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Sulforaphane Inhibits LPS-induced Macrophage PANoptosis via TLR4/NFκB Pathway: A Potential Therapeutic Strategy for Acute Lung Injury

Yanwei Wang,[1,*] Huifan Liu,[2,*] Yali Feng,[3,*] Shujuan Wu,[4] Jingxuan He[5] and Lei Cao[1]

[1]The first hospital of Hebei Medical University, Shijiazhuang, Hebei, China
[2]Department of Anesthesiology, Research Centre of Anesthesiology and Critical Care Medicine, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China
[3]Hebei Province Hospital of Traditional Chinese Medicine, Shijiazhuang, Hebei, China
[4]Department of Respiratory and Critical Care Medicine, Renmin Hospital of Wuhan University, Wuhan, Hubei, China
[5]General Hospital of the Yangtze River Shipping, Wuhan, Hubei, China

Sepsis-induced acute lung injury (ALI) has a high mortality rate, and cytokine storm is its feature. PANoptosis is a new type of cell death including apoptosis, pyroptosis and necroptosis. The aim of this study is to detect the PANoptosis level of lung macrophages, and to elucidate the new mechanism of sulforaphane (SFN) in sepsis-induced ALI. In septic animal model, the fluorescent staining of Caspase-8, GSDMD and p-MLKL and ASC/Caspase-8/RIPK3 PANoptosome in lung macrophages was performed. Lipopolysaccharide (LPS) was used to induce macrophages to construct cell model of sepsis. The proportion of dead cells was detected by PI staining, and the expression of Bax, GSDMD-N, NLRP3 and p-MLKL was detected by western blotting. Search for the target genes of SFN and sepsis by network pharmacology. Molecular docking analysis confirmed the binding between SFN and TLR4. The protein levels of TLR4, P65 and p-P65 were detected by western blotting. The transcriptional levels of inflammatory factors were detected by qPCR. The expression of Caspase-8, GSDMD, p-MLKL and PANoptosome in septic lung macrophages was significantly increased, suggesting PANoptosis was up-regulated. LPS induced macrophages death and increased protein levels of Bax, GSDMD-N, NLRP3 and p-MLKL, which were reversed by pretreatment with SFN. Network pharmacology and molecular docking demonstrated that SFN could bind to TLR4 and inhibit NFκB pathway. The mRNA levels of pro-inflammatory factors IL6, CXCL16, iNOS and IL18 were down-regulated by SFN. SFN might alleviate LPS-induced macrophage PANoptosis through TLR4/NFκB pathway, thereby inhibiting macrophage inflammation and becoming a potential therapeutic drug for sepsis-induced ALI.

Keywords: inflammation; PANoptosis; sepsis-induced ALI; sulforaphane; TLR4/NFκB pathway
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Introduction

Sepsis is a clinical syndrome caused by infection or injury, which is pathologically characterized by an excessive inflammatory response (Chousterman et al. 2017; Wang et al. 2019). Sepsis can cause severe cell and tissue damage and organ dysfunction, such as acute lung injury (ALI) (Li et al. 2022a). As an important part of the immune system, macrophages are involved in the key link of ALI (Hamidzadeh et al. 2017). Accumulating evidence sug-

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*These authors contributed equally to this work.
Correspondence: Lei Cao, The first hospital of Hebei Medical University, No. 89, Donggang Road, Shijiazhuang, Hebei 050023, China.
e-mail: 18511807110@163.com
Jingxuan He, General Hospital of the Yangtze River Shipping, No. 5, Huiji Road Wuhan, Hubei 430015, China.
e-mail: hjx850822@126.com
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gested that death of lung macrophage played an important role in the progression of pulmonary inflammation by affecting other immune cell populations in the lung. Cell death formed a positive feedback loop with tissue inflammation, ultimately leading to hyperinflammation and disease development (Yang et al. 2016; Fan and Fan 2018). Although traditional clinical drugs, including glucocorticoids, isofylline, and statins, have been used to treat sepsis, they cannot effectively reduce the mortality rate of patients. Therefore, there is an urgent need to fully elucidate the pathophysiological mechanisms of ALI and explore new effective treatments to alleviate and prevent the development of the disease.

PANoptosis, a unique new form of programmed cell death (PCD), is characterized by pyroptosis, apoptosis, and necroptosis, but it cannot be explained by pyroptosis, apoptosis or necroptosis alone (Pandian and Kanneganti 2022; Qi et al. 2023). As a unique mode of innate immune inflammatory cell death, PANoptosis is associated with the pathogenesis of a variety of systemic diseases, including cancer (Lin et al. 2022), autoinflammatory diseases (Sun et al. 2023), and neurodegenerative diseases (Zeng et al. 2023). In recent years, some studies have found that PANoptosis is involved in the pathogenesis of sepsis. The study by Zhou et al. (2023) showed that NINJ1 could regulate PANoptosis to participate in disseminated intravascular coagulation in sepsis. ALI is also closely related to the occurrence of PANoptosis. MiR-29a-3p improved ALI by reducing PANoptosis of alveolar epithelial cells (Cui et al. 2022). Ursodeoxycholic acid exerted its therapeutic effect on sepsis by blocking PANoptosis, too (He et al. 2023). Therefore, inhibition of PANoptosis may be a therapeutic target for sepsis-induced ALI.

