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ORIGINAL RESEARCH

Xuebijing Injection Alleviates Sepsis-Induced Acute Lung Injury by Inhibition of Cell Apoptosis and Inflammation Through the Hippo Pathway

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Background: Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is a critical complication of sepsis, strongly associated with poor prognosis. Xuebijing (XBJ) injection, a standardized multi-herbal formulation containing five active components (safflower, red peony, Chuanxiong, Salvia miltiorrhiza, and Angelica sinensis), has demonstrated clinical efficacy in sepsis management through its multimodal pharmacological actions. While XBJ is increasingly used as an adjunctive therapy for sepsis-induced ALI/ARDS, its specific protective mechanisms remain incompletely understood. The purpose of this study was to evaluate the improvement effect of XBJ injection on ALI in sepsis and its undefined molecular mechanism.

Methods: Sepsis-induced ALI (SALI) murine animal model was established in rats by cecum ligation and puncture (CLP), and these rats were treated with or without XBJ injection. Lung injury across different groups was assessed by HE staining, W/D ratio, and BALF analysis. ZO-1 and CD31 immunofluorescence were used to evaluate endothelial damage. To illustrate the mechanism of the protective effect of XBJ on SALI, human umbilical vein endothelial cells (HUVECs) stimulated with lipopolysaccharide (LPS) were used to establish an in vitro endothelial inflammation model. Inflammatory cytokines and apoptotic proteins were measured in LPS-stimulated HUVECs to evaluate endothelial inflammation. Lung tissue transcriptomic analysis was performed to explore downstream pathway, and key Hippo pathway related proteins were assessed in both rat lung tissue and HUVECs.

Results: In SALI animal models, treatment with XBJ significantly alleviated lung injury. Meanwhile, a substantial amelioration of endothelial damage was observed. In vitro, XBJ substantially mitigated apoptosis and inflammatory response of LPS stimulated HUVECs. Meanwhile, transcriptomic analysis revealed that XBJ significantly upregulates the gene expression of the Hippo-related signaling pathway, and we further validated these findings in both rat lung tissues and HUVECs.

Conclusion: Our study establishes the preventive role of XBJ injection in SALI by alleviating apoptosis and inflammatory response partially through regulating the Hippo pathway.

Keywords: sepsis, acute lung injury, Xuebijing, inflammatory response, cell apoptosis, Hippo signaling pathway

Introduction

Sepsis is a life-threatening condition characterized by organ dysfunction resulting from infection, often with significant involvement of the lungs. Sepsis-associated acute lung injury (SALI) is a common and severe complication,[1,2] with approximately 50% of sepsis patients developing acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and a mortality rate of 35.5%.[3,4] Despite the widespread use of various clinical interventions, such as mechanical ventilation,

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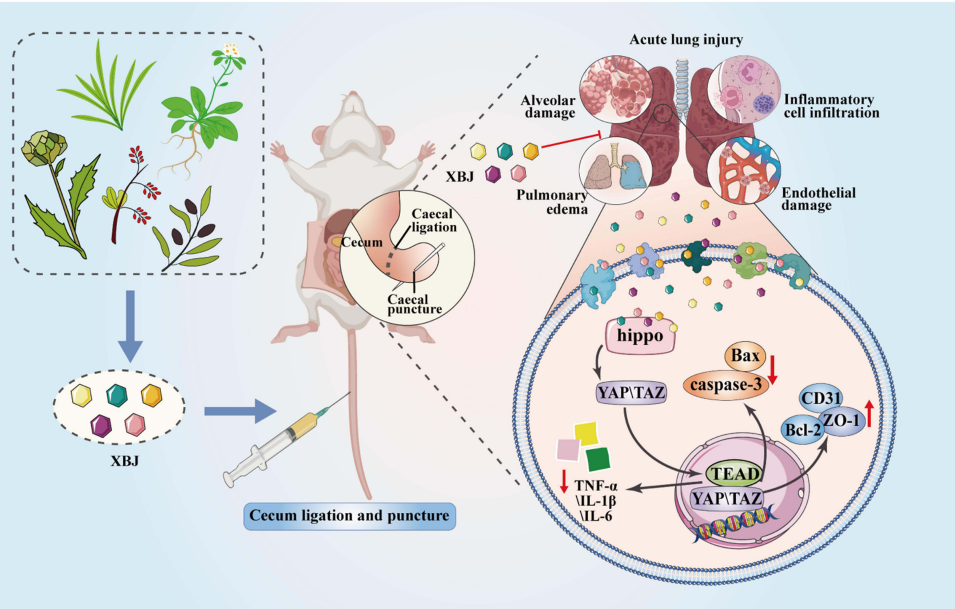
Graphical Abstract



fluid resuscitation, and antibiotic therapy, the high mortality rate associated with SALI has not been substantially reduced.[5,6] Therefore, a better understanding of the underlying mechanisms of SALI and the development of more effective therapeutic strategies are urgently required.

The disruption of the alveolar-capillary barrier, which is a key pathogenic mechanism of SALI, impairs fluid exchange between the alveoli and bloodstream, thereby contributing to pulmonary edema, and, in severe cases, can progress to ARDS and ultimately result in respiratory failure.[7,8] Endothelial cells (ECs) are essential for maintaining the integrity and function of the alveolar-capillary barrier.[9] Early in SALI, alveolar endothelial cells can remain functionally stable,[10,11] however, as the disease progresses, they undergo apoptosis and dysfunction, leading to reduced alveolar surfactant synthesis, impaired gas exchange, and increased pulmonary edema.[12,13] Recent studies have demonstrated that modulating the inflammatory response of ECs and reducing ECs apoptosis can effectively mitigate the onset and progression of SALI in animal models of ALI.[14] These findings highlight the potential of targeting ECs dysfunction as a crucial therapeutic strategy for managing SALI.[15]

Emerging evidence highlights the therapeutic potential of traditional Chinese herbal medicines in modulating inflammatory responses and cellular apoptosis, particularly for multi-organ dysfunction syndromes (MODS) including sepsis.[16] Among these, Xuebijing (XBJ) Injection—a standardized polyherbal formulation—has garnered significant attention due to its regulatory approval and demonstrated efficacy in critical care settings.[17] XBJ Injection, approved by China's National Medical Products Administration in 2004, is a quintessential traditional Chinese medicine (TCM) formula comprising five component herbs: safflower, red peony, Chuanxiong, Salvia miltiorrhiza, and Angelica sinensis[18] Pharmacological investigations characterize XBJ as a polypharmaceutical agent exhibiting "multi-compound, multi-target, multi-pathway" therapeutic properties.[19] Its bioactive matrix—encompassing hydroxysafflor yellow A, paeoniflorin, salvianolic acids, and ligustrazine—orchestrates synergistic modulation of inflammatory cascades, coagulation disorders, and oxidative stress.[20]

XBJ demonstrates multifaceted pharmacological actions including anti-inflammatory, anticoagulant, and microcirculation-improving effects.[21] XBJ has been clinically proven to reduce 28-day mortality in sepsis patients by suppressing early inflammatory responses, while also shortening ICU stays in ARDS cases.[22,23] In septic rat models, XBJ has been shown to protect lung function by alleviating oxidative stress and ferroptosis, as well as maintaining lung permeability

through the upregulation of Toll-interacting protein expression.[24,25] Despite reports indicating that XBJ can mitigate ECs damage and enhance intestinal microcirculation in septic rats,[26] its impact on lung endothelial function in the context of SALI remains largely under-investigated.

