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Knockdown of PHLDA1 alleviates sepsis-induced acute lung injury by downregulating NLRP3 inflammasome activation

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KEYWORDS

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Abstract

Objective: To investigate the regulatory mechanism of pleckstrin homology-like domain, family A, member 1 (PHLDA1) in sepsis-induced acute lung injury (ALI).

Method: Mice model of sepsis were established by cecal ligation and puncture (CLP). The expression of PHLDA1 was reduced by injecting short hairpin RNA (shRNA)-PHLDA1 into the tail vein. The levels of PHLDA1, pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), IL-1 β , IL-18, super-oxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH), molecular mechanism related to pyroptosis, such as caspase 1, adaptor apoptosis-associated speck-like protein containing a CARD (ASC), and gasdermin D (GSDMD)-N, and nucleotide oligomerization domain (NOD)-like receptor family pyrin domain-containing 3 (NLRP3) were tested by Western blot analysis, quantitative real-time polymerase chain reaction, and enzyme-linked-immunosorbent serologic assay. Pathological changes in lung tissues were examined by hematoxylin and eosin staining. Wet-dry weight ratio of lung tissues was observed.

Results: The expression of PHLDA1 was up-regulated in lung tissues from CLP-induced septic mice. Knockdown of PHLDA1 could reduce lung injury and wet-dry weight ratio in mice with sepsis-induced ALI. Moreover, silencing of PHLDA1 decreased the expressions of IL-1 β , TNF- α , IL-18, IL-6, and MDA but increased SOD and GSH expressions in CLP-induced septic mice. The expressions of NLRP3, GSDMD-N, ASC, and caspase 1 were decreased by PHLDA1 silencing.

Conclusion: Knockdown of PHLDA1 inhibited lung inflammation and pyroptosis in mice with sepsis-induced ALI by down-regulating NLRP3.

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Introduction

Sepsis is defined as a life-threatening organ dysfunction caused by host's dysfunctional response to infection, and is a major health care problem with high global mortality rates.¹ According to reports, the lung is one of the vulnerable target organs in pathological changes, and the mortality rate of sepsis-induced acute lung injury (ALI) is as high as nearly 40%.²

As an important component of innate immunity, the inflammasome nucleotide oligomerization domain (NOD)-like receptor family pyrin domain-containing 3 (NLRP3) plays a significant role in the immune response and disease occurrence.³ NLRP3 can be activated by various types of pathogens or danger signals, which may lead to caspase 1-mediated activation of protein hydrolysis of interleukin-1 β (IL-1 β) and IL-18 and induce pyroptosis.⁴ Thus, NLRP3 could provide a new target for various inflammatory diseases treatment. Research has found that NLRP3 is one of the key mechanisms leading to sepsis-induced ALI.⁵ Blocking the activation of NLRP3 may have beneficial effects on lung diseases, thus targeted inhibition of NLRP3 activation in lung tissues could be a possible way for ALI prevention.^{6,7}

Pleckstrin homology like domain, family A, member 1 (PHLDA1) is also known as TDAG51. It is widely expressed in various tissues and participates in physiological processes, such as cell signal regulation, cell adhesion, and apoptosis, and its expression is associated with the occurrence and development of various diseases.⁸ PHLDA1 inhibits the production of pro-inflammatory cytokines by interacting with Tollip.⁹ PHLDA1 promotes the growth of glioblastoma cells by maintaining the activated state of Ras proteins.¹⁰ In addition, TDAG51 was reported to be up-regulated in *lipopolysaccharide* (LPS)-stimulated RAW264.7 cells.¹¹ PHLDA1 expression was up-regulated in a mouse lung contusion model, and inhibition of PHLDA1 expression could inhibit lung injury, neutrophil infiltration, and production of inflammatory factors.¹² PHLDA1 blocker effectively alleviates ischemia/reperfusion-induced brain injury by altering M1/M2 polarization of microglia and inhibiting NLRP3 inflammasome activation.¹³ However, the effect of PHLDA1 in sepsis-induced lung injury is still unclear.

In this research, we investigated whether PHLDA1 plays a role in sepsis-induced ALI by detecting PHLDA1 expression in sepsis-induced ALI mice and its regulation of NLRP3 expression.