Sulforaphane (SFN) is an isothiocyanate derived from glucoraphanin, occurring in large amounts in Brassica genus plants (Russo et al. 2018). SFN is well known for its promising anticancer activity (Iahtisham-Ul-Haq et al. 2022). The main molecular mechanism is to act as an activator of nuclear factor erythroid-2-related factor 2 (Nrf2) and inhibit cellular oxidative stress (Bose et al. 2020; Ma et al. 2023). However, a few studies have reported the therapeutic effects of SFN on sepsis. Lee et al. (2017) reported that SFN inhibited high mobility group box 1 (HMGB1) release and reduced mortality and lung injury in septic mice. Zhao et al. (2017) revealed that SFN attenuated ALI by inhibiting oxidative stress via Nrf2/ Heme oxygenase-1 (HO-1) pathway in a rat sepsis model. However, the relationship between SFN and PANoptosis has not been reported until now.

In this study, we found that PANoptosis in lung macrophages was significantly increased in a mouse model of LPS-induced sepsis. PANoptosis might be a potential therapeutic target for sepsis-induced ALI. LPS was used to treat macrophages to mimic sepsis, and the results showed that SFN could significantly block macrophage PANoptosis. Based on network pharmacology and molecular docking analysis, we found the therapeutic effects of SFN might be closely related to toll-like receptor 4 (TLR4). Our study demonstrated that SFN might treat sepsis-induced ALI by blocking macrophage PANoptosis through inhibiting TLR4/ nuclear factor kappa-B (NFκB) signaling pathway, which provided a new possible mechanism for the treatment of ALI by SFN.

Materials and Methods

Animal models

In this experiment, male C57BL/6 mice (8-12 weeks old) obtained from Cyagen (Suzhou, China) were utilized. The mice were housed (5 mice per cage) in a temperature-controlled (24 ± 2°C) specific-pathogen-free facility under 12-h light/dark cycles with free access to food and water. All animal studies were approved by the Animal Care and Use Committee of Renmin Hospital at Wuhan University (IACUC Issue No. 20220501 A). All the mice were randomly divided into two groups: the normal saline group (Normal), the lipopolysaccharide group (Sepsis). The mice were intraperitoneally injected with LPS (Sigma-Aldrich, USA, L2630, 5 mg/kg). 24 h after the injection of LPS, the mice were euthanized using 75 mg/kg ketamine and 10 mg/kg phenothiazine to obtain the lung tissues for further analysis.

Cell culture and treatments

The human mononuclear cell line THP-1 cell line was cultivated in Roswell Park Memorial Institute- (RPMI-) 1640 culture medium (Gibco, USA) supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin solution (Servicebio, Wuhan, China). The cells were cultured at 37°C with 5% CO_2. THP-1 cells were added 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 24 h to differentiate into M0 macrophages. Then, pretreated macrophages with 20 uM SFN (MCE, Shanghai, China) for 12 h. After that, cells were added with 1 ug/mL LPS 24 h to simulate the cell model of sepsis.

Hematoxylin-eosin (H&E) staining

Lung tissues were fixed in 4% polyformaldehyde (Servicebio) and then dehydrated and embedded in paraffin. Tissue sections were cut, plated on the slide and were dried in a 45°C oven, dewaxed, and rehydrated in descending series of alcohol. Following washing with distilled water for 5 min, the slices were stained with hematoxylin for 5 min, hydrolyzed with 1% hydrochloric acid-ethanol for 3 s, and dyed by 5% eosin solution for 3 min. Thereafter, the slices were dehydrated, cleared, and sealed before microscopic observation.

Immunofluorescence

Lung tissues were fixed with 4% polyformaldehyde for 72 h, embedded in paraffin, and sectioned at 3 μm. Lung sections were dewaxed and rehydrated, then heated 20 min at 80°C in citrate buffer 10 mM pH = 6 for antigen retrieval.

Lung sections were permeabilized in 0.5% Triton, blocked with 5% BSA for 1 h at room temperature, and then incubated overnight with primary antibodies to Mouse anti-F4/80 (1:200; Abcam, UK) and Rabbit anti-caspase-8 (1:200; Proteintech, Wuhan, China), Rabbit anti-GSDMD (1:200; Affinity, Jiangsu, China) and Rabbit anti-p-MLKL (1:200; Affinity), respectively. After washing with PBST, the sections were incubated with Alexa Fluor 488-labeled goat anti-mouse and Alexa Fluor 594-labeled goat anti-rabbit secondary antibodies. Following washing, lung sections were stained with 4′, 6-diamindino-2-phenylindole (DAPI; Sigma) for 10 min, washed with PBST, and mounted onto microscope slides (Olympus, Japan).

For immunofluorescence staining of cells, BAL-macrophages were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton for 15 min, and blocked with 5% BSA at room temperature for 1 h. Rabbit anti-ASC (1:200; Proteintech), Rabbit anti-caspase-8 (1:200; Proteintech) and Rabbit anti-RIPK3 (1:200; Proteintech) antibodies were added for overnight incubation, respectively. After incubating a kind of primary antibody, the cells were incubated with Alexa Fluor 594-labeled goat anti-rabbit, Alexa Fluor 488-labeled goat anti-Rabbit or Alexa Fluor 628-labeled goat anti-bodies (1:200; Proteintech) for 1 h under light-proof conditions. The nuclei were stained with 4′,6-diamindino-2-phenylindole (DAPI; Sigma). Images were acquired by a laser scanning confocal microscope (Olympus).