Given XBJ's established efficacy in sepsis-related organ protection and its regulatory role in endothelial dysfunction —a key pathological feature of SALI—we selected this drug to explore its potential in SALI management. Furthermore, the lack of mechanistic studies on XBJ's direct effects on pulmonary ECs underscores the novelty of this investigation. Therefore, this study aims to investigate XBJ's protective effects on SALI by examining endothelial function modulation, analyzing associated pathways through transcriptomics, and experimentally validating the underlying mechanisms.

Materials and Methods

Rats

Sprague-Dawley (SD) rats (male, average weight 280–300 g), which were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (certificate no. SCXK (Liao) 2020–0001), were housed in Anhui Medical University's Experimental Animal Center, a controlled environment (20–22°C, 50–60% relative humidity) with a 12-hour light-dark cycle. Rats were allowed free access to food and water ad libitum. All animal experiments were approved by the Animal Ethics Committee of Anhui Medical University (certificate no. LLSC-20242423) and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Groups and Treatments

SD rats (n = 8, sham group) that did not receive the cecal ligation and puncture (CLP) were euthanized after 24 hours. Other SD rats (n = 24) that underwent CLP were fasted for 12 hours, anesthetized with 4% isoflurane and were ligated and punctured twice in two-thirds of the ileocecal region with an 18-gauge needle. The cecum was carefully compressed to extrude a small amount of its contents. After surgery, the incision was sutured, and 10 mL sterile saline was administered subcutaneously for fluid resuscitation. These rats were randomly divided into 3 groups according to whether to receive XBJ injection and injection dose: CLP group, CLP + low-dose XBJ group (4 mL/kg, XBJ(L)) and the CLP + high-dose XBJ group (8 mL/kg, XBJ(H)). In the XBJ group, CLP rats were injected with XBJ (4 mL/kg or 8 mL/kg) immediately and every 12 hours thereafter. Serum, lung tissue and BALF were collected from all rats after 24 hours.

Cell Cultivation and Treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from Yifei Biotech. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Thermo Scientific, USA) and 1% penicillin and streptomycin (Beyotime, China). Cells were grown in an incubator at 37°C supplemented with 5% CO_2 and were divided into three groups according to whether or not to receive lipopolysaccharide (LPS) (1 μg/mL, Sigma, USA) and XBJ (50 μL/mL) treatment: control, LPS, and LPS+XBJ. Cells were collected after 24 hours of treatment.

Hematoxylin and Eosin Staining

Lung tissues were deparaffinized in xylene and rehydrated through a series of graded alcohol solutions (100%, 95%, 70%). The sections were then stained with Hematoxylin for 5–10 minutes, followed by washing in running tap water. Next, the tissue was stained with Eosin for 1–3 minutes. After staining, the sections were dehydrated through a series of graded alcohol solutions (70%, 95%, 100%), cleared in xylene, and mounted with a coverslip using a suitable mounting medium.

Wet/Dry Ratio

The upper lobe of the right lung was harvested from the rats to measure its wet weight. It was then dried at 80°C until a stable weight was achieved. The wet-to-dry (W/D) ratio was subsequently calculated using the formula: wet weight divided by dry weight.

BALF Collection

The trachea of rats were exposed and cannulated after euthanasia, and the lungs were lavaged with 4 mL of sterile normal saline three times, achieving a recovery rate of over 85%. The bronchoalveolar lavage fluid (BALF) was then centrifuged at 2000 rpm for 15 minutes to separate the cell pellet from the supernatant. The supernatant was subsequently stored at −80°C for future analysis and the cell pellet was used immediately for neutrophil analysis.

BALF Analysis

The supernatant was measured by BCA Protein Assay Kit (Beyotime, China) to evaluate total protein content in the BALF. The cell pellet was resuspended in PBS and cells were measured by a fully automated hematology analyzer (SYSMEX XT-2000i, Japan).

RNA Sequencing Analysis

Lung tissues were collected from three rats in the CLP and CLP + XBJ (H) groups. Total RNA from lung tissue was subjected to RNA sequencing (RNA-seq) using the BGISEQ-500 platform (Huada Genomics). The sequencing reads were aligned to RGSC Rnor_6.0 using Bowtie2. Gene expression levels were normalized using RSEM to obtain FPKM values, which were subsequently used to analyze the expression levels in the tissues. Differentially expressed genes (DEGs) were identified based on the false discovery rate (FDR) and log fold change (logFC), with the following thresholds: Fold change≥2, FDR<0.05. Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the DEGs were performed using the clusterProfiler R package.

Cell Toxicity Assay

HUVECs were seeded into 96-well microplates at a concentration of 8,000 cells per well and maintained at 37°C for a 12-hour period to ensure adherence. They were then treated with XBJ at concentrations from 50 to 350 μL/mL for 24 hours. After treatment, CCK-8 reagent was added in a light-protected area and incubated for 1 hour. Absorbance was measured at 450 nm, and the procedure was repeated three times. Cell viability was calculated based on the absorbance readings.

ELISA Analysis

ELISA was performed to measure the concentrations of IL-6, TNF-α, and IL-1β in rat lung tissues, serum samples and HUVEC cell supernatants according to the manufacturer's instructions (Elabscience, Wuhan, China).

RNA Extraction and Quantitative Real-Time RCR (qRT-PCR)

Total RNA (1 μg) was extracted from lung tissues and HUVEC cells using a total RNA extraction kit (GOONIE, China) followed by reverse transcription of mRNA using cDNA Synthesis SuperMix (NovoProtein, China) per the standard manufacturer's instructions. qPCR was performed with SYBR Green qPCR Master Mix (GLPBIO, USA), with gene expression normalized to GAPDH. Gene expression was calculated using the $2^{\wedge-\Delta\Delta Ct}$ method. Primer sequences are listed in Tables 1 and 2.

Table 1 Primer Information for PCR in Rat

Gene Name	Forward Primer	Reverse Primer
YAP	GGAGAAGGAGAGGCTGCGATTG	GGCAACTGGCTGCGGAGAG
TAZ	TGGACGGCTGATTGCTGAGTG	AGGGTGGACTGTTAGGGAGGAC
GAPDH	AGCCCTCCCTTCTCTCGAAT	CCCCACAACACTGCATTCAC

Table 2 Primer Information for PCR in HUVEC

Gene Name	Forward Primer	Reverse Primer
YAP	GGAGAAGGAGAGGCTGCGATTG	GGCAACTGGCTGCGGAGAG
TAZ	TGGACGGCTGATTGCTGAGTG	AGGGTGGACTGTTAGGGAGGAC
GAPDH	AGCCCTCCCTTCTCTCGAAT	CCCCACAACACTGCATTCAC

Immunoblotting Analysis

Rat lung tissues and HUVEC cells were dissected and homogenized in ice-cold RIPA lysis solution (P0038, Beyotime, China). Proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Primary antibodies were used in this study: anti-Bax (WL01637, 1:1000, Wanleibio, China), Bcl-2 (WL01556, 1:1000, Wanleibio, China), Caspase-3 (WL02117, 1:1000, Wanleibio, China), YAP (WL03624, 1:1000, Wanleibio, China), p-YAP (13008T, 1:1000, CST, USA), TAZ (23306-1-AP, 1:1000, Proteintech, China), MST2 (ET1610-8, 1:1000, HUABIO, China), p-MST1/2 (HA721737, 1:1000, HUABIO, China), LATS1/2 (YT6125, 1:2000, Immunoway, China), p-LATS1/2 (YP1222, 1:1000, Immunoway, China) and β-actin (20536-1-AP, 1:10000, Proteintech, China). After incubation with HRP-conjugated secondary antibodies, protein bands were visualized using chemiluminescent detection (PK10002, Proteintech, China).

