin-17 causes Rho-kinase-mediated endothelial dysfunction and hypertension, Cardiovasc. Res. 97 (4) (2013) 696–704.
[18] Q.J. Han, et al., Fasudil prevents liver fibrosis via activating natural killer cells and suppressing hepatic stellate cells, World J. Gastroenterol. 27 (24) (2021) 3581–3594.
[19] J. Rao, et al., The RhoA/ROCK pathway ameliorates adhesion and inflammatory infiltration induced by AGEs in glomerular endothelial cells, Sci. Rep. 7 (2017), 39727.
[20] L. Zhang, et al., Protective effects of a Rho kinase inhibitor on paraquat-induced acute lung injuries in rats, Inflammation 41 (6) (2018) 2171–2183.
[21] L.D. Maston, et al., Central role of T helper 17 cells in chronic hypoxia-induced pulmonary hypertension, Am. J. Physiol. Lung Cell Mol. Physiol. 312 (5) (2017) L609–L624.
[22] Y. Ge, et al., IL-38 alleviates inflammation in sepsis in mice by inhibiting macrophage apoptosis and activation of the NLRP3 inflammasome, Mediators Inflamm 2021 (2021), 6370911.
[23] L. Li, et al., CB2R deficiency exacerbates imiquimod-induced psoriasiform dermatitis and itch through the neuro-immune pathway, Front. Pharmacol. 13 (2022), 790712.
[24] D. Droppa-Almeida, et al., Peptide vaccines designed with the aid of immunoinformatic against Caseous Lymphadenitis promotes humoral and cellular response induction in mice, PLoS One 16 (11) (2021), e0256864.
[25] Z. Luo, et al., Ropivacaine mesylate exerts neurotoxicity via up-regulation of Fas/FasL expression in rat pheochromocytoma PC12 cells, Am J Transl Res 11 (3) (2019) 1626–1634.
[26] F. Winterbottom, Treating sepsis in patients with heart failure, Crit Care Nurs Clin North Am 34 (2) (2022) 165–172.
[27] Y. Li, L. Suo, J. Zhang, [Role of autophagy in pancreatic injury in sepsis], Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 32 (4) (2020) 504–507.
[28] P. Liu, et al., Serum amylase and lipase for the prediction of pancreatic injury in critically ill children admitted to the PICU, Pediatr. Crit. Care Med. 22 (1) (2021) e10–e18.
[29] Y. Li, et al., Hydrogen treatment prevents lipopolysaccharide-induced pulmonary endothelial cell dysfunction through RhoA inhibition, Biochem. Biophys. Res. Commun. 522 (2) (2020) 499–505.
[30] X.X. Yan, et al., Protective effect of pantoprazole against sepsis-induced acute lung and kidney injury in rats, Am J Transl Res 11 (8) (2019) 5197–5211.
[31] Z. Li, et al., Naringin attenuates MLC phosphorylation and NF-kappaB activation to protect sepsis-induced intestinal injury via RhoA/ROCK pathway, Biomed. Pharmacother. 103 (2018) 50–58.
[32] W. Pan, et al., The regulatory subunit PPP2R2A of PP2A enhances Th1 and Th17 differentiation through activation of the GEF-H1/RhoA/ROCK signaling pathway, J. Immunol. 206 (8) (2021) 1719–1728.
[33] W. Zhang, Z. Wang, [Research progress on the dyshomeostasis of effector T cell subsets related to immunosuppression in sepsis], Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 34 (1) (2022) 95–99.
[34] S. Huang, et al., Tim-3 regulates sepsis-induced immunosuppression by inhibiting the NF-kappaB signaling pathway in CD4 T cells, Mol. Ther. 30 (3) (2022) 1227–1238.
[35] C. Luo, et al., Mesenchymal stem cells attenuate sepsis-associated acute kidney injury by changing the balance of Th17 cells/Tregs via Gal-9/Tim-3, Curr. Stem Cell Res. Ther. 18 (4) (2023) 540–-550.
[36] M. Shen, et al., Taxifolin ameliorates sepsis-induced lung capillary leak through inhibiting the JAK/STAT3 pathway, Allergol. Immunopathol. 50 (2) (2022) 7–15.