Assay of dead cell staining

Propidium iodide (PI) staining was used to distinguish between live cells and dead cells according to the manufacturer's instructions. Briefly, PI reagents (Beyotime, Shanghai, China) were added to macrophages from different group. The cells were then incubated at 37℃ for 10 min. The labeled cells were viewed under a fluorescence microscope later.

Western blotting

Total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime) and the protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime) as previously described (Wu et al. 2023). Almost 40 ug protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking membranes with protein-free rapid blocking solution (Yazyme, Shanghai, China), they were incubated with appropriate dilutions of specific primary antibodies overnight in 4℃. TBST was used to wash the blots on the second day. Then incubating it with HRP-conjugated second antibodies (1:5000, Abmart, Shanghai, China) and visualized using an electrochemiluminescence (ECL) system (Monad, Jiangsu, China). Antibodies against NLRP3 (1:1000; ABclonal; A5652; Wuhan, China), p-MLKL (1:1000; Affinity; AF7420), Bax (1:1000; Proteintech; 50599-2-Ig), GSDMD-N (1:1000; Abmart; TA4012), TLR4 (1:1000; Santa Cruz Biotechnology; sc-293072; USA), P65 (1:1000; Abmart; T55035) and p-P65 (1:1000; Abmart; TP56372) and *β*-actin (1:5000; abmart; T40104) were used according to the manufacturer's instruction. Image J software was used to analyze the gray value of the blots. The gray value of Control group was set to 1, and the variation of LPS or LPS+SFN group relative to Control group was statistically analyzed.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using AG RNAex Pro Reagent (ACCURATE BIOTECHNOLOGY (HUNAN) CO., LTD, Changsha, China) and reverse-transcribed using mRNA Reverse Transcription Kit (ACCURATE) according to the manufacturer's instructions. Gene expression was determined by real-time PCR using SYBR Green Premix Pro Taq HS qPCR Kit (ACCURATE) with a CFX96TM Real-Time system (Bio-Rad, USA). The primer sequences were shown in Table 1 and *β*-actin were selected as the internal control and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The value of Control group was set to 1, and the variation of LPS or LPS+SFN group relative to Control group was statistically analyzed.

Microarray-based gene expression profiling

The PubChem database was employed to predict the molecular structure of SFN. The target gene of SFN was then retrieved using the database SuperTarget and SwissTargetPrediction databases. "Sepsis" was used as a keyword to predict the target genes associated with sepsis by the OMIM, GeneCards and CTD databases and dataset GSE54514, and then analyzed employing the jvenn tool. Next, the predicted target genes of SFN and sepsis-related genes were subjected to Venn diagram analysis using the jvenn. Gene symbol of 26 target genes with intersection was imported into the String (https://string-db.org/) service

Table 1. The sequences of the primers for qRT-PCR.

Gene	Primer Sequence (5′ to 3′)	
β-actin	F	GATCAAGATCATTGCTC
	R	TTGTCAAGAAAGGGTGTA
IL6	F	AATAACCACCCCTGACCCAAC
	R	ACATTTGCCGAAGAGCCCT
CXCL16	F	GACATGCTTACTCGGGGATTG
	R	GGACAGTGATCCTACTGGGAG
iNOS	F	TCCCGAGTCAGAGTCACCAT
	R	TCCATGCAGACAACCTTGGG
IL18	F	AACAAACTATTTGTCGCAGGAAT
	R	TGCCACAAAGTTGATGCAAT
TGF-*β*	F	GACTCGCCAGAGTGGTTATCT
	R	CGGTAGTGAACCCGTTGAT
CCL17	F	GAGCCATTCCCCTTAGAAAG
	R	AGGCTTCAAGACCTCTCAAG

platform, and the limited species was Homo sapiens to construct a protein–protein interaction (PPI) network. The PPI network was visualized by Cytoscape 3.10.1 software and the top 4 Hub target genes were obtained.

Molecular docking analysis

The TLR4 protein crystal structures used for docking were obtained from the PDB database and the small molecule 3D structures were obtained from PubChem and energy minimization was performed under the MMFF94 force field. AutoDock Vina 1.1.2 software was used to perform molecular docking. PyMol 2.5.2 was used to remove water molecules, salt ions and small molecules. The docking box is then set so that the box wraps the entire protein. In addition, all processed small molecules and receptor proteins were converted into PDBQT format required for docking by AutoDock Vina 1.1.2 using ADFRsuite 1.0. For docking, the exhaustiveness of the global search was set to 32, and the rest of the parameters were kept at default Settings. The output docking conformation with the highest scoring was considered by us as the bound conformation, and finally the PyMol 2.5.2 docking results were used for visual analysis.

Statistical analysis

We used Graph Pad Prism 8 software to conduct statistical analyses. The data were presented as the mean ± SD. One-way ANOVA coupled with the Tukey's post-hoc test was used to assess differences among the three groups. $p < 0.05$ was statistically significant.

