Shikonin ameliorates lipoteichoic acid-induced acute lung injury via promotion of neutrophil apoptosis

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Abstract. Shikonin is the major active component in *Lithospermum erythrorhizon* and has pharmacological effects including reducing inflammation, aiding resistance to bacteria and promoting wound healing. However, the effect of shikonin on lipoteichoic acid (LTA)-induced acute lung injury (ALI) remains to be elucidated. ALI is a serious illness resulting from significant pulmonary inflammation caused by various diseases, such as sepsis, acid aspiration and trauma. The present study found that shikonin significantly attenuated LTA-induced ALI. Following shikonin treatment, the accumulation of pulmonary neutrophils and expression of TNFα, IL-1β and IL-6 were decreased in mice with LTA-induced ALI. Furthermore, Shikonin promoted neutrophil apoptosis by increasing the activation of caspase-3 and reducing the expression of the antiapoptotic myeloid cell leukemia-1 (Mcl-1) protein. However, shikonin treatment did not influence the expression of B-cell lymphoma-2. The findings of the present study demonstrated that shikonin protected against LTA-induced ALI by promoting caspase-3 and Mcl-1-related neutrophil apoptosis, suggesting that shikonin is a potential agent that can be used in the treatment of sepsis-mediated lung injury.

Introduction

Acute lung injury (ALI) is a serious illness resulting from pulmonary inflammation caused by various conditions, including sepsis, acid aspiration and trauma (1-3). Infection by gram-positive bacteria is one of the main causes of pulmonary inflammation (4). Although extensive research on the mechanism and treatment of gram-positive-bacteria-induced ALI has been carried out, the specific mechanism remains to be elucidated and effective drugs and treatments remain unavailable, resulting in high ALI-associated morbidity and mortality worldwide (5-8). Lipoteichoic acid (LTA) is expressed on the surface of gram-positive bacteria (9). Mice treated with LTA develop gram-positive-bacteria-induced pneumonia, a common and serious type of pneumonia that affects humans of all ages (7,10).

Shikonin is a major active ingredient isolated from the roots of *Lithospermum erythrorhizon* (11). The antitumor and anti-bacterial properties of shikonin have been studied *in vitro* and *in vivo* (12,13). Previous studies have indicated that the anti-inflammatory effect of shikonin results from the reduction of oxidative stress (14), the inhibition of Th2 cytokine production and the release of histamine from mast cells (15). Recently, the protective role of shikonin against lipopolysaccharide (LPS)-induced ALI was demonstrated in a mouse model (16). However, the effect of shikonin on LTA-induced ALI and the underlying mechanisms have not been studied.

Neutrophils are the first line of immune-defense cells against pathogen infection (17). However, the defensive response by overactivated neutrophils can also have a damaging effect on the lung tissue (18) due to the release of excessive amounts of inflammatory factors such as TNF-α, IL-1β and IL-6 causing serious lung injury (17-19). Previous studies have shown that apoptosis is caused by bacterial infection (20-22). The continuous release of inflammatory factors in ALI is in part ascribed to the inhibition or delay of neutrophil apoptosis in the lung tissue (23,24). This suggests that promoting neutrophil apoptosis could reduce lung inflammation and damage. Apoptosis is an orderly cell death process controlled by multiple genes (25). Caspase-3 is a key regulatory protein involved in apoptosis. Poly (ADP-ribose) polymerase (PARP) DNA-repairing enzyme can be cleaved into different fragments by caspase-3, resulting in abnormal DNA repair and apoptosis (26). Caspase 3-mediated apoptosis can be inhibited by myeloid cell leukemia-1 (Mcl-1) and Bcl-2 (27). Therefore,

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identification and action on targets related to neutrophil apoptosis is an alternative strategy for preventing and treating ALI.

In the present study, the protective effect of shikonin in LTA-induced ALI was identified. Pretreatment with shikonin in LTA-induced ALI mouse model markedly reduced lung inflammation and decreased pro-inflammatory cytokine expression. The findings of the present study confirmed that shikonin promoted neutrophil apoptosis mediated by increased caspase-3 and decreasing Mcl-1 expression, consequently reducing inflammation in gram-positive-bacteria-induced ALI.

Materials and methods

Animals. Male C57BL/6 mice (6-8 weeks old, 22±3 g, specific pathogen-free, n=20) were purchased from SLAC Laboratory Animal Corporation and acclimated to laboratory conditions (25˚C, 50-60% humidity) for two weeks under a 12-h light/dark cycle and with free access to food and water before the experiments. The present study adhered to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1996) (28). All experimental protocols described in the present study were approved by the Animal Care and Use Committee of Bengbu Medical College (approval no. LABMT-019).

Compound and reagents. Shikonin was prepared at 20 mM in DMSO (Sigma-Aldrich; Merck KGaA) and stored at -20˚C. The primers used in the present study were synthesized by Shanghai HuaGen Biotech Co., Ltd. Cleaved caspase-3 (Asp175; cat. no. 9664), cleaved PARP (cat. no. 5625), Mcl-1 (cat. no. 94296), Bcl-2 (cat. no. 15071), p53 (cat. no. 2527) and β-actin (cat. no. 4970) primary antibodies were purchased from Cell Signaling Technology, Inc. The peroxidase AffiniPure goat anti-rabbit IgG (H+L) (cat. 111-035-003) and peroxidase AffiniPure goat anti-mouse IgG (H+L) (cat. 115-035-003) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. and diluted in EZ-Buffers (cat. no. C520011-0100; Sangon Biotech Co., Ltd. Other chemical reagents were obtained from Sigma-Aldrich (Merck KGaA).

