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ORIGINAL RESEARCH

Icariside II regulates TLR4/NF-κB signaling pathway to improve septic lung injury

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Abstract

Sepsis is caused by the inadequate response to infection and may eventually lead to fatal organ dysfunction and a high mortality rate. Acute lung injury (ALI) caused by sepsis is an important cause of its high mortality, so effective treatment drugs are urgently needed. Icariside II (ICA II) is derived from Epimedii, a ubiquitous biological flavonoid compound. ICA II has shown multiple biological activities. ICA II alleviates LPS-induced neuroinflammation by inhibiting the TLR4 / MyD88 /NF-κB pathway, however, the possible role of icaridinin II in sepsis induced acute lung injury remains unclear. Herein, we developed a sepsis-related ALI mice model induced by LPS treatment, and found Icariside II ameliorated sepsis-related acute lung injury of mice induced by LPS. Our data further confirmed that Icariside II inhibited the inflammatory response in sepsis-related ALI mice, and ameliorated oxidative stress injury. We further revealed Icariside II inhibited the apoptosis of lung cells via TLR4-NF-κB axis. Our data therefore provided a promising therapeutic drug for the treatment of sepsis-induced ALI.

Keywords

Sepsis; Acute lung injury (ALI); Icariside II (ICA II); Apoptosis; Inflammatory response; TLR4-NF-κB

1. Introduction

Sepsis is caused by the inadequate response to infection and may eventually lead to fatal organ dysfunction and a high mortality rate [1, 2]. In sepsis related to multiple organ damage, lung is one of the most vulnerable target organs, and acute lung injury (ALI) due to its early onset and high incidence [3, 4]. Sepsis-associated ALI is one of the important causes of mortality, and most treatments failed to reduce the mortality of severe sepsis and ALI [5]. Previous studies have reported that endothelial injury leads to neutrophil aggregation, inflammatory response, and oxidative stress, ultimately leading to lung injury [6]. In order to improve the survival rate of patients, it is still necessary to further clarify the pathogenesis of Sepsis induced ALI and find effective therapeutic drugs.

Icariside II (ICA II) is derived from *Herba Epimedii*, a ubiquitous biological flavonoid compound [7]. ICA II has shown multiple biological activities, such as antioxidant, anti-inflammatory, and anti-apoptotic properties [8, 9]. Studies have shown that ICA II alleviates LPS-induced neuroinflammation by inhibiting the TLR4/MyD88/NF-κB pathway in rats [10]. Icariside II alleviates eosinophilic airway inflammation and remodeling by inhibiting the NF-κB and STAT3 pathways in a mouse model of asthma [11]. ICA II can inhibit several pathways, such as COX-2/PGE 2 pathway, and induce mitochondria-dependent apoptosis in tumor cells [12]. However, the role of icaridinin II in sepsis induced acute lung injury remains unclear.

A large number of studies have reported that NF-κB played an important role in the pathogenesis of organ damage caused by sepsis [13, 14]. Toll-like receptor 4 (TLR4)-NF-κB pathway is a classical signaling pathway triggered by pathogen-associated molecular pattern (PAMP) or risk-associated molecular pattern (DAMP) in sepsis [14]. It was reported that TLR4-NF-κB axis was a potential target for the treatment of sepsis.

In this study, we found Icariside II could ameliorate acute lung injury induced by LPS. Our data further confirmed that Icariside II inhibited the inflammatory response in sepsis-related ALI mice, and ameliorated oxidative stress injury. We further revealed Icariside II inhibited the apoptosis of lung cells via TLR4-NF-κB axis. Our data therefore provided a promising therapeutic drug for the treatment of sepsis -induced ALI.

2. Materials and methods

2.1 Sepsis-induced acute lung injury (ALI) model

All animal experiments were approved by the Ethics Committee of Wuhan Hankou Hospital. Icariside II (CAS: 113558-15-9) was bought from Sigma (USA). 32 C57BL/6 mice were bought from the Shanghai Laboratory Animal Center (SLAC; Shanghai, China) and maintained in a room with access to tap water and normal chow diet in 12 hours light–dark cycle.