Results

PANoptosis of lung macrophages increases in septic mice

First, we used LPS to stimulate mouse model for sepsis, which was widely used in the induction of animal models of sepsis (Dang and Leelahavanichkul 2020; Li et al. 2022b). The results of H&E staining showed that the normal group exhibited normal pulmonary alveolar structure, whereas the lung tissues in the sepsis group showed significant histopathological changes, including severe inflammatory cell infiltration, thickened alveolar walls, alveolar collapse, and alveolar hemorrhage, which suggested a successful mice model (Fig. 1A). Most studies have shown that PANoptosis occurs mainly in immune cells, especially macrophages (Karki et al. 2022). To investigate the level of PANoptosis in macrophages of septic mice, macrophages were de-labeled with a mouse macrophage-specific marker F4/80, IF staining showed that the expressions of caspase-8, GSDMD and p-MLKL were significantly increased in the sepsis group (Fig. 1B-D). Caspase-8 is a marker of apoptosis, GSDMD mediates the classical pyroptosis pathway, and p-MLKL is the executive protein of necroptosis. At the same time, the number of PANoptosome composed of ASC, caspase-8 and RIPK3 in the alveolar macrophages of BALF with sepsis was significantly higher than that in the normal group (Fig. 1E). These results indicated that the PANoptosis in lung macrophages of septic mice was significantly increased.

SFN inhibits LPS-induced PANoptosis of macrophages

SFN has been shown to treat sepsis-induced acute liver and kidney injury, but its role in lung injury remains unknown. Next, we induced THP-1-derived macrophages with LPS to construct a cell model of sepsis. As shown in Fig. 2A,B, LPS significantly increased the number of cells with positive PI staining, which meant dead cells. Pretreatment with SFN reduced the number of PI positive cells. Meanwhile, Western blotting results showed that LPS up-regulated the protein levels of Bax, GSDMD-N, NLRP3 and p-MLKL. Bax, an important pro-apoptotic protein, mainly plays a regulatory role in the upstream of several caspases (Schmitt et al. 1997). Bai et al. (2024) studied that inhibiting Bax with zinc ions could effectively reduce PANoptosis after spinal cord injury, which proved the induction effect of Bax on PANoptosis. However, SFN reversed the protein changes (Fig. 2C-G). These results indicated that LPS could induce PANoptosis of macrophages, which could be inhibited by SFN.

Network pharmacology analysis reveals the potential mechanism of SFN in the treatment of sepsis

In order to explore the possible mechanisms of SFN in the treatment of sepsis, we searched for sepsis-related genes using OMIM, CTD, GeneCards and GEO databases, and included genes that appeared at least twice. A total of 374 genes were identified as target genes associated with sepsis (Fig. 3A). Next, the Smile version of SFN was obtained on Pubchem, and the Smile version was imported into SwissTargetPrediction and SuperTarget databases to obtain a total of 199 therapeutic targets of SFN after removing duplicate targets. Almost 26 target genes of SFN in the treatment of sepsis were obtained by intersection of the two (Fig. 3B). The 26 target genes were input into the string database, and Cytoscape software was used to construct the PPI network. A total of 4 Hub targets were obtained, including TLR4, NFκB1, SRC and CXCR4 (Fig. 3C). GO analysis of these genes showed that they were mainly related to response to external stimulus, regulation of multicellular organismal process, inflammatory response and so on (Fig. 3D). KEGG analysis showed that VEGF signaling pathway, HIF-1 signaling pathway, AGE-RAGE signaling pathway, and Toll-like receptor signaling pathway were involved in the process of SFN treatment of sepsis (Fig. 3E). TLR4, sensing of LPS, the most potent pathogen-associated molecular pattern of gram-negative bacteria, activates NFκB and Irf3, which induces inflammatory cytokines and interferons that trigger an intense inflammatory response, which is critical for the pathogenesis of sepsis (Bai et al. 2023). Considering the importance of TLR4 in sepsis, it was identified as our core target.