LTA-induced ALI mouse model. Shikonin was dissolved in a vehicle (castor oil:ethanol:PBS=1:1:8). Mice were randomly divided into four groups and anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium before intratracheal injection. Each group contained 5 mice. The LTA group mice were intratracheally challenged with 5 mg/kg LTA (Sigma-Aldrich; Merck KGaA). The LTA + shikonin group mice were intraperitoneally injected with shikonin at 10 mg/kg for 30 min, then administered an intratracheal injection of 5 mg/kg LTA. The vehicle group mice were intraperitoneally injected with the same volume of castor oil:ethanol:PBS (1:1:8) as the LTA + shikonin group mice and, 30 min later, intratracheally challenged with the same volume of PBS as used for the LTA + shikonin group mice. The vehicle + shikonin group mice were intraperitoneally injected with 10 mg/kg shikonin for 30 min, then administrated an intratracheal injection of the same volume of PBS as the LTA + shikonin group mice. Animal health and behavior were monitored all the time during the experiment. The four group mice appeared healthy without obvious abnormal behavior during the experiment. After 6 h, all the mice were sacrificed with CO_2 inhalation (30% cage volume/min, 5-6 min) until the mice ceased breathing and had faded eyes. The samples were then collected. The experiments were conducted in March 2019.

Acquisition and analysis of bronchoalveolar lavage fluid (BALF). The BALF of mice were collected according to a previous study (29). Briefly, the lungs were lavaged three times with 50 μM EDTA. The BALF was centrifuged at 300 x g at 4˚C for 5 min. The cell-free supernatants were harvested and analyzed for the total protein content using a BCA protein assay kit (Beyotime Institute of Biotechnology). Neutrophils were incubated with Gr-1(Ly6G)-FITC antibody (1:200; cat. no. 11-5931-82; eBioscience; Thermo Fisher Scientific, Inc.) and analyzed by flow cytometry (LSRFortessa X-20; BD Biosciences) to determine the percentage of neutrophils in BALF.

Histopathology. Lung tissues (left lobe) were fixed with 4% paraformaldehyde overnight in 4˚C. Following dehydration in 80% alcohol for 1 h, 90% alcohol for 2 h and 100% alcohol for 2 h at room temperature, then washing in 100% xylene for 1 h in room temperature, the lung tissues were embedded in paraffin and cut into 5-μm sections with a microtome (cat. no. RM2235; Leica Microsystems GmbH). Prior to staining, the sections were heated in a drying oven to 90˚C for 30 min and then washed with 100% xylene for 15 min following immersion in a descending series (100, 95, 95, 80 and 70%) of alcohol for 3 min at room temperature, at each concentration. Then, the sections were stained with 100% hematoxylin for 5 min and 100% eosin for 1 min, both at room temperature (Beyotime Institute of Biotechnology) and images were captured by a light microscope (RX51; Olympus Corporation).

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Frozen lungs (one of the right upper lobe) were homogenized and the total RNA was isolated using TRIzol® Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was prepared using ReverTra Ace qPCR RT kit (Toyobo Life Science) according to the manufacturer's protocol and amplified by qPCR with a TOROGreen® Qpcr Master Mix (TOROIVD TECHNOLOGY COMPANY LIMITED) and primer sets for TNF-α (forward, 5'-TTCTCATTCCTGCTTGTGG-3' and reverse, 5'-ACTTGGTGGTTTGCTACG-3'); IL-1β (forward, 5'-CCAGCTTCAAATCTCACAGCAG-3' and reverse, 5'-CTTCTTTGGGTATTGCTTGGGATC-3'); IL-6 (forward, 5'-CTTCTTGGGACTGATG-3' and reverse, 5'-CTGGCTTTGTCTTTCT-3'); and GAPDH (forward, 5'-TGCGACTTCAACAGCAACTC-3' and reverse, 5'-CTTGCTCAGTGTCCTTGCTG-3)'. Thermocycling of RT-qPCR was: 95˚C for 2 min, 95˚C for 10 sec, 58˚C for 30 sec and 72˚C for 20 sec, repeated for 40 cycles. The $2^{-\Delta\Delta Cq}$ method (30) was used to analyze the expression of mRNAs normalized to the GAPDH internal reference gene. The experiments were independently repeated in triplicate.

Myeloperoxidase (MPO) activity assay. The MPO activity was determined according to a previous study (31). Briefly, lung tissues were collected and subjected to three freeze-thaw

cycles. Supernatants were collected at 4˚C. Protein concentration in supernatants was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). After adding the substrate and catalyst to the supernatants, changes in absorbance at 655 nm were measured using a microplate reader (FlexStation 3; Molecular Devices, LLC). MPO activity was defined as the absorbance change per min per gram of protein.

Isolation of neutrophils and cell culture. Mouse bone-marrow neutrophils were obtained by flushing the bone marrow from mouse tibias and femurs. Neutrophils were purified (>95% purity) using a one-step Nycoprep/Percoll gradient (TBD, Tianjin Haoyang Biological Products Technology Co., Ltd.). According to the manufacturer's protocol, cells were suspended and cultured in RPMI-1640 (HyClone; Cytiva) with 10% fetal bovine serum (FBS; Biological Industries) and treated with the indicated concentrations of shikonin according to the experimental requirements. DMSO (1%) was added to the culture medium as the solvent control.