Animals were divided into four groups (6 mice per group): (1) Control (sham group, same level of saline was aspiration via the airway); (2) Model group (LPS induced ALI group), and LPS (1.5 mg/kg) was administered via nostrils at concentration of 1.5 mg/kg body weight; (3) ICA II-treated control group with the concentration of 20 mg/kg body weight, dissolved in 10% tween 80 + 90% PBS; (4) 20 mg/kg ICA II-treated model group, and LPS (1.5 mg/kg) was aspiration via the airway (8 mice for each group). Mice received intragastrically administration of ICS II 20 mg/kg dissolved in 10% tween 80 + 90% PBS, 2 days before sepsis stimulation. The NF-κB pathway agonist phorbol myristate acetate (PMA) was purchased from Selleck and used in mice at the concentration of 2.5 mg/kg.

2.2 HE staining

The lung tissues collected from all groups were cut into slices. Then slices were dehydrated through absolute alcohol and rehydrated. Slides were stained with hematoxylin for 4 minutes, rinsed, differentiated in 70% alcohol and stained in eosin Y, and cleared in xylenes before mounting. The hyperemia/congestion in lung and alveolar collapse were recognized as lung injury.

2.3 Cytokine measurement

The concentration of the pro-inflammatory cytokines MIP, MPO, IL-10, and TNF-α in the lung tissues was detected with enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (Dakewei, Beijing, China).

2.4 Assessment of antioxidant activity

The levels of GSH-px, MDA, and CAT were assessed by the detection kits of Nanjing Jiancheng Bio-engineering Institute (Jiangsu, China) through ELISA in accordance to the manufacturer's instructions.

2.5 TUNEL assay

The slices were deparaffinized in xylene for 5 minutes, hydrated and rinsed in distilled water. Then the sections were incubated with 3% H2O2 to block endogenous peroxidase activity and TdT Reaction Mixture for 1–2 hours in humidified chamber. Then stop reaction in stop wash buffer. The signals were detected after incubation with Streptavidin-HRP and DAB under light microscope.

2.6 Immunoblot

The total proteins from lung tissues were collected with RIPA lysis buffer and separated by SDS-PAGE. After transferred onto PVDF membrane, membranes were blocked with 2% BSA in TBST buffer at room temperature and subsequently incubated using the specific antibodies against GAPDH (1:2000 dilution, ab8245), cleaved caspase-3 (1:500 dilution, ab32042), cleaved caspase-9 (1:500 dilution, ab2324), TLR4 (1:500 dilution, ab22048), p-p65 (1:200 dilution, ab76302), and p65 (1:500 dilution, ab16502) purchased from Abcam (Cambridge, UK) for 2 hours at room temperature. Then the membranes were subjected to HRP-conjugated secondary antibodies for 1 hour at room temperature. Signals were visualized by an ECL kit (GLPBIO, USA).

2.7 Statistical analysis

Data are displayed as mean $\pm$ SD. The data was analyzed by GraphPad Prism (ver.5.04, GraphPad Software Inc., San Diego, CA, USA). p value < 0.05 was considered statistically significant.

3. Results

3.1 Icariside II alleviates LPS-induced acute lung injury (ALI) in mice

To explore the therapeutic effect of Icariside II on LPS-induced lung injury in mice, we first constructed four group of mice with or without Icariside II pretreatment. After construction of LPS-induced acute lung injury (ALI) model, histological change in all groups were analyzed through HE staining. Model group revealed obvious lung hyperemia/congestion and alveolar collapse compared with sham group (Fig. 1). Importantly, we noticed Icariside II treatment alleviated phenomenon of lung hyperemia (Fig. 1). Taken together, these results indicate Icariside II could attenuate lung injury induced by LPS treatment.