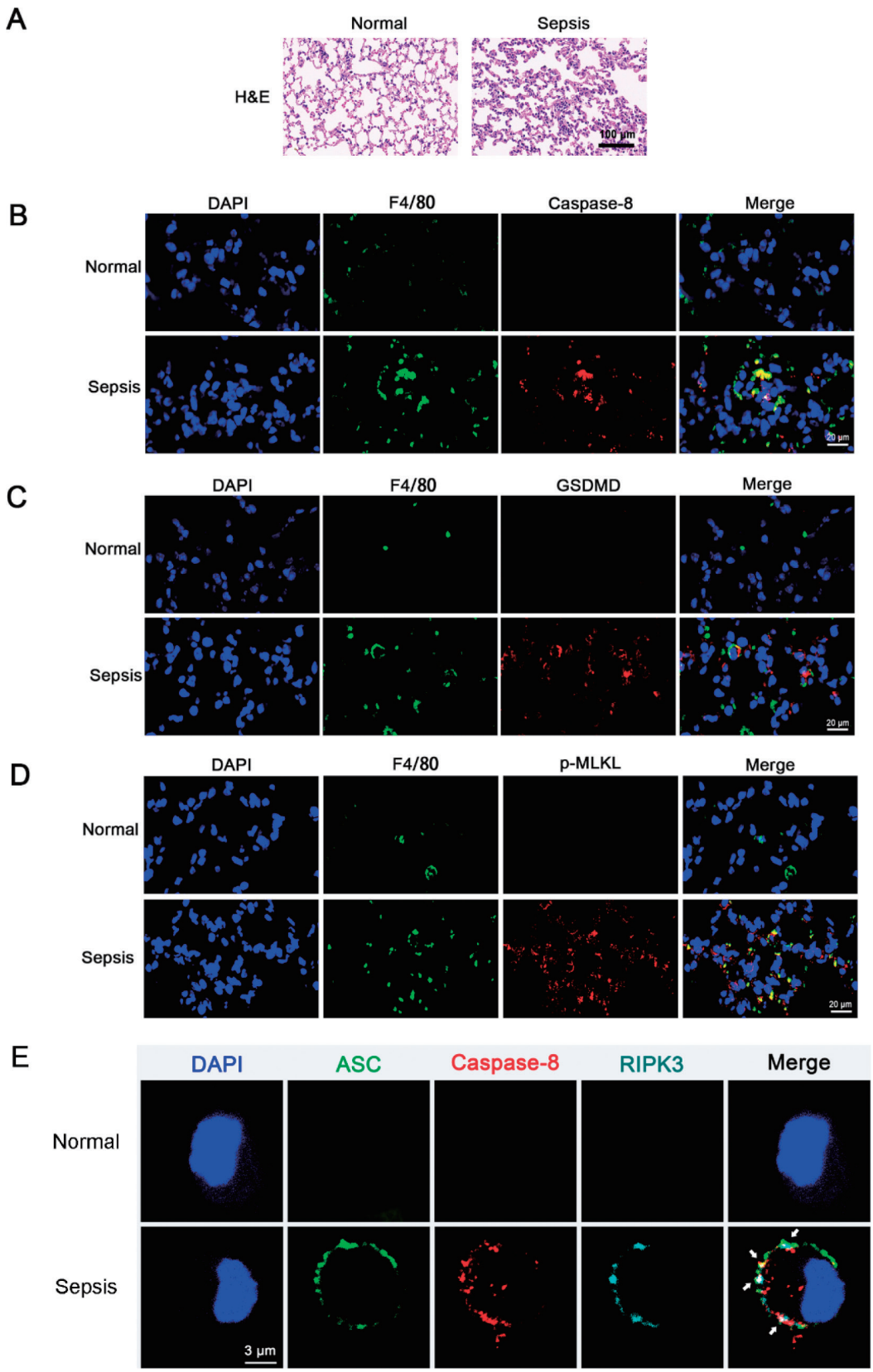

Fig. 1. PANoptosis of lung macrophages increases in septic mice.
(A) H&E staining of lung tissue in normal and sepsis mouse. Scale bars = 100 um. (B-D) Immunofluorescence staining was used to evaluate the expression of Caspase-8, GSDMD and p-MLKL in mouse lung macrophages. Scale bars = 20 um. (E) Immunofluorescence detection of PANoptosome composed of ASC, Caspase-8 and RIPK3 in alveolar macrophages in normal or sepsis mouse. Arrow: PANoptosome. Scale bars = 3 um. n = 3.