Annexin-V/PI-binding apoptosis assay. The neutrophil is the terminal differential cell with short survival time (32). The primary cultured neutrophils *in vitro* can be preserved for only 1-2 days due to activation from culture environment (32). Given the short survival time of primary-cultured neutrophil, 0.3, 1 and 3 μM shikonin was used to treat neutrophils for 24 h according to previous studies (33-37). The neutrophils were collected, centrifuged at 300 x g for 5 min at 4˚C and washed with cold PBS. Then, neutrophils were resuspended with 400 μl PBS and incubated with 2 μl Annexin V-FITC and 4 μl propidium iodide for 30 min at room temperature according to the manufacturer's instructions of the Annexin V-FITC apoptosis assay kit (Beyotime Institute of Biotechnology). Samples were analyzed by flow cytometry (LSRFortessa X-20; BD Biosciences). Data were analyzed using FlowJo 7.6 software (FlowJo LLC).

Western blot analysis. Following treatment with shikonin for 24 h, neutrophils were collected and lysed in RIPA buffer (Xi'an Weiao Biotechnology Co., Ltd.) with 1 mM PMSF and quantified using a BCA assay (Beyotime Institute of Biotechnology). Following heating at 99˚C for 10 min, each sample (15 μg) was loaded on 10% SDS/PAGE gels and then transferred to nitrocellulose filter membranes. Blots were blocked with 5% skim milk at room temperature for 1 h and then incubated with primary antibodies (1:1,000) overnight at 4˚C. Subsequently, blots were probed with the corresponding secondary antibodies (1:10,000) at room temperature for 1 h in dark. The protein signals were detected using an ECL kit (Shanghai Share-Bio Biotechnology Co., Ltd.). Semi-quantification of western blots was performed using ImageJ software (1.4.3.67; National Institute of Mental Health).

Statistical analysis. Data are presented as mean ± standard error of the mean obtained from ≥3 independent experiments. One-way analysis of variance (ANOVA) and Bonferroni's Multiple Comparison Test was performed using GraphPad Prism 6 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Shikonin attenuates LTA-induced ALI and inflammatory response. An LTA-induced ALI mouse model was established to determine the effect of shikonin on gram-positive bacterial-infection-induced ALI. As shown in Fig. 1A, the administration of LTA for 6 h induced inflammatory cell infiltration, inter-alveolar septal thickening and alveolar collapse. However, the infiltration of inflammatory cells was significantly reduced following pretreatment with shikonin (Fig. 1A). The total protein concentration in the BALF of the LTA-challenged group was ~10-fold higher compared with the vehicle group (Fig. 1B). Pretreatment with shikonin significantly reduced the total protein concentration in BAL cells (Fig. 1B). The expression of pro-inflammatory cytokines in lung tissues was measured following LTA treatment for 6 h; compared with that in the mice that received vehicle treatment, the expression of TNF-α, IL-1β and IL-6 was significantly elevated (Fig. 1C-E). However, the expression of TNF-α (Fig. 1C), IL-1β (Fig. 1D) and IL-6 (Fig. 1E) was markedly decreased following pretreatment with shikonin. These results indicate that shikonin protects against LTA-induced ALI and the corresponding inflammatory response.

Shikonin inhibits LTA-induced infiltration of pulmonary neutrophils. Infiltration of pulmonary neutrophils is one of the most important symptoms of pneumonia that directly leads to lung-tissue damage (38). As shown in Fig. 2, neutrophil infiltration increased ~20 times compared with the vehicle group following a 6-h LTA challenge. However, the neutrophil percentage (Fig. 2A and B) and amounts (Fig. 2C) were significantly reduced following pre-injection of shikonin compared with those in mice receiving LTA and vehicle. The activity of MPO-specific marker representing neutrophil infiltration is associated with the severity of ALI (39). MPO activity in lung tissue was tested. LTA-challenged mice had higher MPO activities compared with the mice that received the vehicle (Fig. 2D). However, the activity was significantly reduced by pretreatment with shikonin for 6 h (Fig. 2D). These results indicated that shikonin inhibits the accumulation of pulmonary neutrophils.

Shikonin accentuates neutrophil apoptosis. Neutrophils serve an important function in infection-induced inflammation (20). Moderately activated neutrophils protect against pathogen infection (40,41) but inflammatory factors released by overactivated neutrophils induce tissue injury (24). To determine the effect of shikonin, neutrophils were isolated from bone marrow and treated with 0.3, 1 or 3 μM shikonin. After 24 h of incubation, neutrophils were stained with Annexin V/PI and analyzed by flow cytometry. As shown in Fig. 3, the proportion of apoptotic cells increased significantly with increased shikonin concentration (Fig. 3A and B). The effect of shikonin on neutrophil apoptosis was examined further by determining the fractional DNA content (sub-G1) in the late stage of apoptosis (Fig. 4A). The results showed that shikonin dose-dependently enhanced DNA fragmentation (Fig. 4B). The proportion of sub-G1 neutrophils was markedly increased by 10 μM Shikonin from 25.33±2.72% (untreated) to