Methods

Experimental animals

C57BL/6 mice (8-10 weeks old, male) were obtained from Guangdong Medical Laboratory Animal Center (Foshan, Guangdong, China), and divided into the following four groups (n = 6): control, cecal ligation puncture (CLP), CLP+short hairpin RNA (shRNA)-PHLDA1 (sh-PHLDA1), and CLP+shRNA-non-coding (sh-NC). Mice in CLP+sh-PHLDA1 and CLP+sh-NC groups were treated with sh-PHLDA1 (AmyJet Scientific, Wuhan, Hubei, China) and sh-NC, respectively, by tail vein injection (1 mg/kg body

weight). Ethical approval was obtained from the Ethics Committee of Nanjing Medical University and all procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals*.

Cecal Ligation Puncture model

As mentioned earlier, CLP was used to establish sepsis model.¹⁴ Briefly, mice were fasted for 12 h prior to operation. Subsequently, 10% chloral hydrate solution (3.5 mL/1 kg body weight) was used to anesthetize mice through intraperitoneal route. The skin was disinfected and the cecum was exposed by making a 1-cm incision in the middle of the abdomen; a 4-0 silk suture was used to ligate the cecum. The ligated cecum was punctured using a 21-gauge needle. To induce infection, a small amount of intestinal content was squeezed out, and then the cecum was reset and incision was closed. In mice of the control group, the abdomens were simply opened and incisions were closed. After 16 h of operation, the lungs of mice were removed and washed with cold physiological saline. The left lung lobe was taken out for histological analysis. Other lung tissues were stored at -80°C.

Wet-dry weight ratio of lung tissues

After dissection, lung tissues were weighed promptly. This weight was recorded as wet weight of lung tissues. Then, the lung tissues were placed in an oven at 60°C for 48 h and dry lungs were weighed. This weight was recorded as dry weight of lung tissues. Finally, the ratio of wet-dry weight of lung tissues was calculated.

Hematoxylin and eosin (H&E) staining and lung injury score

For histological analyses, lung tissues were dehydrated, embedded in paraffin, sectioned, and stained with H&E. A semi-quantitative scoring system was used for evaluating the severity of lung injury.¹⁵ Hemorrhage, edema, inflammation, and alveolar septal thickening were recorded on a scale of 0-4. Hemorrhage and edema score = 0 (no alveolar involved), score = 1 (mild; <10% alveolar involvement), score = 2 (moderate; 10-30% alveolar involvement), score = 3 (severe; 30-50% alveolar involvement), score = 4 (very severe; >50% alveolar involvement). Inflammation score: count the number of inflammatory cells as 100 \times field. Cells were counted randomly in 5-7 fields in each slice. Alveolar septal thickening score: the vertical distance at the thickest part of the alveolar septum was measured using a 400 \times magnification photograph and divided by 400, with the following evaluations. Score = 0 (absent; alveolar septal thickness < 15 μ m); score = 1 (mild; alveolar septal thickness 15-30 μ m), score = 2 (moderate; alveolar septal thickness 30-45 μ m), score = 3 (severe; alveolar septal thickness 45-60 μ m), and score = 4 (extremely severe; alveolar septal thickness > 60 μ m). Average scores for alveolar septal thickening, hemorrhage, edema, and inflammation in each group were calculated. An overall assessment of these four injury parameters was represented as the final score.

Western blot analysis

Total proteins were obtained from lung tissues by using radioimmunoprecipitation analysis (RIPA) buffer (Invitrogen, Carlsbad, CA). BCA protein assay kit (Invitrogen) was used to measure protein concentrations. The blots were observed using the enhanced chemiluminescence (ECL) detection system.^{16,17} Primary antibodies (Abcam, Cambridge, UK) used in the experiments included antibodies against NLRP3 (1:1000), PHLDA1 (1:10,000), adaptor apoptosis-associated speck-like protein containing a CARD (ASC; 1:1000), gasdermin D (GSDMD-N; 1:1000), caspase 1 (1:1000), IL-1 β (1:1000), IL-18 (1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs from lung tissues were obtained by Trizol reagent (Invitrogen), and reversely transcribed into complementary DNA (cDNA) by reverse transcriptase kit (Invitrogen). SYBR Green PCR Master Mix (Takara, Otsu, Japan) on an ABI StepOnePlus RT-PCR system (Applied Biosystems, Foster City, CA, USA) was used to carry out qRT-PCR. The relative expression of PHLDA1 was measured by $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 1. Each sample measure was performed in triplicate.