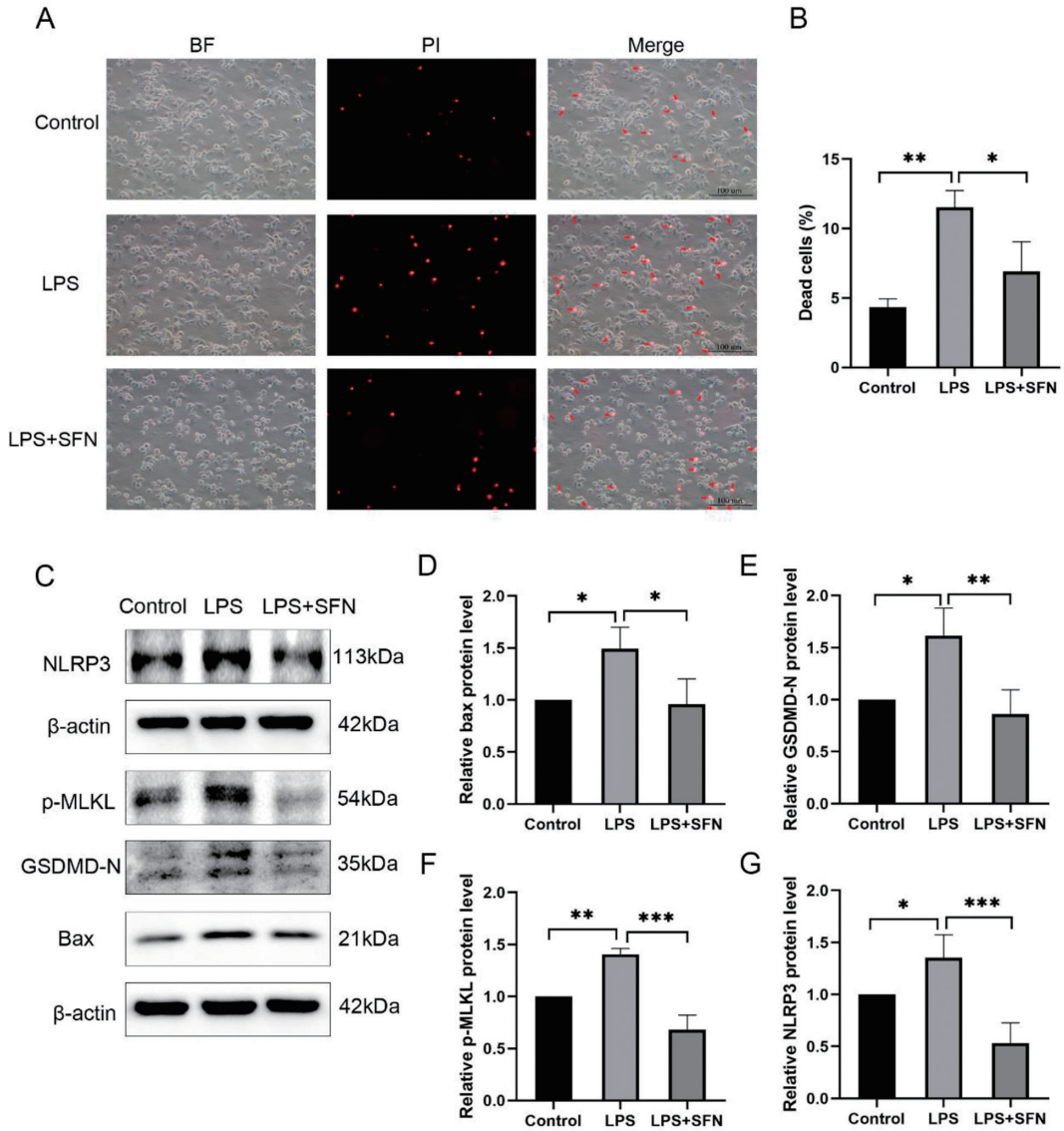

Fig. 2. SFN inhibits LPS-induced PANoptosis of macrophages.
(A-B) PI staining to observe dead macrophages in Control, LPS and LPS+SFN groups. Scale bars = 100 um. (C) Western blotting assay was used to detect the protein expression including Bax, p-MLKL, NLRP3, and GSDMD-N of macrophages in Control, LPS and LPS+SFN groups. (D-G) Western blotting assay results were quantified. n=3. Data represent the mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

SFN inhibits TLR4/NFκB signaling pathway in macrophages

To further prove the effects of SFN on TLR4, we examined the potential interaction between SFN and TLR4. The computational modeling results revealed that SFN formed a hydrogen bond with amino acid HIS179. The binding score was -3. Representative images were depicted in Fig. 4A-C. The inhibitory effect of SFN on TLR4 was confirmed by western blotting results. TLR4 usually upregulates the expression of myeloid differentiation factor 88 (MyD88), activates the NFκB signaling pathway, and induces inflammatory responses (Ju et al. 2018). Then we examined the expression of NFκB pathway. As showed in Fig. 4D-F, LPS significantly promoted the activation of phosphorylated P65, while SFN inhibited the expression of p-P65. These results suggested that SFN inhibited TLR4/NFκB signaling pathway in macrophages, which might be the potential mechanism for treatment of sepsis.

SFN inhibits LPS-induced inflammatory response in macrophages

The early feature of sepsis is a storm of inflammatory factors. Given the excellent inhibition of TLR4/NFκB by SFN, we finally examined the anti-inflammatory effect of SFN on macrophages. PCR results showed that LPS-induced proinflammatory cytokines interleukin (IL) 6, CXC chemokine ligand 16 (CXCL16), inducible Nitric Oxide Synthase (iNOS) and IL-18 were significantly down-regulated by SFN (Fig. 5A-D). However, the mRNA expression of anti-inflammatory cytokines transforming growth

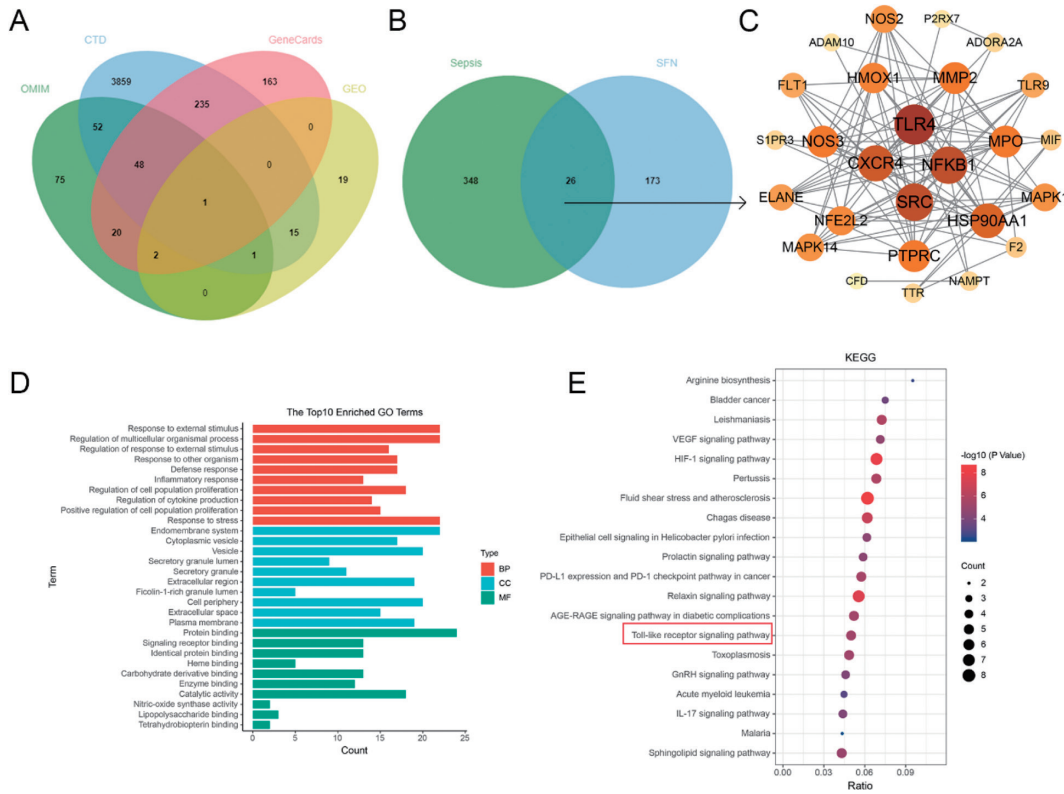

Fig. 3. Network pharmacology analysis reveals the potential mechanism of SFN in the treatment of sepsis. (A) Venn diagram analysis of sepsis-related genes predicted by the GeneCards, CTD, OMIM and GEO databases. (B) Venn diagram analysis of sepsis-related genes and targets of SFN predicted by SuperTarget and SwissTargetPrediction databases. (C) PPI network analysis of target genes of SFN and sepsis by Cytoscape. The color changes gradually from yellow to orange; the closer to the orange target gene, the more important it is. (D) The top10 enriched GO terms of target genes. (E) KEGG pathway enrichment analysis of the target genes of SFN and sepsis.