3.2 Icariside II relieves LPS-induced inflammation response in mice

Sepsis was accompanied with activation of NF-κB and transcription of MIP, MPO, IL-10, and TNF-α. To delineate the role of Icariside II on sepsis-induced inflammatory cytokine production, we detected the concentrations of MIP, MPO, IL-10, and TNF-α in the lung tissues of mice respectively. As shown in Fig. 2, sepsis stimulation significantly induced elevated level of MIP, MPO, and TNF-α, and decreased level of IL-10 (Fig. 2A–D) in the lung tissues of mice. However, Icariside II administration relieved the increasing of MIP, MPO, and TNF-α and the decreasing of IL-10 in lung tissues (Fig. 2A–D). These results suggest that Icariside II relieves LPS-induced inflammation response in mice.

3.3 Icariside II affects GSH-px, MDA, and CAT activity in mice with lung injury

To examine the antioxidant effect of Icariside II, the level of GSH-px, MDA, and CAT were analyzed. Compared with Control group, GSH-px, and CAT levels in LPS-induced group significantly decreased, whereas that of MDA markedly enhanced (Fig. 3A–C). Icariside II treatment restored antioxidant capacity dramatically, as exhibited by the increasing of GSH-px, and CAT levels and decreasing of MDA content (Fig. 3A–C). These data therefore suggested Icariside II treatment exerted reduced antioxidant property in lung injured mice.

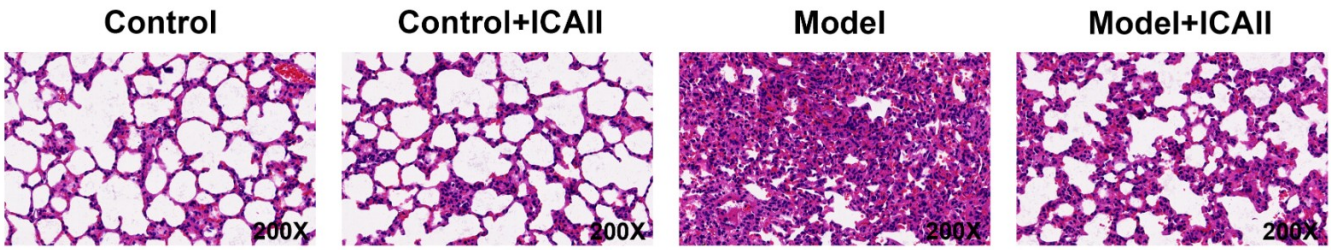

FIGURE 1. Icariside II alleviates LPS-induced lung injury in mice. The histological changes of lung tissue in Control, Model, Control+ 20 mg/kg Icariside II, Model+ 20 mg/kg Icariside II groups.