Immunofluorescence Staining

Lung tissue sections were incubated overnight at 4°C with primary antibodies against YAP (1:500), TAZ (1:500), ZO-1 (GB115686-100, 1:500, Servicebio, China), and CD31 (GB153151-100, 1:500, Servicebio, China). The next day, sections were treated with Cy3-tagged Goat Anti-Rabbit IgG (GB21303, 1:500, Servicebio, China) for YAP and CD31, and Goat Anti-Rabbit IgG Alexa Fluor 488 (GB25301, 1:500, Servicebio, China) for ZO-1 and TAZ for 1 hour at room temperature. After washing, sections were counterstained with DAPI (G1012-10ML, Servicebio, China), mounted, and observed under a fluorescence microscope. For HUVECs, cells at 80% confluence were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% BSA. After overnight incubation with primary antibodies, cells were treated with fluorescent secondary antibodies for 1 hour at room temperature in the dark. Nuclear staining was performed with DAPI, and fluorescence signals were observed using a fluorescence microscope (Zeiss, Axio Scope.A1).

Statistical Analysis

Data analysis was performed using GraphPad 9.0. Student's *t*-test was used for comparisons between two groups, while one-way ANOVA was applied for comparisons between multiple groups. Continuous variables are reported as mean ± SD, and categorical variables as frequencies (%). Statistical significance was set at $P < 0.05$.

Results

XBJ Treatment Can Relieve SALI in Rats

First, the protective effect of XBJ against SALI was evaluated. The CLP group displayed evident lung tissue impairment exhibited by the marked inflammatory cell infiltration, pulmonary edema, hemorrhage, and alveolar damage. Treatment with both XBJ(L) and XBJ(H) significantly lessened these impairments. A more pronounced improvement was observed in the XBJ(H) group (Figure 1A). Lung injury scores corroborated the reduction in tissue damage by XBJ, particularly with XBJ(H) ($P<0.0001$) (Figure 1B). Biochemical analyses demonstrated that XBJ improved the wet-to-dry weight ratio ($P<0.0001$). Besides, it reduced total protein in BALF ($P<0.0001$) and decreased neutrophil percentages ($P<0.0001$) (Figure 1C–E).

Immunofluorescence staining for ZO-1 and CD31 in lung tissue revealed distinct morphological restoration by XBJ. The CLP group showed decreased ZO-1 expression and irregular CD31 distribution, indicating endothelial damage. XBJ(L) treatment partially attenuated these abnormalities, while XBJ(H) treatment exhibited a more pronounced restoration of ZO-1 expression and CD31 organization, aligning with the trend of dose-dependent efficacy observed in

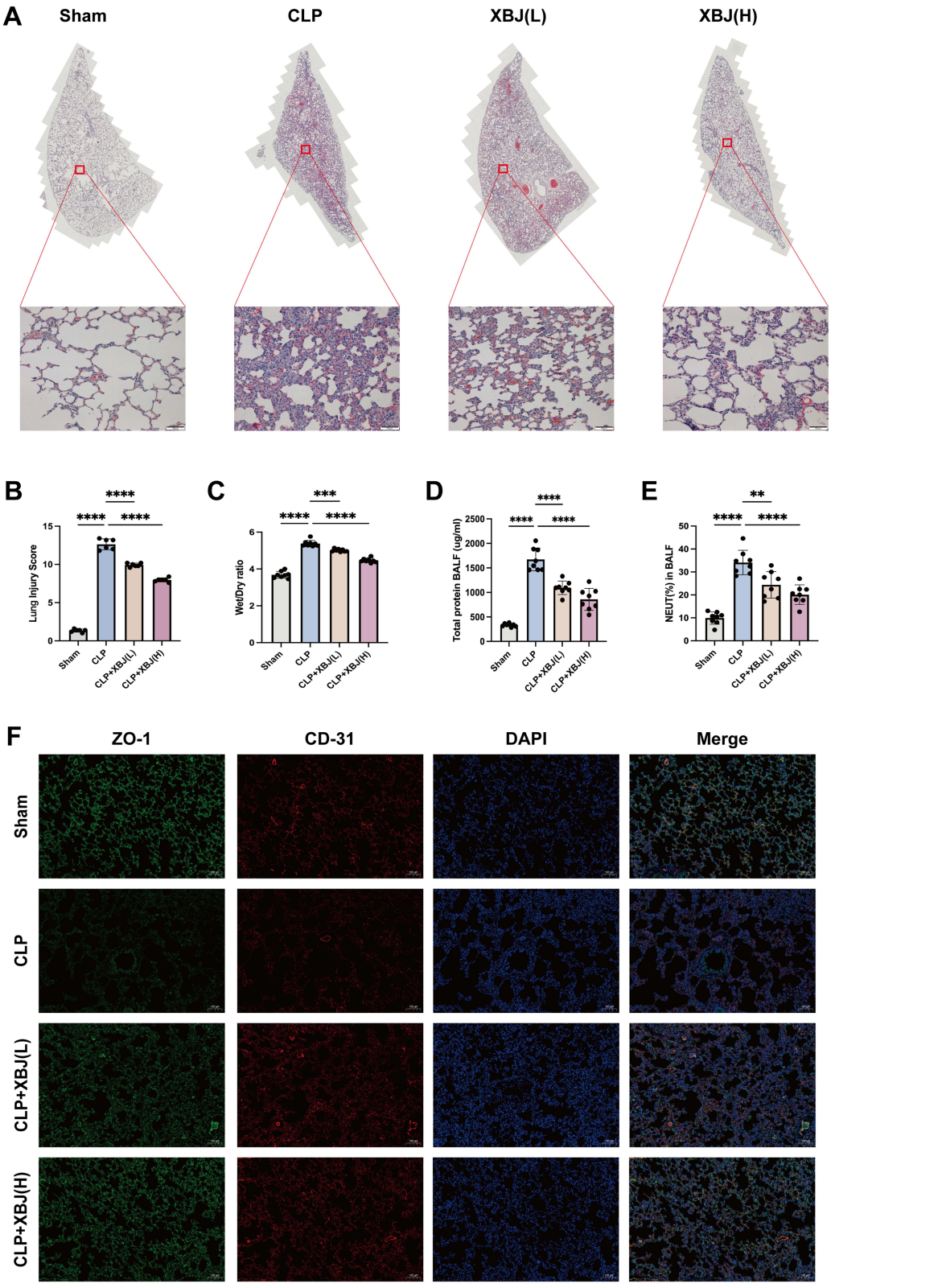


Figure 1 The Effect of XBJ on Rats with SALI. (**A**) Histopathological changes of rat lung tissues observed by HE staining. Scale bar: 50 μm, Magnification: ×400. (**B**) The lung injury score of rats. (**C**) The wet/dry ratio of the lungs of rats with different treatments (N=8). (**D**) The total protein concentration in BALF was evaluated using the BCA protein concentration assay kit (N=8). (**E**) The proportion of neutrophils in each group was detected by a fully automated hematology analyzer (N=8). (**F**) Distribution of ZO-1 (green) and CD31 (red) in rat lung tissues. Nuclei are stained with DAPI (blue). Scale bar: 100 μm, Magnification: ×200. **P < 0.01, ***P < 0.001, ****P < 0.0001.

other assays (Figure 1F). Therefore, XBJ exerts a dose-dependent therapeutic effect against SALI, and XBJ(H) offers the greatest protection. These data suggest that XBJ may limit endothelial inflammation and maintain barrier integrity in a dose-dependent manner, thereby protecting against lung injury.