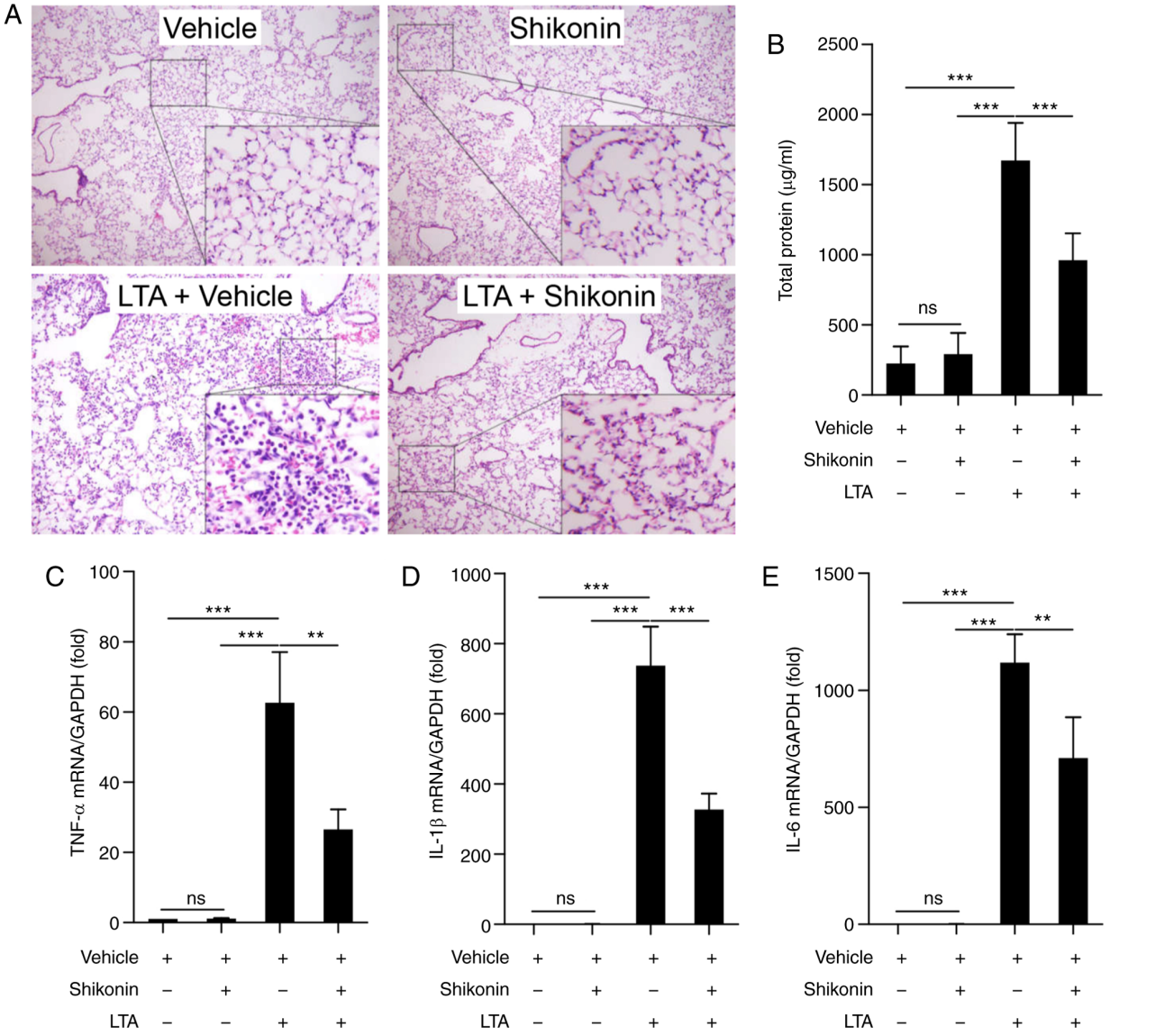

Figure 1. Shikonin attenuates lipoteichoic acid-induced acute lung injury and inflammatory cytokines production. (A) The lung lobes were fixed and hematoxylin and eosin staining was performed to determine the lung injury (original magnification of whole field, x200; enlarged box magnification, x2,000. (B) The lungs were lavaged and the total protein concentration in bronchoalveolar lavage fluid was determined. Total RNAs were isolated from lung homogenates and mRNA expression levels for (C) TNF-α, (D) IL-1β and (E) IL-6 were quantified using reverse transcription-quantitative PCR. Data are presented as mean ± standard error of the mean, n=5, **P<0.01, ***P<0.001. ns, not significant; LTA, lipoteichoic acid.

86.87±1.64% (Fig. 4B). These results suggested that shikonin directly induces neutrophil apoptosis.

Shikonin induces an apoptotic signaling pathway. To further investigate the mechanism by which shikonin promotes neutrophil apoptosis, the activation of caspase-3 (cleaved caspase-3) and PARP (cleaved PARP), which serve important roles in apoptosis, were measured. As shown in Fig. 5, the expression of cleaved caspase-3 and cleaved PARP increased in a dose-dependent manner following treatment with different concentrations of shikonin for 24 h. PARP is a DNA-repair-related enzyme and the major substrate of caspase-3. It can be cleaved into different fragments by cleaved caspase-3 resulting in abnormal DNA repair and apoptosis (26). The content of cleaved PARP was determined and, as expected, the expression of cleaved PARP was elevated in a dose-dependent manner following shikonin treatment (Fig. 5C). As the full-long caspase-3 and PARP were decreased following cleavage, the expression of cleaved caspase-3 and cleaved PARP was normalized by using β-actin (42,43). These data suggested that shikonin induced apoptosis by activating the caspase-3 signaling pathway.