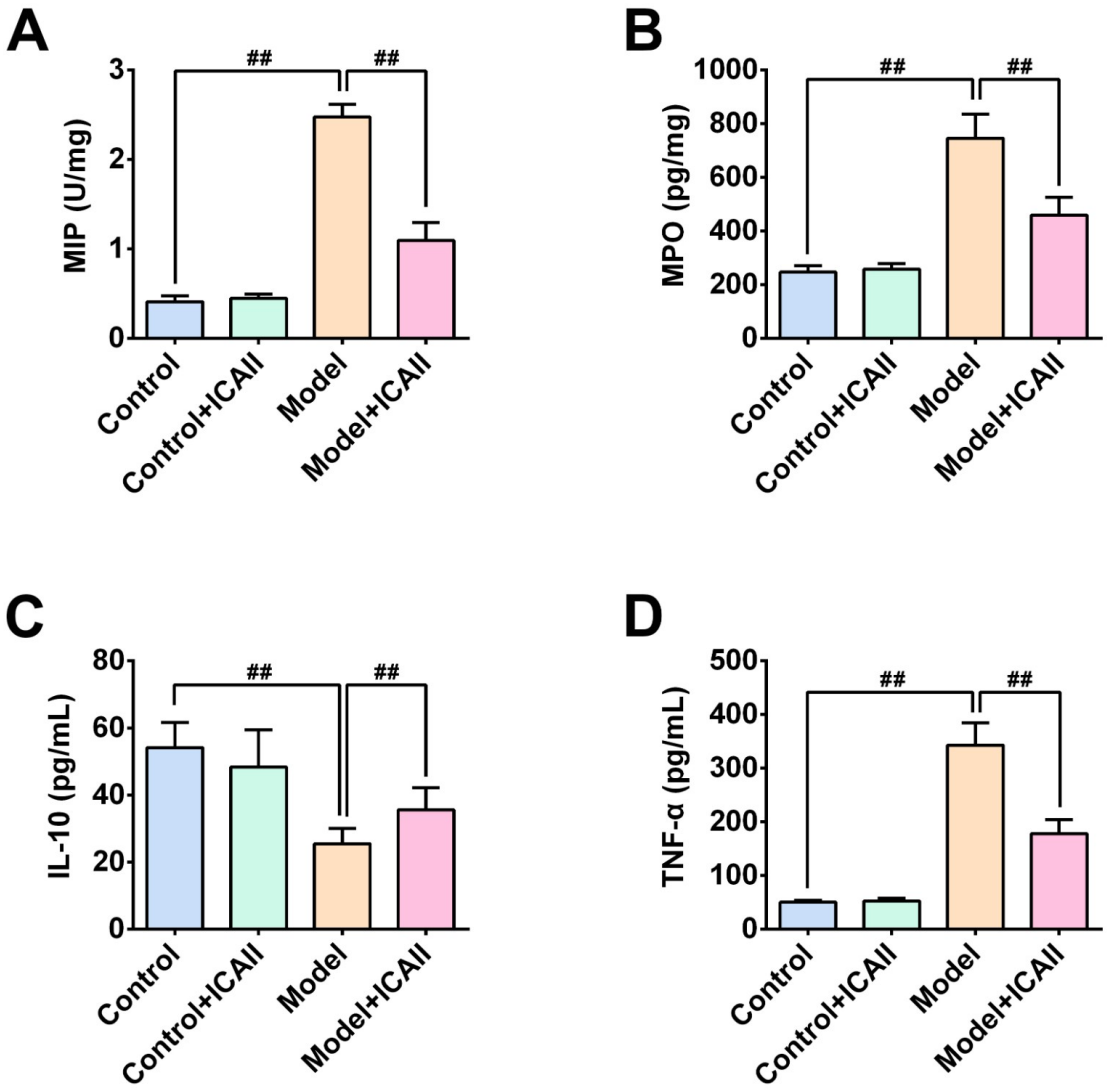

FIGURE 2. Icariside II relieves LPS-induced inflammation response in mice. (A–D) MIP (A), MPO (B), IL-10 (C), and TNF-α (D) levels in lung tissues from the indicated groups. Data are displayed as mean ± SD. ##, $p < 0.01$.

3.4 Icariside II suppresses the apoptosis of lung cells in mice with lung injury

We then detected the effects of Icariside II on the apoptosis of lung cells in mice with lung injury through TUNEL assay and Immunoblot assays. Cleaved caspase-3 and caspase-9 are two markers of apoptosis cells, and we therefore detected their expression levels in lung tissues from the mice. We found compared with control group, Cleaved caspase-3 and caspase-9 levels in LPS-induced group were significantly increased and TUNEL positive cells were accumulated in LPS group. (Fig. 4A–C) Icariside II treatment rescued the increased cleaved caspase-3 and caspase-9 levels dramatically and significantly reduced the TUNEL positive cells, suggesting the inhibition of lung cell apoptosis in mice with lung injury (Fig. 4).