XBJ Alleviates Inflammatory Response in Rats with SALI Partially Through Inhibiting Cell Apoptosis

SALI exhibits excessive apoptosis and dysregulated inflammatory responses that lead to lung tissue damage and impaired function. To determine if XBJ's anti-apoptotic properties contribute to its anti-inflammatory effects, we first analyzed apoptosis in lung tissues by measuring the expression of pro-apoptotic markers (Caspase-3 and Bax) and the anti-apoptotic protein Bcl-2. The CLP group demonstrated significant increases in Caspase-3 and Bax expression, coupled with a reduction in Bcl-2, indicating upregulated apoptosis. Treatment with XBJ significantly decreased pro-apoptotic marker expression and restored Bcl-2 levels (Figure 2A–D), suggesting that XBJ reduces apoptosis in SALI. To explore whether XBJ alleviates inflammation through its anti-apoptotic effects, we measured pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) in serum and lung tissues. The CLP group displayed significantly increased cytokine levels, signifying a strong inflammatory response. XBJ treatment inhibited the expression of these cytokine levels (Figure 2E–J), suggesting it lessens the cytokine storm and reduces inflammation. These results suggest that XBJ modulates inflammation, possibly through its anti-apoptotic actions, thereby preserving lung tissue integrity in SALI.

XBJ Alleviates Inflammatory Response and Apoptosis in LPS-Stimulated HUVECs in vitro

Endothelial cells are critical for the development of SALI, especially regarding inflammatory and apoptotic processes. To explore whether XBJ modulates inflammation and alleviates endothelial cell injury in SALI through anti-apoptotic mechanisms, we analyzed the effects of XBJ on LPS-stimulated HUVECs. We measured cell viability utilizing the CCK-8 assay across a concentration range of (50–350 μL/mL). XBJ enhanced cell viability at (50 μL/mL); however, higher concentrations (≥150 μL/mL) significantly decreased viability, suggesting toxicity at high doses (Figure 3A). Therefore, 50 μL/mL was selected for further experiments.

We found that XBJ decreased LPS-induced release of IL-6 ($P<0.01$), TNF-α ($P<0.05$), and IL-1β ($P<0.05$) (Figure 3B–D). Besides, we demonstrated that LPS increased the pro-apoptotic proteins Bax and Caspase-3 and decreased the anti-apoptotic protein Bcl-2, while XBJ countered these effects ($P<0.05$) (Figure 3E–H), indicating its protective effect against apoptosis. Hence, these results suggested that XBJ reduces inflammation in LPS-stimulated HUVECs partially through regulating endothelial cell apoptosis.

XBJ Upregulates the Expression of the Hippo Pathway in Lung Tissues of Rats with SALI

To further explore the underlying molecular mechanisms of XBJ in SALI, we performed RNA-seq on lung tissues from animal models. DESeq2 analysis indicated 1633 DEGs, including 1184 upregulated and 449 downregulated genes in the XBJ(H) group that suggests a broad effect on gene expression. A volcano plot and heatmap (Figure 4A and B) illustrate these gene expression differences.

GO and KEGG pathway analyses were employed to study the mechanisms behind XBJ's protective effects. GO analysis demonstrated a significant over-representation of biological processes relevant to SALI, such as the inflammatory response, cell adhesion, and endothelial cell apoptosis (Figure 4C). These findings indicate that XBJ may control inflammation, facilitate tissue repair, and preserve endothelial barrier function, processes that are critical for lessening SALI severity. KEGG analysis demonstrated significant enrichment of DEGs in the Hippo signaling pathway (Figure 4D). This pathway, implicated in cell proliferation, apoptosis, and inflammation, plays a key role in SALI progression. Its enrichment implies a potential role in mediating how XBJ modulates inflammation and apoptosis in lung tissues. While specific regulators in the Hippo pathway were not identified, its involvement in endothelial cell homeostasis and inflammation offers a plausible mechanistic link. In conjunction with ELISA and Western blot data indicating XBJ's effectiveness in reducing pro-

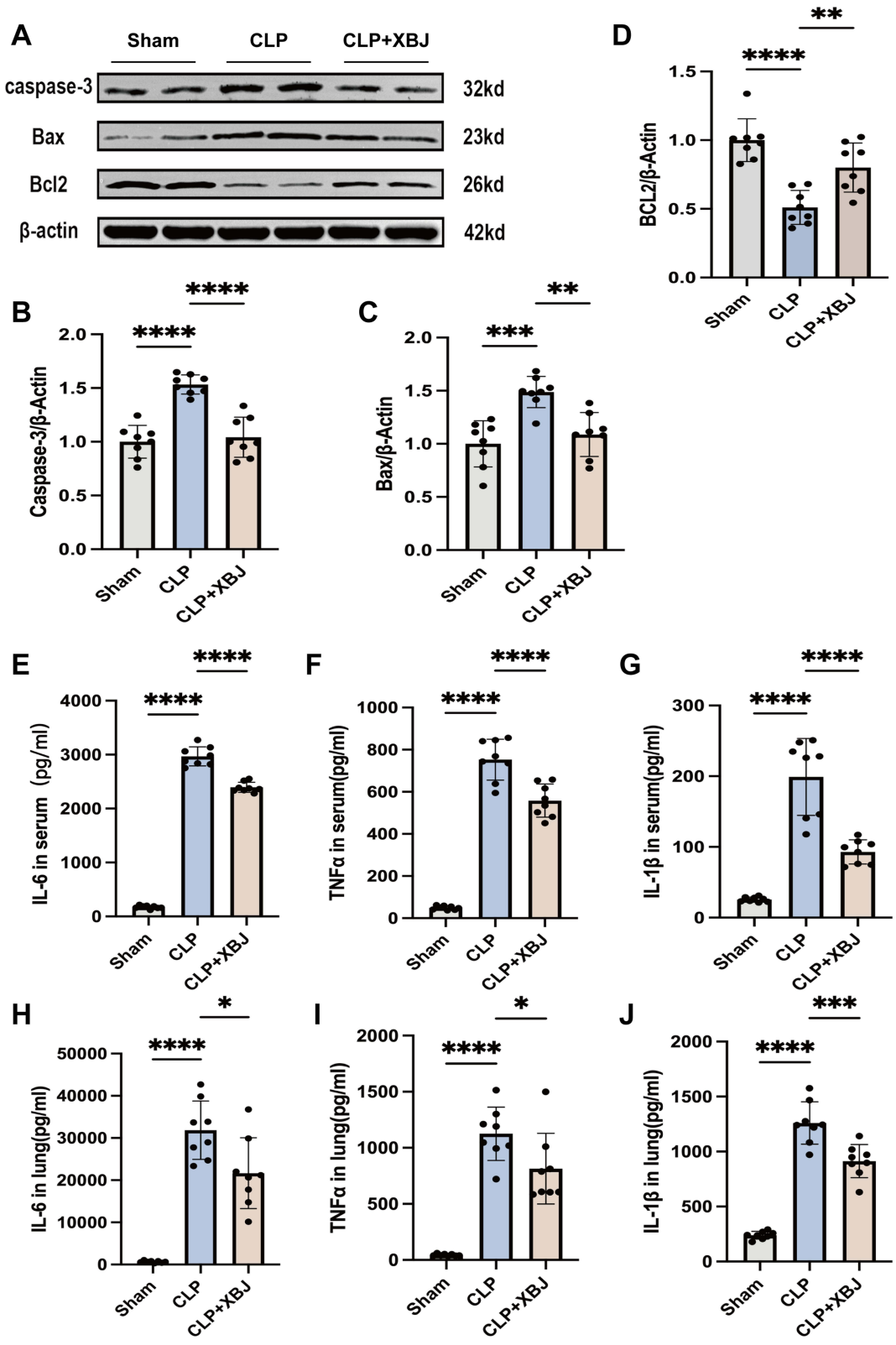


Figure 2 The Effect of XBJ on Inflammatory Response and Cell Apoptosis in Lung Tissue of Septic Rats. (**A–D**) The expressions of caspase-3, Bax, and Bcl-2 proteins in lung tissues were detected by Western blot (N = 8). The levels of IL-6 (**E**), TNF-α (**F**), and IL-1β (**G**) in serum and the levels of IL-6 (**H**), TNF-α (**I**), and IL-1β (**J**) in lung tissues were detected by ELISA (N = 8). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