Shikonin inhibits the expression of Mcl-1 and increases the expression of p53. Studies have shown that the apoptosis caused by caspase-3 can be inhibited by the Bcl-2 family proteins (27), with Mcl-1 and Bcl-2 being important proteins of the BCL-2 group (44). To confirm the pro-apoptosis mechanism of shikonin, the effect of shikonin on the expression of the antiapoptotic proteins Mcl-1 and Bcl-2 was examined. Following treatment with different concentrations of shikonin for 24 h, the expression of Mcl-1 was significantly reduced in a dose-dependent manner (Fig. 6A and B); the expression of Mcl-1 was four times lower compared with untreated neutrophils following treatment with 3 μM shikonin for 24 h

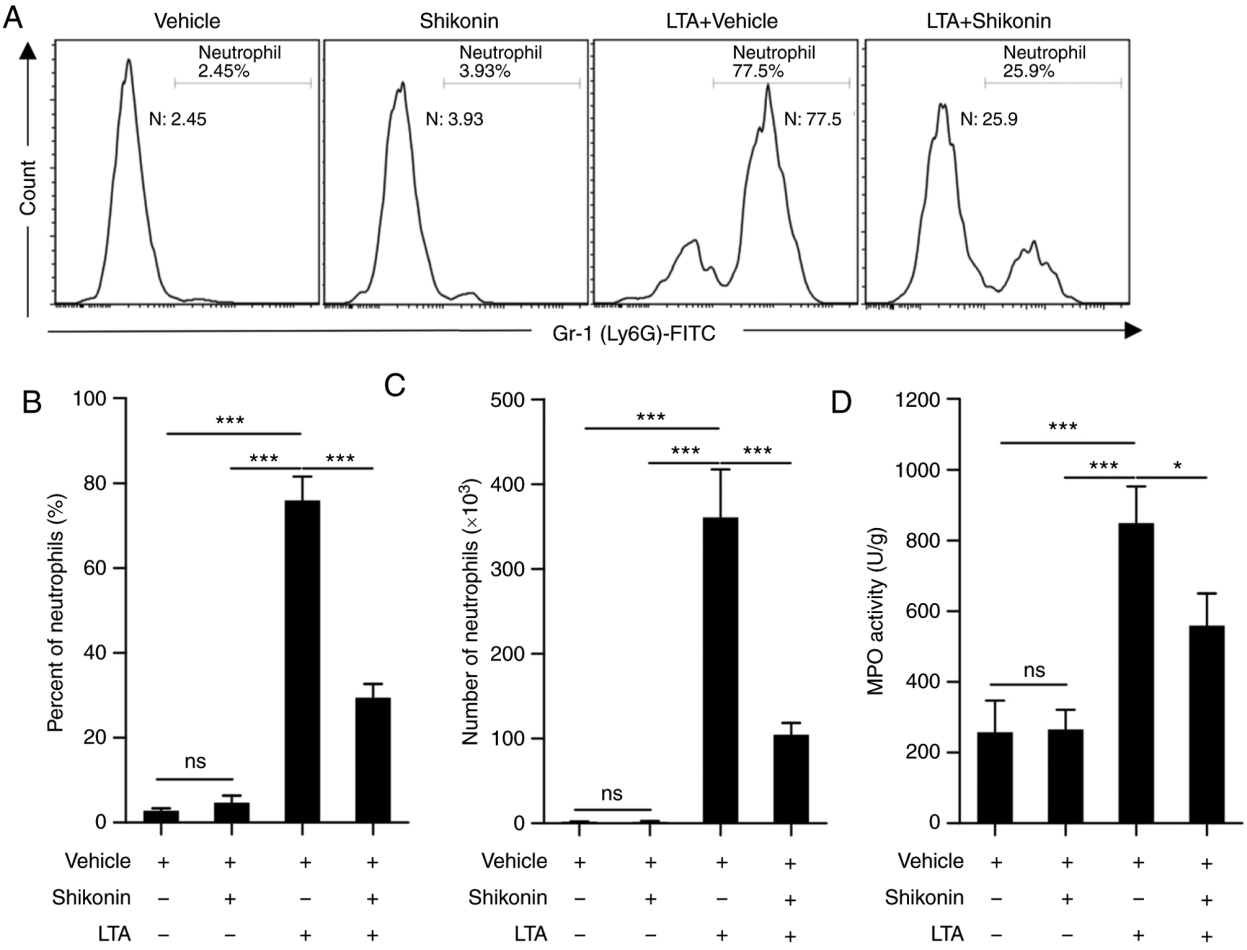

Figure 2. Shikonin inhibits lipoteichoic acid-induced pulmonary neutrophil infiltration. (A) The infiltrated cells in BALF were stained with FITC-conjugated anti-Gr-1(Ly6G) antibody and analyzed by flow cytometry. (B) The percentage of neutrophils (Gr-1+) was quantified. (C) The total number of infiltrated neutrophils in BALF was determined. (D) The lung tissue was homogenized and MPO activity was measured. Values are presented as mean ± standard error of the mean, n=5, *P<0.05, ***P<0.001. BALF, bronchoalveolar lavage fluid; ns, not significant; LTA, lipoteichoic acid; MPO, myeloperoxidase.