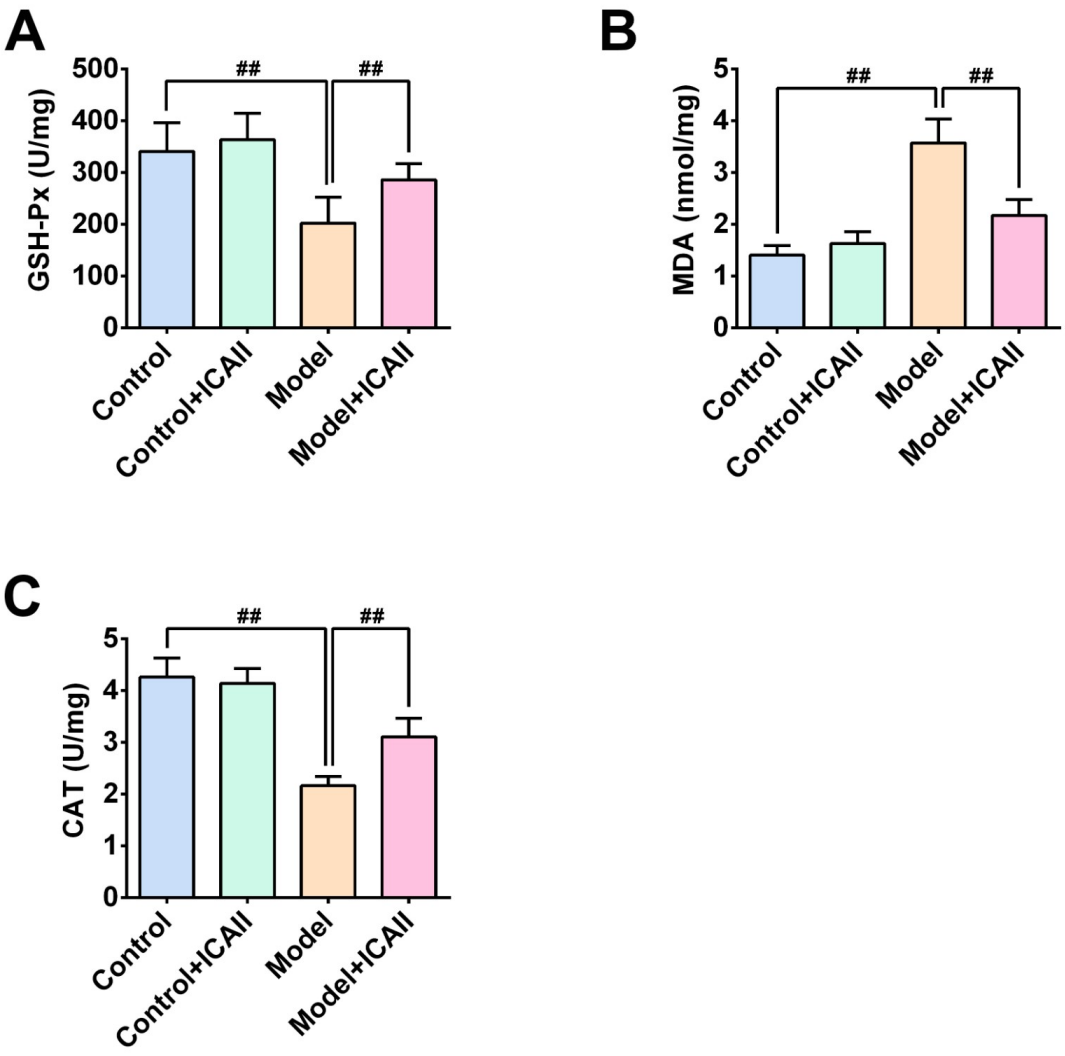

FIGURE 3. Icariside II affects SOD, GSH, MDA level and MOP activity in mice with lung injury. (A–C) GSH-px (A), MDA (B), and CAT (C) level in the four indicated groups were detected with respective kit. Data are displayed as mean ± SD. ##, $p < 0.01$.