factor-β (TGF-β) was also down-regulated and chemokine (C-C motif) ligand 17 (CCL17) showed no difference (Fig. 5E, F). These results suggested that SFN might only inhibit the pro-inflammatory factors, but not mediate the upregulation of anti-inflammatory factors.

Discussion

This study proved that PANoptosis of lung macrophages was significantly elevated in LPS-induced ALI. SFN could inhibit macrophage PANoptosis. Furthermore, network pharmacology analysis was used to search for the possible targets of SFN and sepsis, and TLR4 was found to be involved in this process. The results of molecular docking and western blotting confirmed our conclusion. SFN also inhibited the downstream NFκB signaling pathway and inflammatory response. Therefore, SFN might treat sepsis-induced ALI by inhibiting macrophage PANoptosis.

Sepsis-induced ALI has a high mortality rate (Cecconi et al. 2018), and inflammatory factor storm is its prominent feature (Zhang et al. 2023). Therefore, inhibiting the excessive activation of inflammatory response may be an important therapeutic strategy for ALI. Although PANoptosis is a new type of death that combines apoptosis, pyroptosis and necroptosis, it is essentially an inflammatory cell death (Karki et al. 2022). Our study demonstrated a significant increase in PANoptosis of lung macrophages in LPS-induced ALI mice. Although PANoptosis occurs mainly in immune cells. However, positive signals of Caspase-8, GSDMD, and p-MLKL were observed in both F4/80 positive and non-positive regions in our fluorescence images. A few researches had reported that PANoptosis was involved in the pathogenesis of sepsis-induced ALI, the target cells were focused on alveolar epithelial cells (Cui et al. 2022; Guo et al. 2023). Our results also demonstrated that PANoptosis could occur in both pulmonary macrophages and alveolar epithelial cells in sepsis. Given the important role of macrophages played in the immune response, we profoundly explored lung macrophages in ALI (Aegerter et al. 2022; Chen et al. 2022a). Furthermore, THP-1-derived macrophages were used to establish a sepsis cell model. Consistent with our expectation, LPS significantly induced macrophage death, and the protein levels of Bax, GSDMD-N, NLRP3 and p-MLKL associated with PANoptosis were also significantly up-regulated. These changes were reversed by the addition of SFN.

During sepsis, LPS binds to the TLR4/myeloid differentiation 2 (MD2) complex to form homodimers. Then, the adaptor protein MyD88 is recruited, which leads to the activation of NFκB and mitogen-activated protein kinases (MAPKs) (Park et al. 2009). It had been suggested that

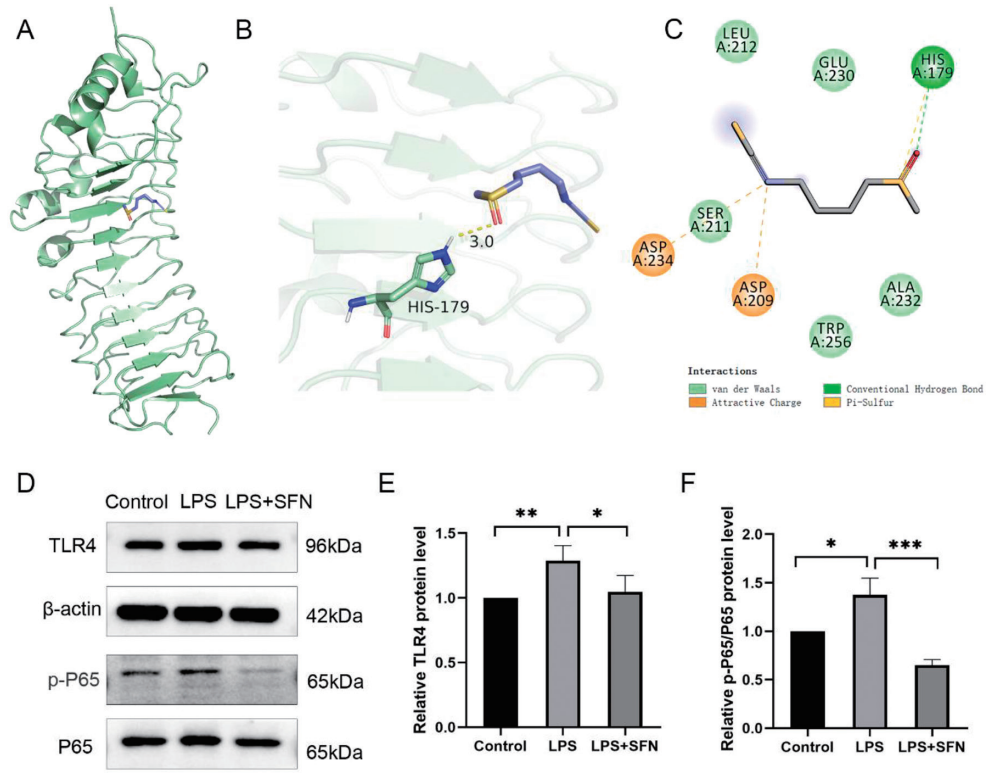

Fig. 4. SFN inhibits TLR4/NFκB signaling pathway in macrophages.
(A, B) Representative images of the docking mode of SFN binding to TLR4. (C) The potential connection sites between SFN and TLR4. (D-F) The protein expression of TLR4, P65 and p-P65 was detected by western blotting. n=3. Data represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