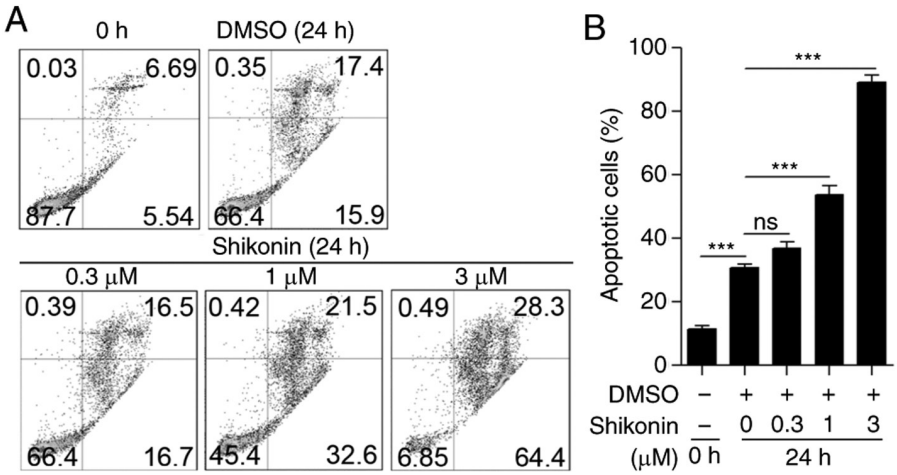

Figure 3. Shikonin induces neutrophil apoptosis. (A) Apoptotic neutrophils were stained with Annexin V/PI and detected by flow cytometry. (B) The proportion of apoptotic cells (Annexin V positive) was quantified. The experiments were independently repeated 3 times. Values are presented as mean ± standard error of the mean, ***P<0.001. ns, not significant.

(Fig. 6B). However, shikonin did not affect the expression of Bcl-2 (Fig. 6A and C). The expression of p53 in neutrophils treated with 0.3, 1 or 3 μM shikonin was also analyzed. The expression of p53 was increased significantly following treatment with 3 μM shikonin for 24 h compared with the DMSO control group (Fig. 6A and D). These results indicated that shikonin inhibited the expression of Mcl-1 and increased the expression of p53 in neutrophils.

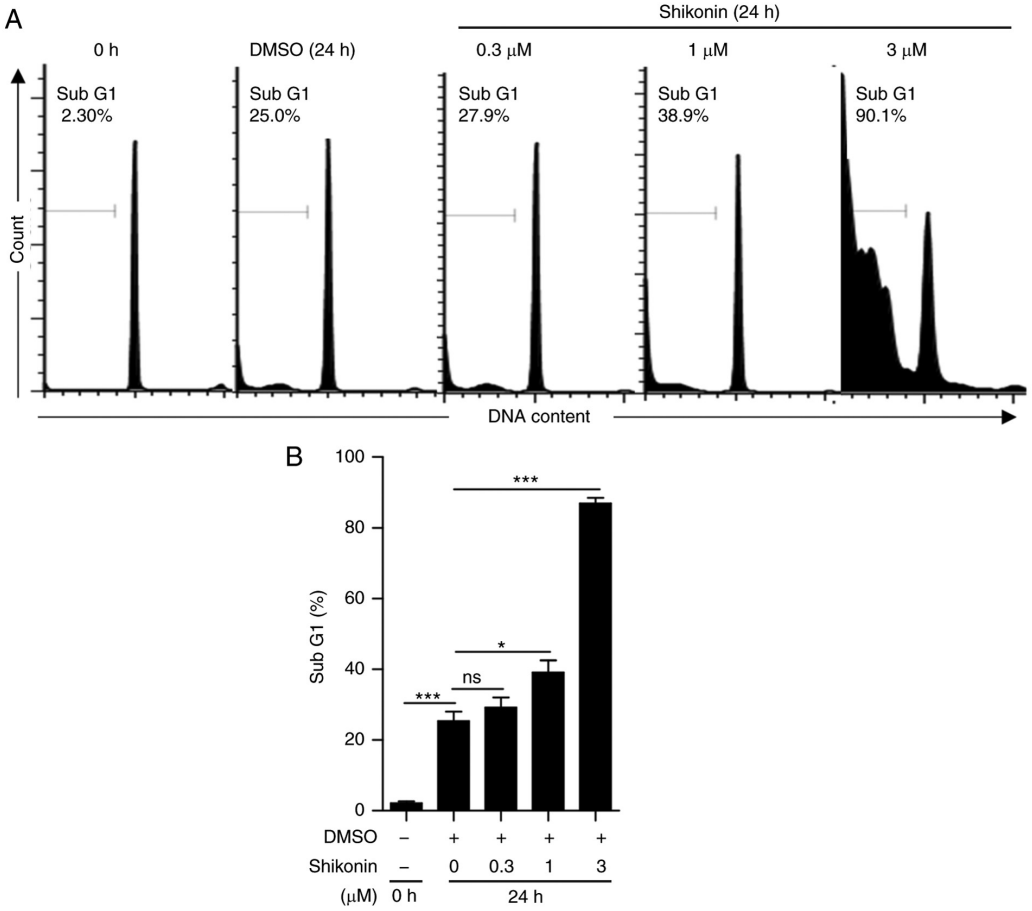

Figure 4. Shikonin increases neutrophil-DNA fragmentation in late apoptosis. (A) The sub-G1 analysis was performed by flow cytometry. (B) The proportion of sub-G1 cells was quantified. The experiments were independently repeated 3 times. Values are presented as mean ± standard error of the mean, *P<0.05, ***P<0.001. ns, not significant.