Enzyme-linked immunosorbent serologic assay (ELISA)

Following the instructions, the levels of tumor necrosis factor α (TNF- α), IL-6, IL-1 β , IL-18, malondialdehyde (MDA), super-oxide dismutase (SOD), and glutathione (GSH) in lung tissues were tested using their corresponding ELISA kits (R&D Systems, Minnesota, USA). The absorbance was measured by an enzyme-labeled colorimeter at 450 nm.

Statistical analysis

All data were analyzed with SPSS 20.0 (SPSS, Chicago, IL, USA) and presented as mean \pm standard deviation (SD). One-way ANOVA followed by Bonferroni post hoc test and *t*-test was used to evaluate statistical significance. All assays were performed in triplicate, and $P < 0.05$ was considered as statistically significant.

Table 1 List of primer sequences used in the study.

Name	Primer	Sequence (5'-3')
PHLDA1	Forward	5'-TCATCCACCACTCCAG-3'
	Reverse	5'-ATGCACTCTCCCACCTCC-3'
GAPDH	Forward	5'-CCAGGTGGTCTCCTGACTT-3'
	Reverse	5'-GTTGCTGTAGCCAAATTCGTTGT-3'

Results

PHLDA1 was highly expressed in CLP-induced septic mice

In order to verify whether PHLDA1 was involved in sepsis-induced ALI, we discovered the expression of PHLDA1 in lung tissues of CLP-induced septic mice. The results of PCR (Figure 1A) and Western blot analysis (Figures 1B and 1C) showed that PHLDA1 messenger RNA (mRNA) and PHLDA1 protein expressions were significantly increased in lung tissues of mice in CLP group.

Knockdown of PHLDA1 alleviated lung injury in CLP-induced septic mice

Here, the experiments of function loss were performed to determine the effect of PHLDA1 on sepsis-induced ALI. Mice were treated with sh-PHLDA1 or shRNA-NC through tail vein injection. As shown in Figure 2A, PHLDA1 protein expression was remarkably down-regulated in lung tissues of mice in the CLP+sh-PHLDA1 group. H&E staining results revealed that silencing PHLDA1 significantly alleviated lung injury in CLP-induced septic mice (Figures 2B and 2C). The wet-dry ratio of lung tissues in the CLP+sh-PHLDA1 group was lower than that in the CLP and CLP+sh-NC groups (Figure 2D).

Silencing of PHLDA1 alleviated lung inflammation in CLP-induced septic mice

In this experiment, we examined the role of PHLDA1 on lung inflammation in CLP-induced septic mice. ELISA showed that the expressions of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β , and IL-18) were significantly reduced in the CLP+sh-PHLDA1 group, compared to the CLP and CLP+sh-NC groups (Figure 3A). Additionally, MDA expression was decreased, but the expressions of SOD and GSH were increased in the CLP+sh-PHLDA1 group (Figure 3B).

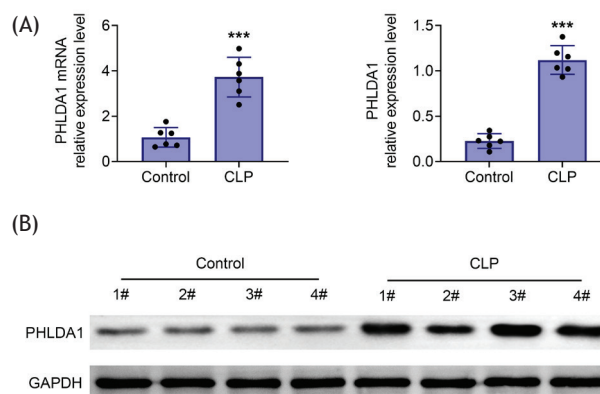


Figure 1 PHLDA1 expression in CLP-induced septic mice. (A) PHLDA1 mRNA expression was measured using qRT-PCR. (B and C) PHLDA1 protein expression was observed by Western blot analysis. *** $P < 0.05$ versus control. PHLDA1: pleckstrin homology-like domain, family A, member 1; CLP: cecal ligation and puncture.