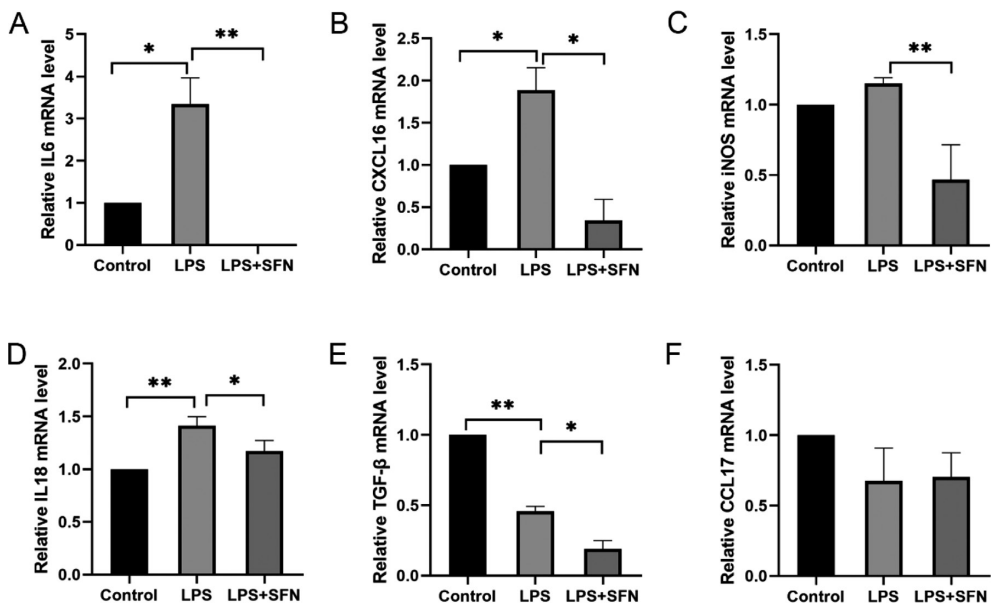

Fig. 5. SFN inhibits LPS-induced inflammatory response in macrophages.
(A-D) QPCR to detect the proinflammatory cytokines including IL6, CXCL16, iNOS and IL18 of macrophages in Control, LPS and LPS+SFN groups. (E-F) QPCR was used to detect the anti-inflammatory cytokines including TGF-β and CCL17 of macrophages in Control, LPS and LPS+SFN groups. n=3. Data represent the mean ± SD. *$p < 0.05$, **$p < 0.01$.

inhibition of LPS interaction with MD2 by SFN by directly binding Cys133 in MD2 was the first step in inhibiting TLR4 activation (Koo et al. 2013). In addition, direct modification of TLR4 cysteine residues was also one of the mechanisms by which SFN inhibited TLR4 (Youn et al. 2010). Interestingly, in this study, we selected TLR4 as a

core target through network pharmacology and molecular docking results demonstrated that SFN also binded to HIS179 of TLR4. As a result, p-P65 was also significantly inhibited.

There are excellent anti-inflammatory effects in SFN. SFN significantly reduced the serum levels of IL-6 and TNF-α, the protein expression of cyclooxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9), and the activity of iNOS in LPS-induced ALI mice (Qi et al. 2016). Consistent with our study, our PCR results also demonstrated the transcriptional repression effect of SFN on pro-inflammatory cytokines IL6, IL18, CXCL16 and iNOS. As a marker of M1 macrophage activation, iNOS often regulates the polarization of M1 and M2 macrophages. Studies have shown that macrophage polarization is unbalanced in sepsis (Jiao et al. 2021; Chen et al. 2022b). Histone methyltransferase SETD2 inhibits M1 macrophage polarization and glycolysis by suppressing HIF-1α in sepsis-induced ALI (Meng et al. 2023). Targeted regulation of the process of macrophage polarization could be a useful approach to normalize the immune balance of the host, offering a new treatment modality for different stages of sepsis (Chen et al. 2021). Our study provided evidence that SFN might have the potential to inhibit M1 macrophage polarization. Interestingly, SFN did not seem to promote the expression of anti-inflammatory molecules, including TGF-β and CCL17, which deserved further investigation.

However, there are still some limitations in our study. Firstly, in vivo experiments with SFN are lacking to more effectively prove our conclusions. What's more, other core targets need to be further validated to explore other potential mechanisms of SFN besides TLR4.

In conclusion, our study demonstrated that SFN inhibited LPS-induced macrophage PANoptosis through TLR4/NFκB signaling pathway and exerted a significant anti-inflammatory effect, which was a possible mechanism of SFN in the treatment of sepsis-induced ALI.

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Author Contributions

Yanwei Wang and Huifan Liu performed the experiments and wrote the manuscript. Yali Feng analyzed the results. Shujuan Wu provided technical assistance and information. Lei Cao and Jingxuan He designed the experiments and edited the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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