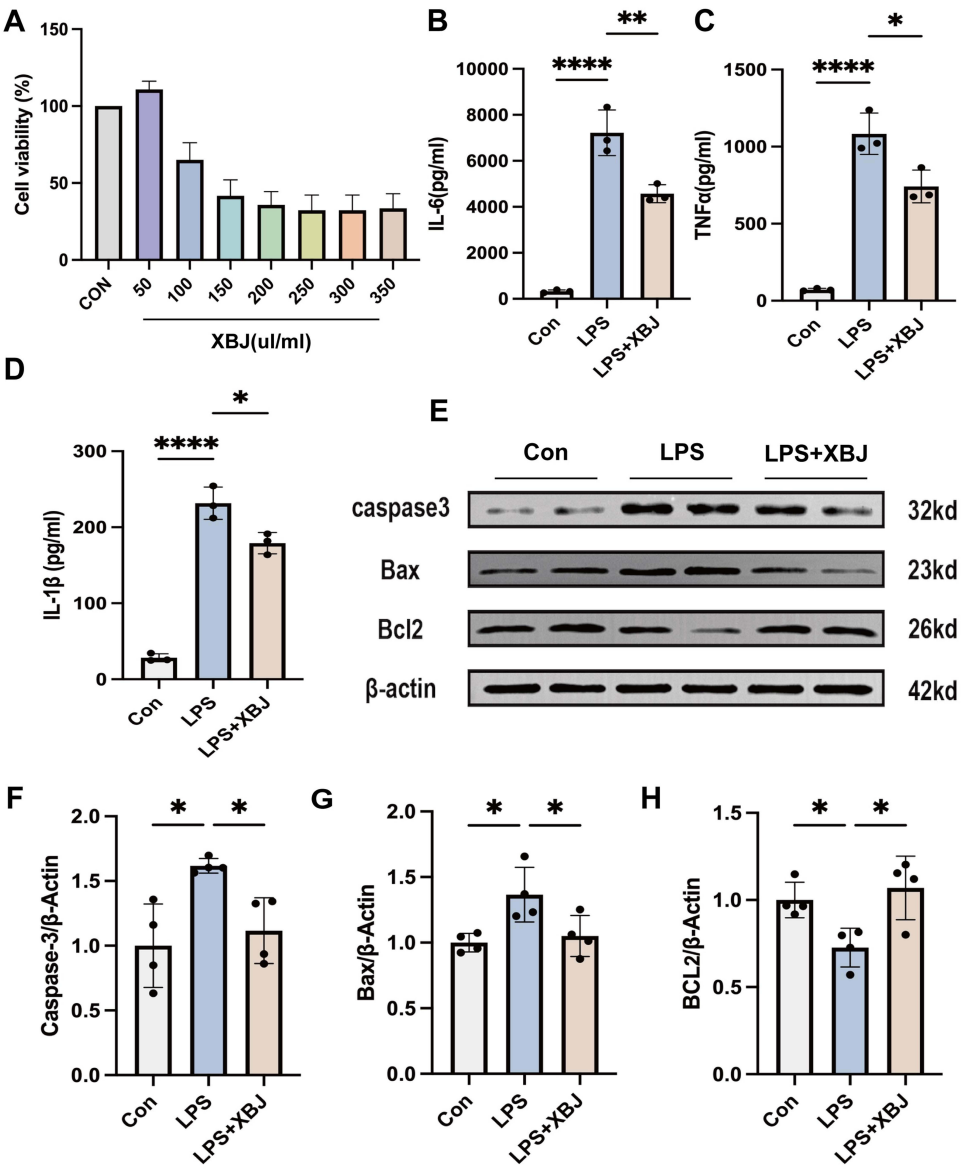


Figure 3 The effects of XBJ on the inflammatory response and apoptosis of HUVEC cells. (**A**) After treatment with different concentrations (50–350 μL/mL) of XBJ for 24h, the viability of HUVEC cells was evaluated by CCK8 assay. The levels of IL-6 (**B**), TNF-α (**C**), and IL-1β (**D**) in the supernatant of HUVEC cells were detected by ELISA. (**E-H**) The expressions of caspase-3, Bax, and Bcl-2 proteins in HUVEC cells were detected by Western blot (N = 4). *P < 0.05, **P < 0.01, ****P < 0.0001.

inflammatory cytokines and decreasing apoptosis, these results suggest XBJ's protective effects may be mediated by influencing pathways such as Hippo, essential for maintaining tissue integrity and regulating inflammation in SALI.

XBJ Upregulates the Expression of the Hippo Pathway in the Lung Tissue of Septic Rats and LPS-Stimulated HUVEC Cell

To determine whether the protective effects of XBJ originate from modulation of the Hippo signaling pathway, the expression of key pathway components was evaluated in both in vivo and in vitro models. In the SALI rat model, Western blot analysis revealed that while the total protein levels of MST2 and LATS1/2 remained relatively unchanged following CLP induction, their phosphorylated forms (p-MST1/2 and p-LATS1/2) were significantly elevated, suggesting stress-induced activation of the Hippo pathway. Concurrently, mRNA and total protein levels of the downstream effectors YAP and TAZ were significantly reduced, while p-YAP expression was markedly increased. Intervention with XBJ reversed these alterations by increasing the total expression of YAP and TAZ, and reducing the phosphorylation levels of