[37] C.L. Yeh, et al., Intravenous Calcitriol Administration Modulates Mesenteric Lymph Node CD4(+) T-Cell Polarization and Attenuates Intestinal Inflammation in Obese Mice Complicated with Polymicrobial Sepsis, JPEN J Parenter Enteral Nutr, 2021.
[38] H. Xia, et al., Maresin1 ameliorates acute lung injury induced by sepsis through regulating Th17/Treg balance, Life Sci. 254 (2020), 117773.
[39] Q. Chen, et al., The emerging roles of T helper cell subsets and cytokines in severe neutrophilic asthma, Inflammation 45 (3) (2022) 1007–1022.
[40] J.Y. Mimpen, et al., Interleukin-17 cytokines and receptors: potential amplifiers of tendon inflammation, Front. Bioeng. Biotechnol. 9 (2021), 795830.
[41] A. Capone, E. Volpe, Transcriptional regulators of T helper 17 cell differentiation in Health and autoimmune diseases, Front. Immunol. 11 (2020) 348.
[42] A.U. Anka, et al., Biological and clinical significance of T helper 17 cell deficiency: insight into monogenic defects, Eur Ann Allergy Clin Immunol 53 (4) (2021) 149–160.
[43] Y. Zhang, et al., RhoA/Rho-kinases in asthma: from pathogenesis to therapeutic targets, Clin Transl Immunology 9 (5) (2020), e01134.
[44] W. Zhou, et al., Inhibition of rho-kinase downregulates Th17 cells and ameliorates hepatic fibrosis by schistosoma japonicum infection, Cells 8 (10) (2019).
[45] Y. Wang, et al., Protective effects of oxymatrine against DSS-induced acute intestinal inflammation in mice via blocking the RhoA/ROCK signaling pathway, Biosci. Rep. 39 (7) (2019).
[46] J.Q. Yang, et al., Ablation of RhoA impairs Th17 cell differentiation and alleviates house dust mite-triggered allergic airway inflammation, J. Leukoc. Biol. 106 (5) (2019) 1139–1151.
[47] F. Abedi, et al., Acute lung injury: the therapeutic role of Rho kinase inhibitors, Pharmacol. Res. 155 (2020), 104736.
[48] V. Fong, et al., Arhgef12 drives IL17A-induced airway contractility and airway hyperresponsiveness in mice, JCI Insight 3 (21) (2018).
[49] Y. Chiba, et al., Interleukin-17A directly acts on bronchial smooth muscle cells and augments the contractility, Pharmacol. Rep. 69 (3) (2017) 377–385.
[50] Y. Chen, et al., Interleukin-17 induces angiogenesis in human choroidal endothelial cells in vitro, Invest. Ophthalmol. Vis. Sci. 55 (10) (2014) 6968–6975.
[51] C. Li, et al., Protective effects of resveratrol and SR1001 on hypoxia-induced pulmonary hypertension in rats, Clin. Exp. Hypertens. 42 (6) (2020) 519–526.
[52] L. Wei, et al., Circular RNAs in the pathogenesis of sepsis and their clinical implications: a narrative review, Ann Acad Med Singap 51 (4) (2022) 221–227.

[53] Z. Jianjun, et al., Exploring the beneficial role of ROCK inhibitors in sepsis-induced cerebral and cognitive injury in rats, Fundam. Clin. Pharmacol. 35 (5) (2021) 882–891.
[54] Y. Wang, et al., Role of the Rho/ROCK signaling pathway in the protective effects of fasudil against acute lung injury in septic rats, Mol. Med. Rep. 18 (5) (2018) 4486–4498.
[55] J. Fu, L. Chang, Fabrication of fasudil hydrochloride modified graphene oxide biocomposites and its defensive effect acute renal injury in septicopyemia rats, J. Photochem. Photobiol., B 186 (2018) 125–130.
[56] L.A. Solt, et al., Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand, Nature 472 (7344) (2011) 491–494.
[57] S. Wang, et al., Blockade of T helper 17 cell function ameliorates recurrent Clostridioides difficile infection in mice, Acta Biochim. Biophys. Sin. 53 (10) (2021) 1290–1299.
[58] J. Wang, et al., RORgammat inhibitor SR1001 alleviates acute pancreatitis by suppressing pancreatic IL-17-producing Th17 and gammadelta-T cells in mice with ceruletide-induced pancreatitis, Basic Clin. Pharmacol. Toxicol. 129 (5) (2021) 357–368.