Original Research

The β-catenin/CBP signaling axis participates in sepsis-induced inflammatory lung injury

Xia Cheng[1], Dandan Liu[2], Xinxin Ren[3], You Nie[2], Yibing Zhao[4], Ruyu Chen[1] and Hongwei Wang[2]

[1]Department of Pathology, Fourth Medical Center, General Hospital of Chinese People's Liberation Army, Jinzhou Medical University, Beijing 100048, China; [2]Department of Pathology, The Fourth Medical Center of PLA General Hospital, Beijing 100048, China; [3]Department of Clinical Laboratory, The Fourth Medical Center of PLA General Hospital, Beijing 100048, China; [4]Department of Oncology, The Fourth Medical Center of PLA General Hospital, Beijing 100048, China
Corresponding author: Hongwei Wang. Email: whw6626@sina.com

Impact Statement

Previous studies have shown that β-catenin, as an important signal transduction molecule, participates in the inflammatory process of sepsis. In addition, as a coactivator, the interaction between CREB-binding protein (CBP) and β-catenin has attracted increasing attention regarding its relationship to diseases such as tissue fibrosis, tumors, and endometriosis. However, this interaction is not understood in sepsis-induced lung injury. In this experiment, in a mouse model of sepsis, the expression of β-catenin was inhibited and the interaction between β-catenin/CBP was blocked through a variety of ways. The levels of lung inflammation were evaluated and compared, indicating the β-catenin/CBP signaling axis to be involved. Inflammatory lung injury during sepsis has a regulatory effect on the transmission of inflammatory signals during sepsis. This opens new future research avenues for the study of the regulation of inflammatory signals in sepsis.

Abstract

Sepsis-induced inflammatory lung injury is a key factor causing failure of the lungs and other organs, as well as death, during sepsis. In the present study, a caecal ligation and puncture (CLP)-induced sepsis model was established to investigate the effect of β-catenin on sepsis-induced inflammatory lung injury and the corresponding underlying mechanisms. C57BL/6 mice were randomly divided into five groups, namely, the sham, CLP, β-catenin knockout (KO) + CLP, XAV-939 + CLP, and ICG-001 + CLP groups; the XAV-939 + CLP and ICG-001 + CLP groups were separately subjected to intraperitoneal injections of the β-catenin inhibitors XAV-939 and ICG-001 for 1 week preoperatively and 2 days postoperatively, respectively. Forty-eight hours after CLP, we measured β-catenin expression in lung tissues and evaluated mouse mortality, histopathological characteristics of hematoxylin and eosin (H&E)-stained lung tissues, serum cytokine (tumor necrosis factor [TNF]-α, interleukin [IL]-10, and IL-1β) levels, lung myeloperoxidase (MPO) activity, and the number of apoptotic cells in the lung tissues. Our results indicated that both the inhibition of β-catenin expression and blockage of β-catenin/CREB-binding protein (CBP) interactions by ICG-001 effectively decreased mouse mortality, alleviated pathological lung injury, and reduced the serum TNF-α, IL-10, and IL-1β levels, in addition to reducing the lung MPO activity and the number of apoptotic cells in lung tissues of the sepsis model mice. Therefore, it can be deduced that the β-catenin/CBP signaling axis participates in regulating sepsis-induced inflammatory lung injury.

Keywords: Sepsis, lung injury, β-catenin, CBP

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Introduction

Sepsis is an uncontrolled systemic inflammatory response syndrome with high incidence rates and a global mortality rate of approximately 20%.[1,2] It is frequently encountered in critical care units and places a significant burden on patients and societies; however, its pathogenesis is yet to be fully elucidated. The lung is the organ most susceptible to sepsis[3–5] and is one of the organs that is most severely affected by injury-induced dysfunction.[6] Sepsis-induced inflammatory lung injury is a critical factor leading to failure of the lungs and other organs, as well as death, in patients with sepsis.