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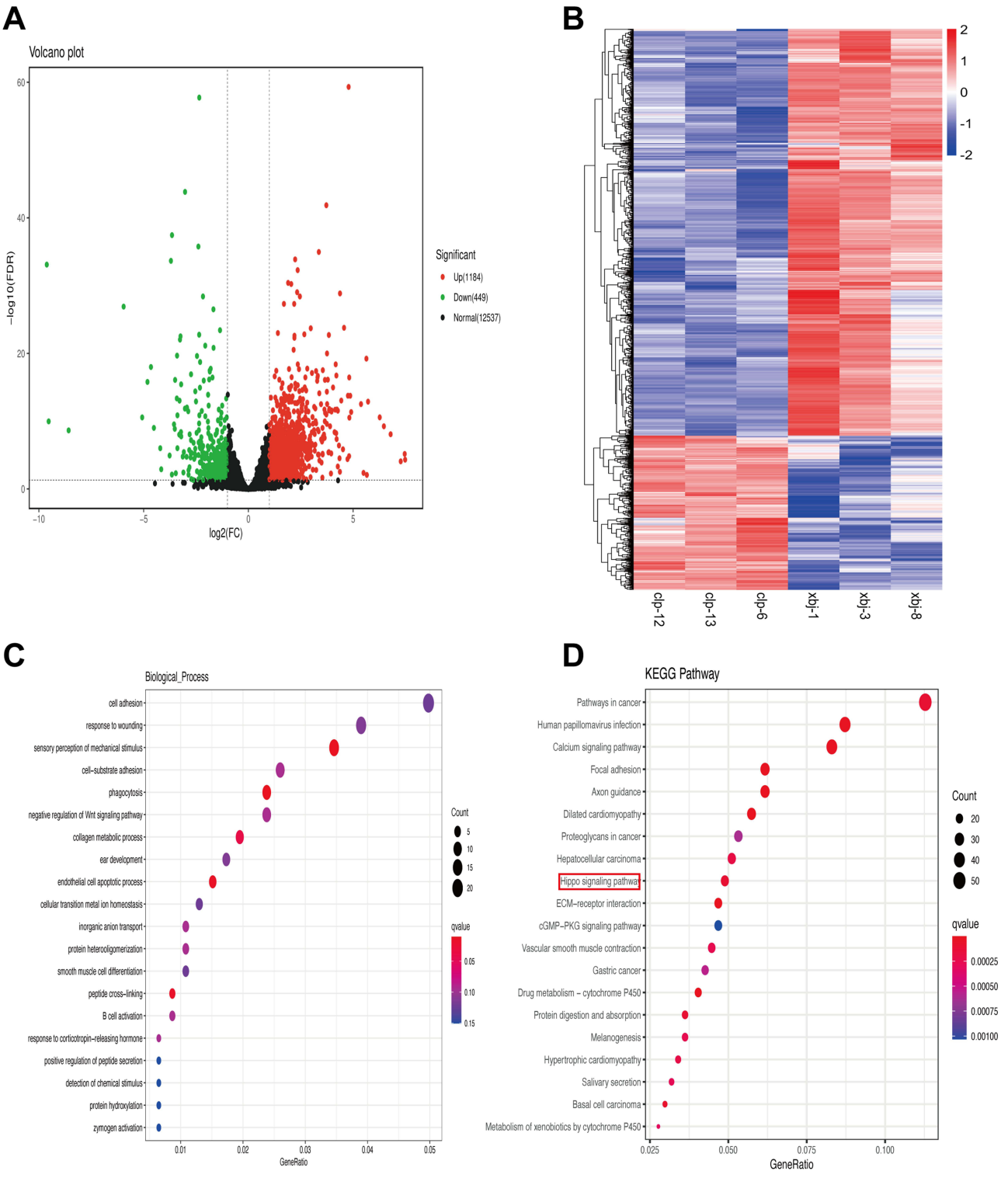

Figure 4 Transcriptomics results of lung tissues in the CLP group and the XBJ group. (**A**) Volcano plot of differential genes in lung tissues of the CLP group and the XBJ group. (**B**) Heat map of differential genes in lung tissues of the CLP group and the XBJ group. (**C**) Gene Ontology (GO) enrichment analysis of differentially expressed genes. (**D**) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of differentially expressed genes. The red box highlights the Hippo signaling pathway, which was identified as a key enriched pathway in the KEGG pathway enrichment analysis.

MST1/2, LATS1/2, and YAP (Figure 5A–H). These findings indicate that XBJ may affect the Hippo pathway. Likewise, in vitro experiments using HUVECs showed that LPS stimulation resulted in a modest increase in p-MST1/2 and p-LATS1/2 levels, while reducing total YAP and TAZ expression and increasing p-YAP levels. Treatment with XBJ counteracted these changes by restoring YAP and TAZ expression and attenuating phosphorylation levels of p-YAP, p-MST1/2, and p-LATS1/2 (Figure 5I–P), further confirming that XBJ regulates the Hippo pathway at the cellular level.

Immunofluorescence staining was employed to evaluate the expression and localization of YAP and TAZ in lung tissues and HUVEC cells. In vivo, robust fluorescence signals for YAP (red) and TAZ (green) were detected in the Sham group, indicative of high expression under normal physiological conditions. In contrast, these signals were reduced in the CLP group, suggesting that sepsis inhibits YAP and TAZ activity. Treatment with XBJ intensified the fluorescence signals of YAP and TAZ (Figure 6A–D), demonstrating its restorative capacity. In HUVEC cells, LPS stimulation significantly weakened the fluorescence signals of YAP and TAZ; however, this effect was reversed by XBJ treatment (Figure 6E–H). These data indicate that XBJ may confer protection against SALI by acting on the Hippo pathway to promote cell survival and preserve tissue integrity.

Discussion

ALI/ARDS is a common and severe complication of sepsis, characterized by pulmonary edema and infiltration of inflammatory cells.[27–29] The pathophysiology of SALI is complex, involving a dynamic interplay between inflammation, endothelial dysfunction, and cell death.[30,31] Currently, clinical interventions remain inadequate in effectively halting disease progression.[32] Our study demonstrated that XBJ attenuates inflammation and apoptosis in lung endothelial cells, thereby effectively alleviating lung injury. This effect is likely mediated through the regulation of the Hippo signaling pathway. These findings provide a foundation for the clinical development of more effective therapeutic strategies for SALI.

XBJ injection is a traditional Chinese medicinal formulation derived from the "Xuefu Zhuyu decoction". Its primary constituents include safflower, red peony, Chuanxiong, Salvia miltiorrhiza, and Angelica sinensis.[33] Previous studies have demonstrated that XBJ significantly alleviates lung inflammation and improves survival in experimental models of sepsis-induced acute lung injury. These protective effects are achieved by regulating various mechanisms, including anti-inflammatory responses, endothelial protection, anticoagulation, and the modulation of ferroptosis pathways. Multiple signaling pathways are involved in mediating these effects.[34–37] The MAPK signaling pathway, whose key molecules including c-Raf, MEK, and ERK are crucial for the activation and migration of inflammatory cells, as well as the release of proinflammatory cytokines is one of the most well-characterized in terms of inflammatory regulation. XBJ has been reported to modulate the MAPK cascade to reduce the production of proinflammatory cytokines.[38,39] In addition to modulating the inflammatory response in sepsis, XBJ also offers direct protection against lung injury through regulating alveolar-capillary dysfunction. This protective effect is mediated through the PI3K/Akt/FOXO1 signaling pathway, which upregulates the expression of claudin-5, thereby preserving pulmonary vascular barrier function and maintaining endothelial barrier integrity.[40] With respect to anticoagulation, compounds in XBJ inhibit ACLY activity, thereby suppressing the expression of RIG-I by reducing the acetylation of the transcription factor MYB, leading to inhibition of coagulation activation.[41] Regarding ferroptosis, XBJ optimizes iron metabolism and lipid peroxidation metabolism by regulating the expression of a series of proteins closely related to iron overload, such as GPX4, ACSL4, x-CT, and FTH1, thereby improving septic acute lung injury.[24] Although XBJ has been shown to mitigate lung injury through multiple mechanisms, including the regulation of cytokine release and the maintenance of alveolar integrity, the precise mechanisms by which it regulates endothelial cells, which constitute the pulmonary vascular barrier, remain unclear. In the present study, we demonstrate that XBJ significantly reduces endothelial cell apoptosis by upregulating Bcl-2 expression and inhibiting the activation of Bax and Caspase-3. Meanwhile, it inhibits the release of inflammatory mediators and ameliorates ALI.