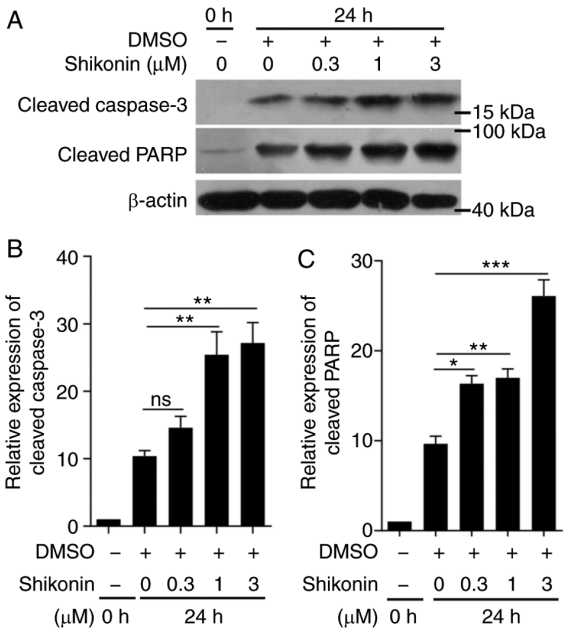

Figure 5. Shikonin induces the activation of caspase-3 and PARP in neutrophils. (A) The cleaved caspase-3, cleaved PARP and β-actin were detected by western blotting. Quantifications of the immunoreactivity of the blots, (B) the cleaved caspase-3 and (C) cleaved PARP were normalized against β-actin. The experiments were independently repeated 3 times. Values are presented as mean ± standard error of the mean, *P<0.05, **P<0.01, ***P<0.001. PARP, poly (ADP-ribose) polymerase; ns, not significant.

Discussion

Sepsis-induced ALI and Acute respiratory distress syndrome are associated with serious inflammation. TNF-α, IL-6 and IL-1β serve important roles in the initiation and development of pneumonia (45,46). TNF-α is the earliest pro-inflammatory cytokine produced mainly by monocytes and can induce the inflammatory cascade in endothelial and epithelial cells thereby accelerating the production of other cytokines, including IL-6 and IL-1β (45,47). The present study identified that the production of TNF-α, IL-6 and IL-1β markedly decreased with shikonin treatment in mice with LTA-induced ALI. A recent study suggested that shikonin serves an anti-inflammatory role in LPS-induced mice by inhibiting the NK-κB signaling pathway *in vitro* and *in vivo* (48). As with LPS stimulation, LTA activates the MAPK and NK-κB signaling pathways by binding to the Toll-like receptor (TLR)4 and TLR2, respectively (49-53). Therefore, the present study hypothesized that shikonin could inhibit LTA-induced cytokine generation *in vitro*. Shikonin is used in traditional Chinese herbal medicine for its various medical properties, including bactericidal activity, promotion of wound healing and anti-cancer effects (54-56). Previous findings have shown that shikonin alleviates LPS-induced ALI (49). LPS is isolated from gram-negative bacteria while LTA is from gram-positive bacteria (57,58). Together with previous research, the findings

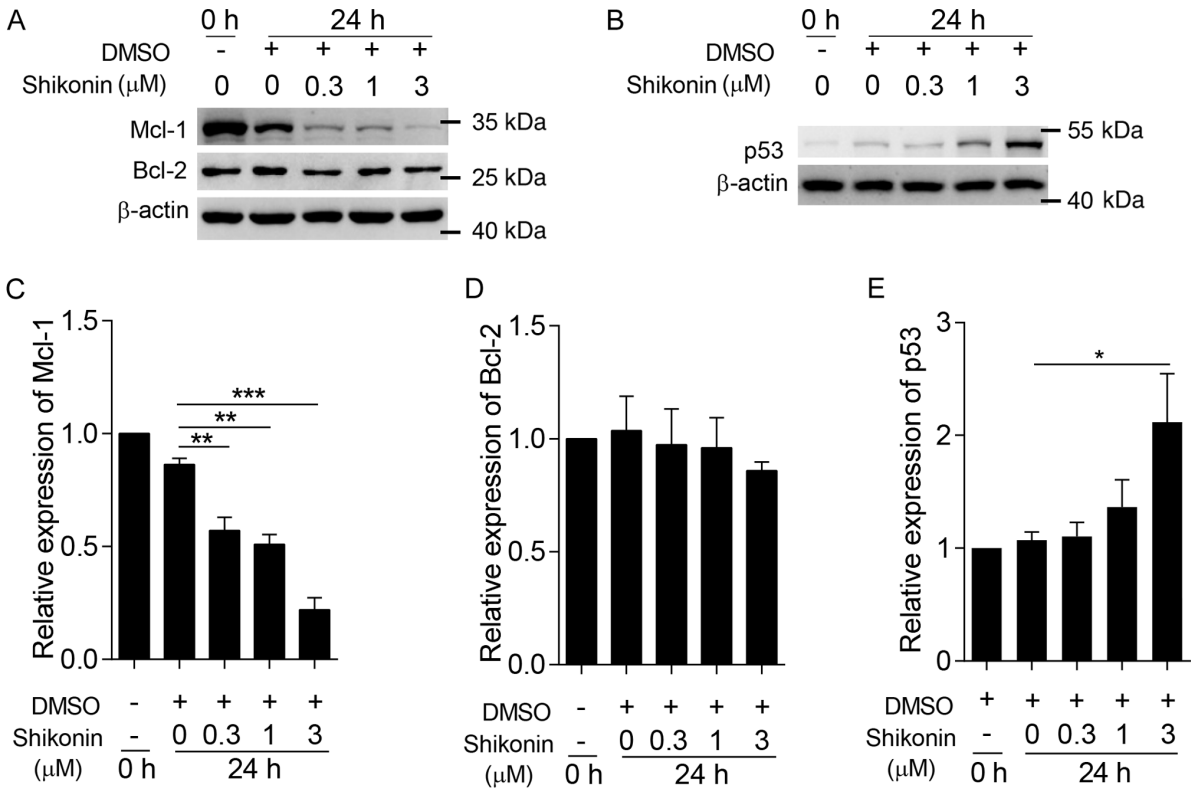

Figure 6. Shikonin reduces the expression of Mcl-1 but has no effect on Bcl-2 expression. The expression of (A) Mcl-1, Bcl-2 and (B) p53 were detected by western blotting. The quantitation of (C) Mcl-1, (D) Bcl-2 and (E) p53 were normalized against β-actin, separately. The experiments were independently repeated for 3 times. Values are presented as mean ± standard error of the mean, *P<0.05, **P<0.01, ***P<0.001. Mcl-1, myeloid cell leukemia-1.

of the present study demonstrated that shikonin is a protective agent against bacteria-induced pneumonia and ALI.