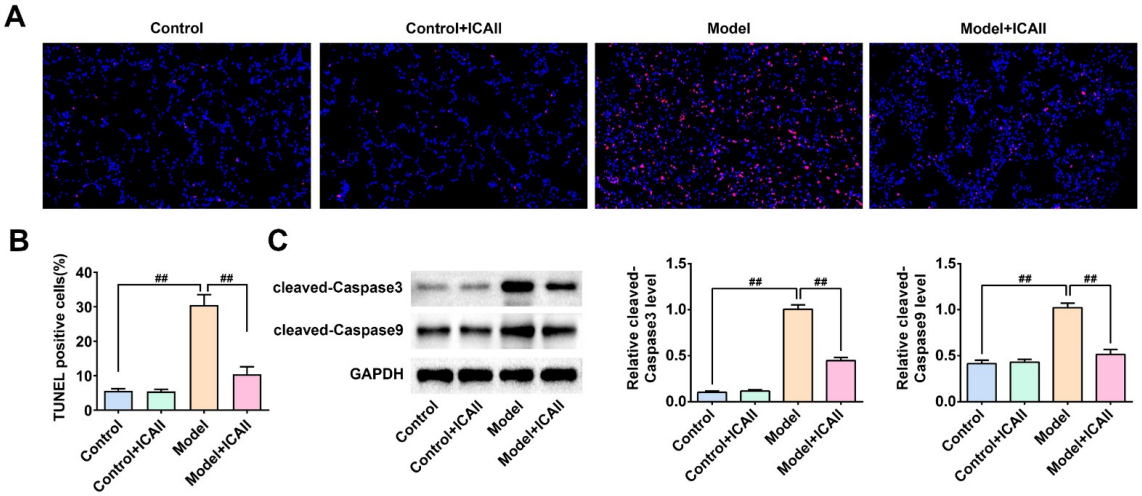

FIGURE 4. Icariside II suppresses the apoptosis of lung cells in mice with lung injury. (A,B) TUNEL assay showed the apoptosis degrees of lung cells upon the indicated treatment. (C) Immunoblot assay detected the protein level of Cleaved caspase-3, cleaved caspase-9, and GAPDH in Control, Model, Control+ 20 mg/kg Icariside II, Model+ 20 mg/kg Icariside II groups. Data are displayed as mean ± SD. ##, $p < 0.01$.