ECs are crucial components of the inner wall of capillaries, where they maintain the interaction and barrier function between the alveoli and capillaries through intercellular junctions, thereby ensuring the integrity of the pulmonary vascular barrier.[42,43] In the context of SALI or ARDS, ECs are activated by pathogen-associated molecular patterns (PAMPs).[44] Under such conditions, ECs release inflammatory mediators and recruit a significant number of leukocytes, resulting in an imbalance between pro-inflammatory and anti-inflammatory responses in the lungs. This imbalance

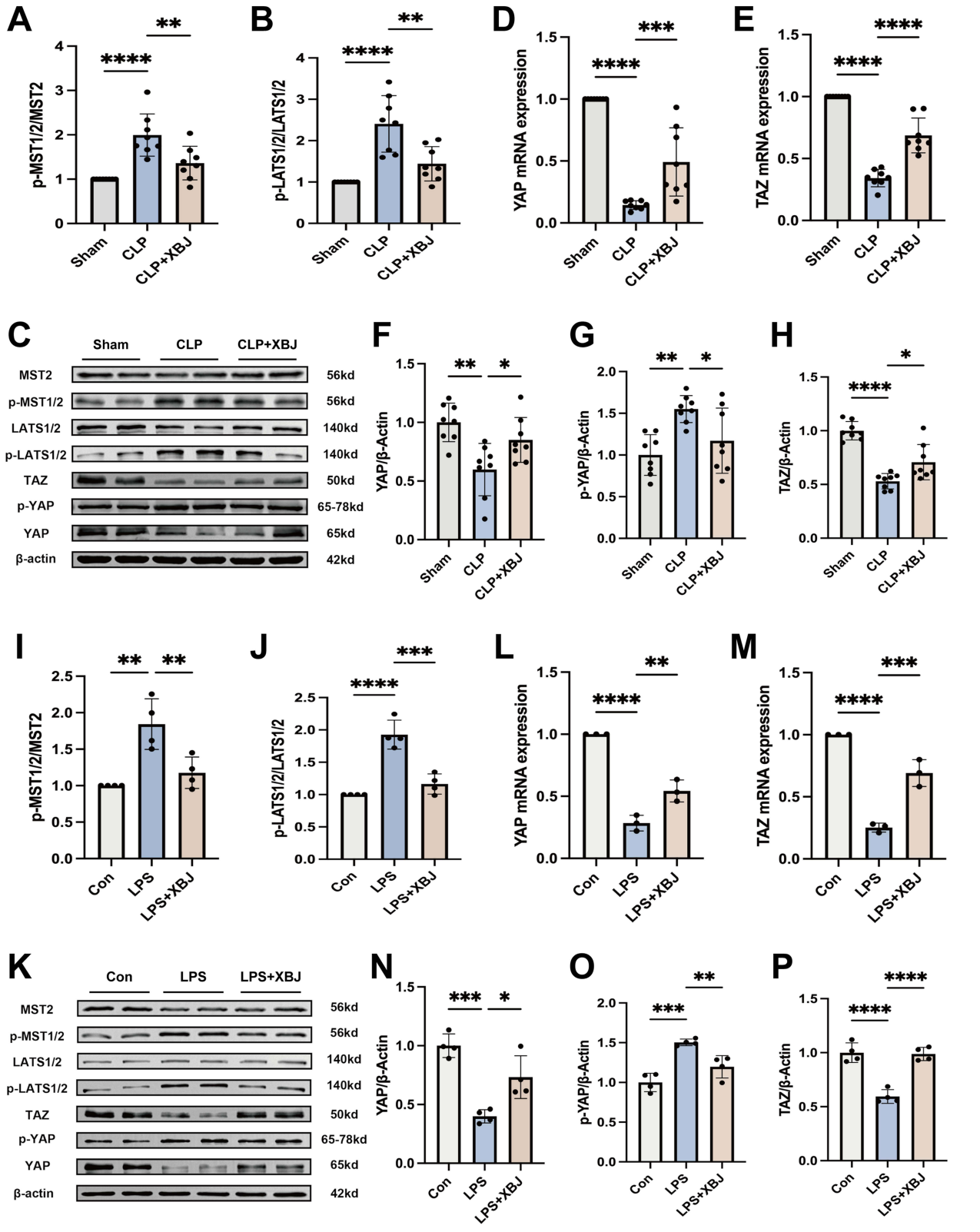

Figure 5 XBJ regulates the expression and phosphorylation of Hippo pathway components in the lung tissues of septic rats and LPS-stimulated HUVEC cells. (**A**–**C**) Protein expression levels of MST2, p-MST1/2, LATS1/2, p-LATS1/2 in lung tissues analyzed by Western blot (N = 8). The mRNA expression levels of YAP (**D**) and TAZ (**E**) in lung tissues (N = 8). (**F–H**) Protein expression levels of YAP, p-YAP, and TAZ in lung tissues analyzed by Western blot (N = 8). (**I**–**K**) Protein expression levels of MST2, p-MST1/2, LATS1/2, p-LATS1/2 in HUVEC cells analyzed by Western blot (N = 4). The mRNA expression levels of YAP (**L**) and TAZ (**M**) in HUVEC cells (N = 3). (**N**–**P**) Protein expression levels of YAP, p-YAP, and TAZ in HUVEC cells analyzed by Western blot (N = 4). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