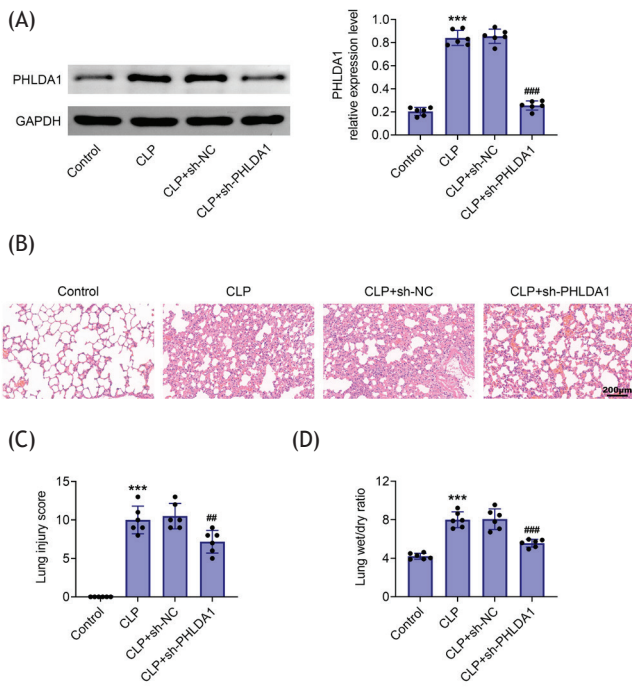


Figure 2 Effect of PHLDA1 knockdown on lung injury in CLP-induced septic mice. Mice were divided into control, CLP, CLP+sh-NC, and CLP+sh-PHLDA1 groups. (A) PHLDA1 protein expression in lung tissues. (B) Pathological changes in lung tissues were examined using H&E staining. (C) A semi-quantitative scoring system was used to score the severity of lung injury. (D) Lung wet-to-dry weight ratio. *** $P < 0.05$ versus control; ### $P < 0.05$ or ## $P < 0.05$ versus CLP+sh-NC. PHLDA1: pleckstrin homology-like domain, family A, member 1; CLP: cecal ligation and puncture; sh-PHLDA1: shRNA-PHLDA1.

Knockdown of PHLDA1 inhibited pyroptosis in lung tissues of CLP-induced septic mice by down-regulating NLRP3

The molecular expression related to pyroptosis in lung tissues was detected by Western blot analysis. Results revealed that the expressions of NLRP3, ASC, caspase 1, IL-18, IL-1 β , and GSDMD-N were reduced in the CLP+sh-PHLDA1 group (Figures 4A and 4B).

Discussion

The mortality rate of sepsis-induced ALI is relatively high in clinical patients. Hence, it is urgent to solve alleviation of sepsis-induced ALI.¹⁸ This study determined that PHLDA1 was involved in sepsis-induced ALI, and change in its expression could regulate lung inflammation and pyroptosis in mice with ALI induced by sepsis.

PHLDA1 was originally discovered as a member of oncogene family, and it participated in the occurrence, development, and metastasis of tumors through a variety of signal pathways.¹⁹ The low expression of PHLDA1 was discovered as related to the progression of breast cancer²⁰ and gastric cancer,²¹ while its high expression was associated with the migration and proliferation of colon cancer cells.²² In addition, PHLDA1 was highly expressed in ovarian cancer patients, and silencing of PHLDA1 could promote the death of human ovarian cancer cells.²³

In recent years, research has established that PHLDA1 is associated with the occurrence of lung diseases, such as lung cancer²⁴ and lung contusion.¹² Moreover, Gong et al. found that PHLDA1 was highly expressed in mice with sepsis, and knockout of PHLDA1 inhibited inflammation and