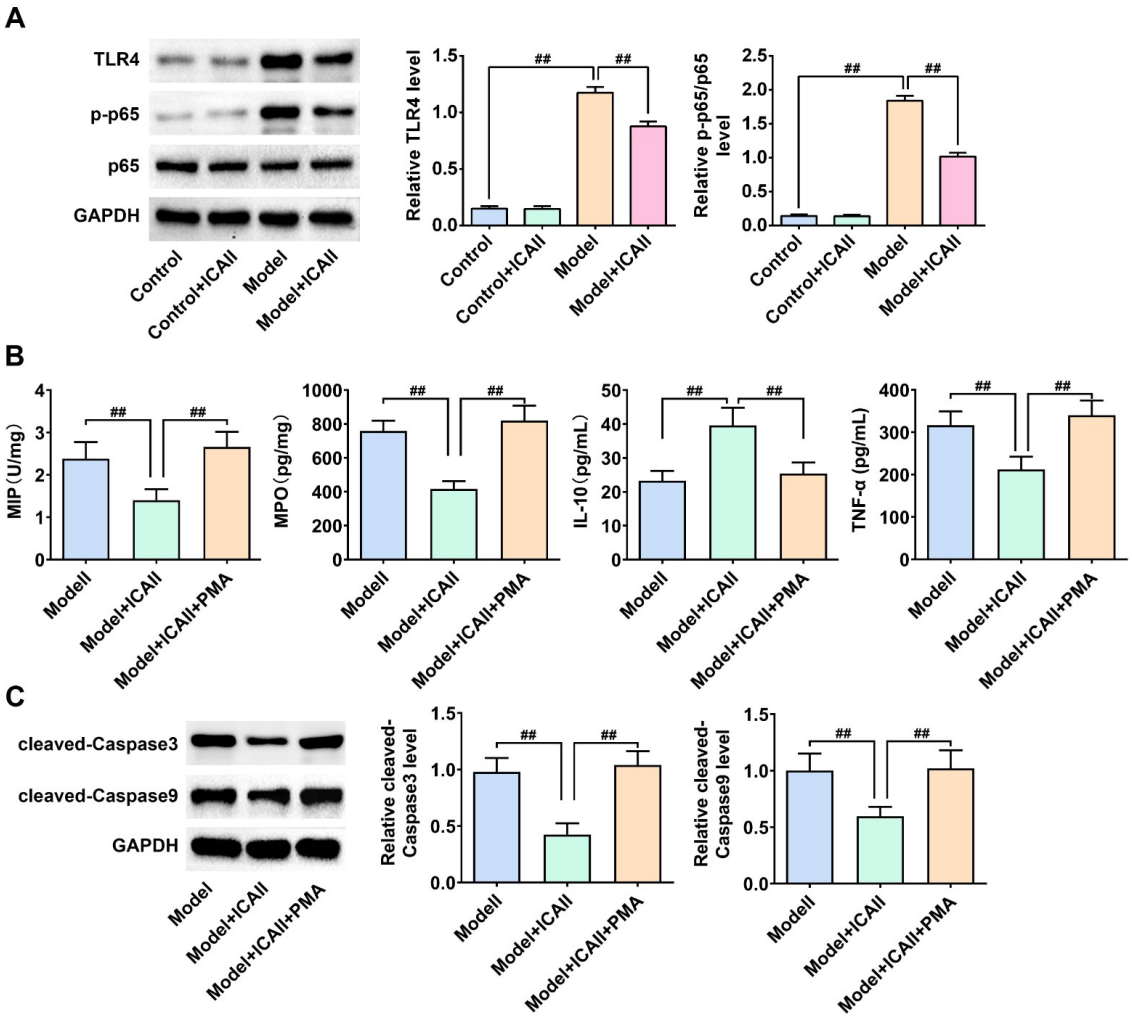

FIGURE 5. Icariside II affected TLR4-NF-κB pathway in mice with lung injury. (A) Immunoblot assay detected the protein level of TLR4, p-p65, p65, and GAPDH in Control, Model, Control+ 20 mg/kg Icariside II, Model+ 20 mg/kg Icariside II groups. (B) ELISA assays showed the levels of MIP, MPO, IL-10, and TNF-α levels in lung tissues from the indicated groups. (C) Immunoblot assays showed the expression of cleaved caspase-3 and cleaved caspase-9 in lung tissues from the indicated groups. Data are displayed as mean $\pm$ SD. ##, $p < 0.01$.

3.5 Icariside II affected TLR4-NF-κB pathway in mice with lung injury

TLR4-NF-κB was involved in sepsis-induced acute lung injury and TLR4 was also a critical regulator in sepsis progression. We aimed to evaluated the potential effect of Icariside II in TLR4 mediated NF-κB pathway. We found LPS-induced lung injury effectively enhanced TLR4 expression. Additionally, the phosphorylation levels of p65 was increased significantly in LPS-induced ALI mice, suggesting the effects on NF-κB pathway (Fig. 5A). However, Icariside II treatment significantly reduced the expression of TLR4 and decreased phosphorylation levels of p65 in the lung tissue of LPS-induced lung injury model (Fig. 5A). Subsequently, the agonist phorbol myristate acetate (PMA) of NF-κB pathway was further used. Through ELISA assays, we noticed the Icariside II treatment decreased the MIP and MPO levels, whereas PMA treatment obviously rescued the decreased levels caused by Icariside II (Fig. 5B). In addition, IL-10 and TNF-α levels were also reversed after the treatment of PMA upon Icariside II treatment (Fig. 5B). We further performed Immunoblot assays and found Icariside II treatment decreased the expression of cleaved caspase-3 and cleaved caspase-9, whereas PMA treatment obviously reversed their expression upon Icariside II treatment (Fig. 5C), further confirming the effects were through NF-κB pathway. Therefore, these findings suggest that Icariside II affected TLR4-NF-κB pathway in mice with lung injury.