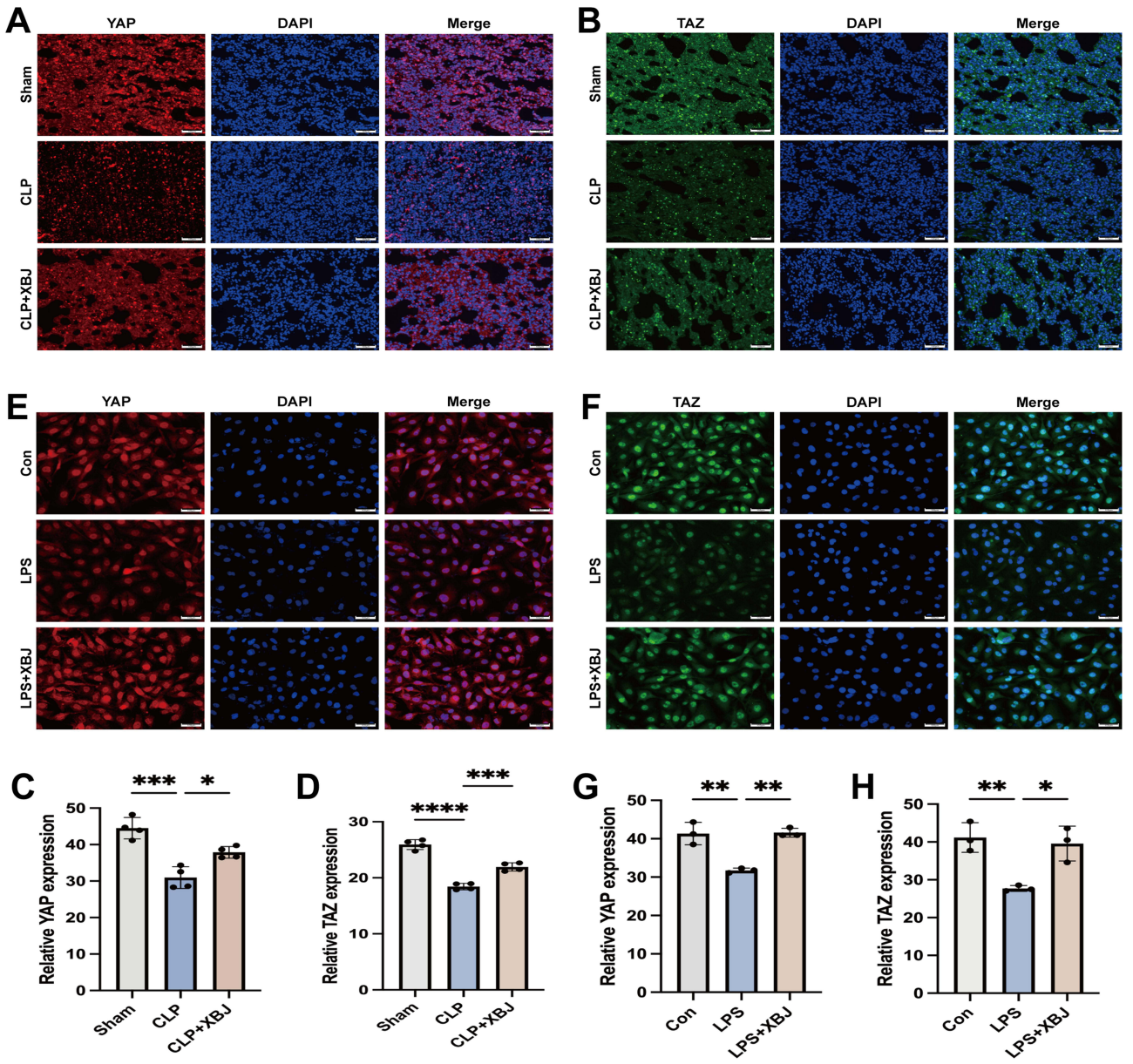

Figure 6 Immunofluorescence staining was used to display the expression of YAP (**A**) (red) and TAZ (**B**) (green) in lung tissues, with the cell nuclei stained by DAPI (blue) (200x, bar = 100μm). (**C** and **D**) Quantitative analysis of the immunofluorescence intensity of YAP and TAZ in lung tissues (N = 4). Immunofluorescence staining was also used to show the expression of YAP (**E**) (red) and TAZ (**F**) (green) in HUVEC cells, with the cell nuclei stained by DAPI (blue) (200x, bar = 100μm). (**G** and **H**) Quantitative analysis of the immunofluorescence intensity of YAP and TAZ in HUVEC cells (N = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

triggers a cytokine storm, further exacerbating vascular and lung tissue damage.[45] Additionally, the excessive release of cytokines induces widespread endothelial cell apoptosis, which in turn leads to the release of damage-associated molecular patterns (DAMPs), thereby amplifying the severity of lung injury.[46] Previous studies have shown that lung damage in a SALI model was significantly alleviated in CircTLK1-knockout mice, with reduced endothelial cell inflammation and apoptosis.[47] Furthermore, research targeting endothelial cell apoptosis has demonstrated that miRNA-146a-5p, as a bioactive molecule, can inhibit LPS-induced Caspase-3 activation in endothelial cells, effectively preventing endothelial cell apoptosis and the progression of SALI.[48] However, despite progress in understanding the regulation of endothelial cell inflammation and apoptosis, there remains a lack of clinically applicable therapeutic agents. In this study, we found that XBJ, a drug approved by the National Medical Products Administration of China, exerts

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a therapeutic effect against endothelial cell inflammation and apoptosis. These findings offer new prospects for the use of traditional Chinese medicine (TCM) in the treatment of SALI.

The Hippo signaling pathway is a highly conserved regulatory network crucial for controlling cell proliferation, apoptosis, and tissue homeostasis.[49] The core components of this pathway, including MST1/2 and LATS1/2 kinases, inhibit the activation of apoptotic and proliferative genes by preventing the nuclear translocation of YAP and TAZ.[50] Previous studies have demonstrated that in CLP-induced sepsis model, depletion of YAP in ECs exacerbates inflammation and promotes apoptosis.[51] In contrast, activation of YAP/TAZ has been shown to alleviate inflammation by inhibiting the Nf-κB signaling pathway, thereby regulating sepsis-related lung injury.[52] Consequently, modulating the Hippo pathway to reduce endothelial cell apoptosis and inflammatory responses offers a promising therapeutic strategy for SALI. However, the role of XBJ in regulating this pathway remains to be fully elucidated. In this study, to investigate the effects of XBJ on endothelial inflammation and apoptosis, we conducted RNA sequencing (RNA-seq) of lung tissue from the CLP and XBJ(H) groups. KEGG pathway analysis revealed significant enrichment of the Hippo signaling pathway in the XBJ(H) group compared to the CLP group. Based on this observation, we further validated the involvement of XBJ in the Hippo pathway through qPCR, Western blotting, and immunofluorescence analyses.

Our results demonstrated that XBJ treatment modulated the Hippo signaling pathway at multiple levels in both in vivo and in vitro models. Specifically, XBJ reduced the phosphorylation levels of upstream kinases MST1/2 and LATS1/2, and restored the expression of the downstream effectors YAP and TAZ, which may contribute to the observed reduction in endothelial cell apoptosis and the restoration of inflammatory homeostasis. These findings provide novel insights into the potential of traditional Chinese medicines, such as XBJ, in the treatment of SALI.

In addition, there may be potential cross-regulatory relationships between the Hippo pathway and the known pathways of XBJ, further emphasizing the important role of the Hippo signaling pathway in XBJ treatment for sepsis-induced lung injury. For example, the activation of YAP/TAZ in the Hippo pathway can inhibit the nuclear translocation of NF-κB,[53] which synergizes with the direct inhibitory effect of XBJ on MAPK phosphorylation, collectively blocking the inflammatory cascade.[54] Meanwhile, ERK in the MAPK pathway can activate the Hippo/YAP/TAZ pathway through mechanical sensing, among other mechanisms,[55] and the inhibitory effect of XBJ on ERK may relieve this negative regulation, amplifying the anti-apoptotic and anti-inflammatory effects mediated by Hippo.[56,57] Moreover, on one hand, the activation of the PI3K/Akt pathway can inhibit the Hippo pathway by promoting YAP nuclear accumulation, and on the other hand, accumulated YAP can further activate the PI3K/Akt pathway by inhibiting its repressor, PTEN,[58] which corresponds with XBJ's mechanism of upregulating claudin-5 via the PI3K/Akt pathway,[40] suggesting that both pathways work together to maintain endothelial barrier function. These cross-regulatory relationships indicate that XBJ may coordinate multiple signaling pathways, such as MAPK, PI3K/Akt, and NF-κB, through a "Hippo-centered" network pharmacology model, ultimately achieving multi-level regulation of processes such as inflammation, apoptosis, and endothelial protection.

Although our study has demonstrated that XBJ can modulate endothelial cell apoptosis and inflammation, and has provided preliminary insights into its underlying mechanism through the Hippo signaling pathway, several key aspects remain unexplored. Specifically, we did not employ gene knockout or pharmacological inhibition strategies to validate the critical role of XBJ in regulating the Hippo pathway. Furthermore, it remains unclear whether the Hippo signaling pathway directly regulates the expression of apoptotic genes to influence endothelial cell apoptosis during XBJ treatment. Therefore, future studies will focus on further elucidating the precise mechanisms by which XBJ improves endothelial cell dysfunction, with the aim of providing solid evidence to support its clinical application.

Conclusion

This research pioneers the discovery that XBJ may protect against SALI by upregulating YAP and TAZ within the Hippo signaling pathway. It may exert its therapeutic effects through its anti-apoptotic action, which in turn regulates the inflammatory response. This novel insight not only enhances our knowledge of the molecular mechanisms behind XBJ's protective effects but also offers a theoretical foundation for its clinical application in sepsis and other inflammatory diseases.

Ethical Approval

The study was approved by Animal Ethics Committee of Anhui Medical University (certificate no. LLSC-20242423).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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