Pathological features of ALI include injured capillary endothelial and pulmonary epithelial cells, increased pulmonary capillary permeability and impaired alveolar gas-exchange (59). Although the pathogenesis of gram-positive-bacteria-induced ALI is not entirely clear, excessive inflammatory responses are considered to be the critical factor in inducing lung injury (60). In addition to cytokine storm, the infiltration of inflammatory cells into lung tissue is widely accepted as a typical characteristic of ALI that leads directly to lung-tissue damage (61). Neutrophils are the dominant leucocytes and provide vital protection against body infection (20). Activated neutrophils release various injurious molecules, including proteolytic enzymes, pro-inflammatory cytokines, oxidants and NO, that can cause damage to the surrounding tissues (17). Primary cultured neutrophils *in vitro* are easily activated by the cultured environment including the Matrigel plate (32). Sustained accumulation of neutrophils contributes to the development of ALI (32). Previous research has confirmed that promoting neutrophil apoptosis is a promising way to treat ALI (62). For example, Rahman *et al* (63) reported that inhibiting erBb (a family of receptor tyrosine kinases) reduces pulmonary inflammation by increasing neutrophil apoptosis in a murine ALI model. Harris *et al* (64) identified that IL-4 accelerates human neutrophil apoptosis through modulated interleukin 4 receptor α-dependent type 2 cytokine signaling that contributes to the ALI resolution. In addition, several compounds, including emodin (65) andrographolide (66) and matrine (67) alleviate ALI by promoting neutrophil apoptosis. The present study indicated that shikonin inhibited LTA-induced ALI by promoting neutrophil apoptosis. Therefore, shikonin is a promising compound for the treatment of neutrophil-related inflammation.

The protective role of shikonin has been researched in previous studies. Lu *et al* (68) identified that shikonin inhibits LPS-induced expression of TNF-α in rat primary cultured macrophages. Prasad *et al* (34) identified that pro-inflammatory mediators including NO, PGE2, TNF-α, inducible nitric oxide synthase and cyclooxygenase-2 were significantly downregulated by shikonin pretreatment in bv-2 microglia. A previous study demonstrates a growth-enhancing effect of shikonin on human dermal fibroblasts (69). Together, those studies suggest that shikonin acts on multiple cell targets. The present study identified that shikonin inhibited inflammation in an LTA-induced ALI mice model by inducing neutrophil apoptosis. Endotoxin-induced apoptosis is partly dependent on the intrinsic mitochondrial pathway (70). The activation of caspase-3 and PARP is the key point of the mitochondrial pathway (26). The effect of shikonin on the mitochondrial pathway was further analyzed. As expected, the levels of cleaved caspase-3 and cleaved-PARP significantly increased with the treatment of shikonin. Previous findings showed that shikonin induces apoptosis of colon cancer (71) and chronic myeloid leukemia cells (72) by reducing the Bcl-2 level. In the present study, however, the expression of Bcl-2 was unchanged but the content of Mcl-1 was significantly increased following treatment with shikonin. This is because the level of Bcl-2 was lower in neutrophils than in cancer cells (27). Thus, its effect is not as important as that of Mcl-1 (73). The decrease

in Mcl-1 contributed to the body's anti-inflammatory response; this result is consistent with that of a previous study (74). Furthermore, a recent study suggested that inhibition of neutrophil apoptosis is closely related to upregulated Mcl-1 and results in increased pulmonary disease (75). Consistent with previous studies, the data from the present study indicated that shikonin enhanced neutrophil apoptosis by increasing cleaved caspase 3 and decreasing Mcl-1 expression. Therefore, Mcl-1 is a more effective target than Bcl-2 in neutrophil apoptosis.

Neutrophils are typically the first leukocytes to be recruited to an inflammatory site and are capable of eliminating pathogens as well as accelerating inflammation (32). Although the present study identified that shikonin inhibited the recruitment of neutrophils to the lung tissues, the mechanisms remain to be elucidated. Furthermore, the results of the present study demonstrated that Mcl-1 was a potential target of shikonin for inducing neutrophil apoptosis. However, it is still unknown how shikonin induces neutrophil apoptosis. The special signal pathways and banding sites of shikonin should to be determined in future studies.

Collectively, the present study demonstrated that pretreatment with shikonin in an LTA-induced murine ALI model alleviated pathological changes in lung tissue, reduced infiltration of inflammatory cells and decreased expression of pro-inflammatory cytokines. Shikonin promoted neutrophil apoptosis by triggering mitochondrial-mediated apoptosis signaling pathways, specifically increasing the level of cleaved caspase-3 and decreasing the expression of Mcl-1. The present study suggested that shikonin is a therapeutic candidate for treating ALI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YoZ and FQ designed the study. YoZ and HZ performed the experiments and drafted the manuscript. MW and SG participated in the animal experiments. LH and TH participated in data analysis. YaZ and YuZ were involved in the interpretation of the data from the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocols of the present study were approved by the Animal Care and Use Committee of Bengbu Medical College (Anhui, China) and conducted in accordance with the guidelines of this committee. The present study adhered to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1996).

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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