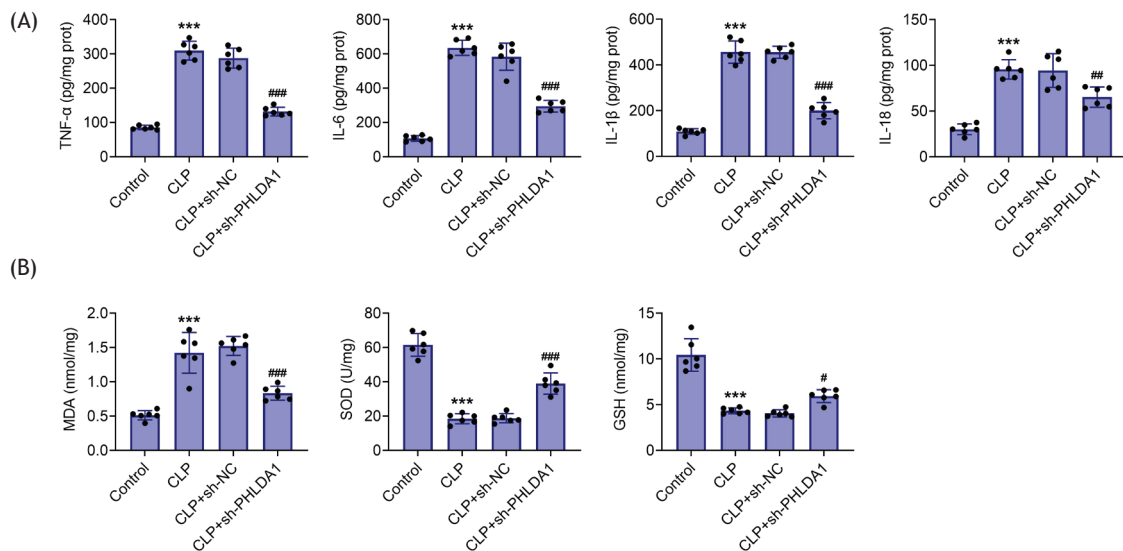


Figure 3 Effect of PHLDA1 knockdown on lung inflammation in CLP-induced septic mice. (A) The expressions of TNF- α , IL-6, IL-1 β , and IL-18 were examined using ELISA. (B) The expressions of MDA, SOD, and GSH were measured by their corresponding ELISA kits. *** $P < 0.05$ versus control; ### $P < 0.05$ or ## $P < 0.05$ or # $P < 0.05$ versus CLP+sh-NC. PHLDA1: pleckstrin homology-like domain, family A, member 1; CLP: cecal ligation and puncture; sh-PHLDA1: shRNA-PHLDA1; TNF- α : tumor necrosis factor- α ; IL-6: interleukin-6; MDA: malondialdehyde; SOD: super-oxide dismutase; GSH: glutathione.