4. Discussion

Sepsis, which is caused by bacterial infection, is known as a clinical process of systemic inflammatory response and an important cause of death [15]. Lung is the most vulnerable target organ in sepsis [5]. Sepsis-induced ALI has a high incidence of morbidity and mortality, and currently common treatments are limited and ineffective [16]. To decrease the mortality of this disease, the development of effective therapeutic drugs is still badly needed [14, 17]. We here found a ubiquitous biological flavonoid compound from Epimedium, Icariside II, could have

significant therapeutic efficiency on this disease. Our data confirmed that Icariside II attenuated LPS induced lung injury in mice, and also reduced the inflammatory response and oxidative stress of mice. Our findings therefore provided a promising drug for the treatment of sepsis induced ALI.

Here, we constructed the sepsis model of C57BL/6 mice by the use of LPS, and the symptoms of ALI were noticed in the model. The first mock exam has been widely used in the pathogenesis and drug screening of sepsis induced acute lung injury [18]. This animal model has several advantages, such as simple steps and no inoculation [18]. Additionally, it could also induce the animal to produce a large number of cytokines, similar to that in patients. Through this model, we identified a promising therapeutic drug for the treatment of Sepsis-induced ALI, and further study should focus on the mechanisms underlying Icariside II alleviating Sepsis-induced ALI.

The therapeutic function of traditional Chinese medicine on Sepsis and ALI have been widely reported [14, 18]. As an effective constituent of traditional Chinese medicine *Epimedii*, ICA II has shown multiple biological activities including antioxidant, anti-inflammatory, and anti-apoptotic [14]. ICA II protected cardiomyocytes from hypoxia-induced injury by upregulating the miR-7-5p/BTG2 axis and activating the PI3K/Akt pathway [7]. ICA II could alleviate LPS-induced neuroinflammation by inhibiting the TLR4 / MyD88 /NF-κB pathway, and also alleviate eosinophilic airway inflammation and remodeling by inhibiting the NF-κB and STAT3 pathways in an asthma mouse model [11]. Notably, many studies showed the effects of ICA II on inflammatory response and oxidative stress [19]. ICA II overcome BRAF inhibitor resistance in melanoma via the induction of ROS production [20]. In this study, we found Icariside II ameliorated LPS-induced ALI, suppressed inflammatory response and oxidative stress injury, and the apoptosis of lung cells. Therefore, we thought ICA II had an effective therapeutic function on the treatment of sepsis-induced ALI. In this study, we found that the effect of ICA II on reducing proinflammatory mediators (MIP, MPO, and TNF-alpha) and increasing IL-10 in lung tissues of the mice with LPS-induced inflammation. Interestingly, IL-10, as a special inflammatory factor, has been proved to be up-regulated in the sepsis model, but it does have a double-edged sword effect on sepsis [21, 22]. Some studies have also shown that the up-regulation of this inflammatory factor has a negative effect on sepsis [23, 24]. Therefore, in-depth understanding of the effect of IL-10 upregulation on sepsis treatment is also a future research direction.

TLR4-NF-κB axis widely affected the occurrence and development of acute lung injury [25]. Multiple studies provided a number of drugs which could improve acute lung injury of patients through TLR4-NF-κB axis [26]. For an example, Madecassoside could protect against LPS-induced ALI through suppressing TLR4/NF-κB activation [27]. Gut microbiota could alleviate LPS-induced ALI by regulating the TLR4/NF-κB pathway [25]. Additionally, miR17 also inhibited the inflammatory response in lipopolysaccharide induced ALI via this pathway [28]. These studies, together with our findings, confirmed TLR4-NF-κB could serve as a promising therapeutic target for the treatment of sepsis-induced ALI.

5. Conclusions

In conclusion, we noticed Icariside II ameliorated LPS-induced ALI. Our data further confirmed that Icariside II inhibited the inflammatory response in sepsis-related ALI mice, and suppressed oxidative stress injury. We further revealed Icariside II inhibited the apoptosis of lung cells via TLR4-NF-κB axis. Our data therefore provided a promising therapeutic drug for the treatment of sepsis-induced ALI and clarified the mechanism.

AUTHOR CONTRIBUTIONS

YL and BL designed the study, supervised the data collection, YL analyzed the data, interpreted the data, BL prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of Wuhan Hankou Hospital (Aprroval No 20180734).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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