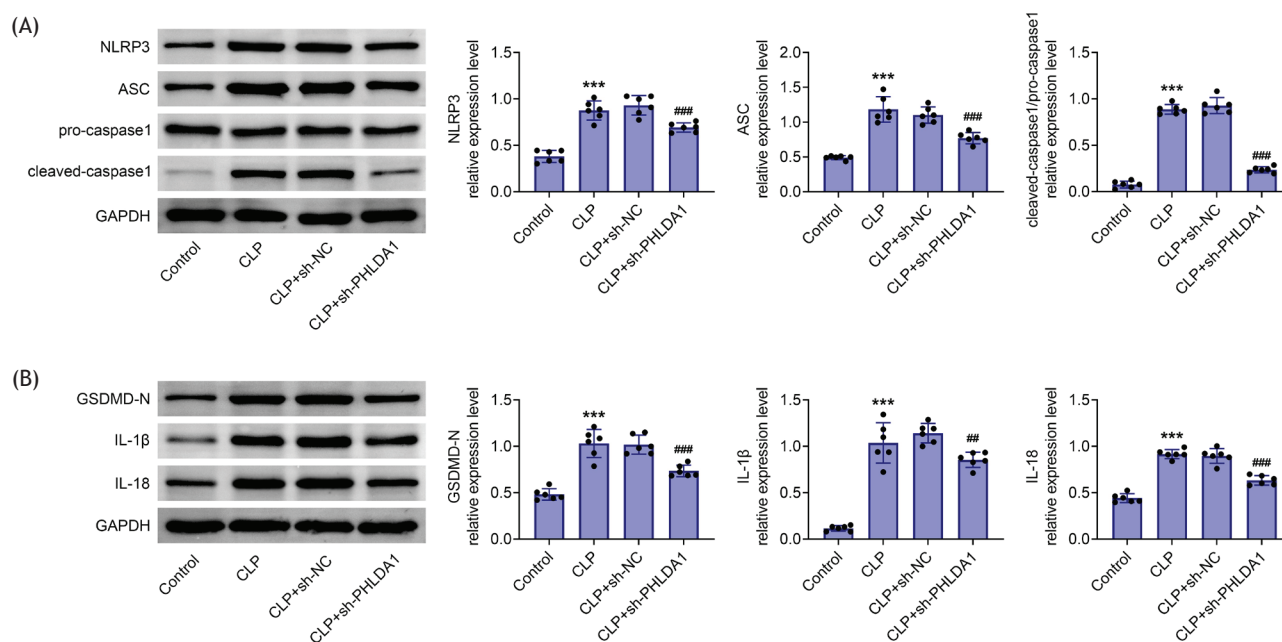


Figure 4 Effect of PHLDA1 knockdown on pyroptosis. (A) The expressions of NLRP3, ASC, and caspase 1 in lung tissues were measured by Western blot analysis. (B) The expressions of GSDMD-N, IL-1 β , and IL-18 in lung tissues were detected by Western blot analysis. *** $P < 0.05$ versus control; ### $P < 0.05$ or ## $P < 0.05$ versus CLP+sh-NC. PHLDA1: pleckstrin homology-like domain, family A, member 1; CLP: cecal ligation and puncture; sh-PHLDA1: shRNA-PHLDA1; ASC: adaptor apoptosis-associated speck-like protein containing a CARD; GSDMD: gasdermin D; NLRP3: NLR family, pyrin domain containing 3.

oxidative stress responses in mice with sepsis-induced acute kidney injury.²⁵ Similar findings were determined in this study. Our research results showed that PHLDA1 expression was extremely raised in the lung tissues of mice with sepsis-induced ALI. Silencing of PHLDA1 could improve lung injury and alleviate lung inflammation in mice with sepsis. We conducted an in-depth study to determine performance of PHLDA1 functions and found that PHLDA1 could regulate pyroptosis and was related to the regulation of NLRP3 expression.

NLRP3 inflammasome is an important member of the nucleotide oligomerization domain (NOD)-like receptor family, and plays a major role in cellular inflammatory response by regulating downstream expressions of pro-inflammatory factors, including IL-18 and IL-1 β .²⁶ Targeting the NLRP3 inflammasome pathway could effectively alleviate inflammatory response.²⁷ Researchers have found that both protopine²⁸ and morroniside²⁹ could alleviate LPS-induced inflammation and oxidative stress in inflammatory bowel disease by inhibiting NLRP3 and NF- κ B signaling pathways. Licochalcone B plays a protective role in peritonitis and hepatitis by specifically inhibiting NLRP3.³⁰ Knockout of NLRP3 can control the progression of acute myeloid leukemia.³¹

In addition, NLRP3 inflammasome also has a significant effect in the pathogenesis of rheumatic diseases, such as systemic lupus erythematosus and dermatomyositis/polymyositis.³² NLRP3 inflammasome was also found to be involved in sepsis.³³ Inhibiting NLRP3/IL-1 β axis could prevent sepsis-induced cardiomyopathy.³⁴ Platelets could lead to sepsis-induced kidney injury through the activation of NLRP3.³⁵ Down-regulation of macrophage migration

inhibitory factor could alleviate NLRP3 inflammatory mediated pyroptosis in sepsis-induced acute kidney injury.³⁶ We found that silencing of PHLDA1 inhibited pyroptosis by down-regulating NLRP3 in mice with sepsis-induced ALI. However, investigation on the mechanism of PHLDA1 is still in the early stage and has not been thoroughly studied in this paper. In the future research, we would delve into the mechanism of PHLDA1 regulating sepsis-induced ALI.

Conclusion

We discovered that PHLDA1 was highly expressed in mice with ALI caused by sepsis. Knockdown of PHLDA1 inhibited lung inflammation and pyroptosis in mice with sepsis-induced ALI by down-regulating NLRP3. This suggested that a decrease in PHLDA1 expression could alleviate ALI in sepsis. This provided a new strategy for treating sepsis-induced ALI.

Competing interests

The authors stated that they had no conflict of interest to disclose.

Consent to participate statement

Written informed consent was obtained from legally authorized representative(s) of anonymized patient information to be published in this article.

Data availability

The authors declare that all data supporting the findings of this study are available in the paper, and any raw data can be obtained from the corresponding author upon request.

Author Contributions

Lijun Meng and Chao Nan designed and carried out the study. Lijun Meng, Tijun Gu, Jinhai Wang, and He Zhang supervised collection, analysis, and interpretation of data. Lijun Meng and Chao Nan prepared and reviewed draft of the manuscript for publication. All authors read and approved the final manuscript.

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