During the presepsis period, the immune system becomes overactive because of factors such as infection, leading to the occurrence of a series of excessive immune responses, including uncontrolled inflammatory responses, cytokine storms, and the release of a multitude of inflammatory mediators, which form a part of the pathophysiological basis for the onset of sepsis. The resultant uncontrolled systemic inflammatory environment causes damage to lung endothelial and epithelial cells, thereby inducing inflammatory lung injury.[7] Consequently, numerous inflammatory cells infiltrate the lungs and release inflammatory mediators, resulting in abnormal apoptosis[8] and extensive lung tissue damage.

As anti-inflammatory treatments do not significantly affect sepsis-induced inflammatory lung injury, interventions for excessive inflammation that target the mechanisms underlying sepsis development are a key research topic and crucial for improving the prognosis of patients with sepsis.[9]

Uncontrolled inflammatory responses in sepsis are the result of the cross-regulation of various signaling pathways.[10–12] One such pathway is the highly conserved Wnt/β-catenin signaling pathway, which plays a pivotal role in the differentiation, proliferation, and functional changes of cells and intercellular signal transduction.[13,14] β-catenin is a central effector molecule of the canonical Wnt/β-catenin signaling pathway, and its activation is essential for maintaining immune homeostasis. In normal cells, β-catenin is localized to the cell membrane and is rarely expressed in the cytoplasm and nucleus.[15] However, when inflammation occurs, Wnt ligands are activated and transmit signals into cells via the cell membrane. Consequently, the activity of β-catenin is no longer inhibited and it is no longer phosphorylated, leading to its accumulation in the cytoplasm.[16] As a result, β-catenin eventually translocates into the nucleus, forming a transcriptional coactivator complex with T-cell factor (TCF)/lymphoid enhancer factor that regulates the expression of downstream target genes, such as genes that induce inflammation and cause tissue damage.[17–19]

In the present study, the expression levels of β-catenin in mice were reduced by gene knockout (KO) and injections of specific inhibitors. A sepsis model was established by caecal ligation and puncture (CLP) to investigate the effects of β-catenin expression on sepsis-induced inflammatory lung injury and the potential regulatory mechanisms underlying these effects. Forty-eight hours after CLP, the levels of sepsis-induced lung injury-associated inflammation markers in the various groups of mice were evaluated. Our results showed that the inhibition of β-catenin expression or the blockage of the binding between β-catenin and CREB-binding protein (CBP) led to lower inflammation marker levels in sepsis-induced inflammatory lung injury. Therefore, it can be deduced that the β-catenin/CBP signaling axis may alleviate the symptoms of lung injury by participating in the regulation of inflammatory lung injury and blocking β-catenin/CBP interactions.

Materials and methods

Reagents

The inhibitors XAV-939 and ICG-001 were purchased from MedChemExpress LLC (MCE; Monmouth Junction, NJ, USA). β-catenin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining reagents were purchased from Beijing ZSGB-BIO Co., Ltd. (Beijing, China). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kits were purchased from Roche Holding AG (Basel, Switzerland). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Biosciences (Franklin Lakes, NJ, USA), and lung myeloperoxidase (MPO) activity assay kits were purchased from Abcam (Cambridge, UK).

Animals

SPF grade β-catenin KO C57BL/6 and wild-type (WT) mice from the same litter were purchased from Cyagen Biosciences (Suzhou) Inc. (Jiangsu, China). Mice were reared under a 12-h light/dark cycle at 21–25°C and 40–70% relative humidity. Standard feed was provided, and mice were allowed *ad libitum* access to food and water. All mice were acclimatized to the rearing environment for 1 week prior to CLP. This research was approved by the Animal Experiment Ethics Committee of the Fourth Medical Center of the General Hospital of the People's Liberation Army and implemented in accordance with the Animal Health and Ethical Guidelines of the National Institutes of Health of China.

Experimental protocol

According to pre-experimental results, 6- to 8-week-old male β-catenin KO and WT mice from the same litter were randomly divided into five groups, 15–30 mice per group: the sham, CLP, β-catenin KO + CLP, XAV-939 + CLP, and ICG-001 + CLP groups. Mice from the XAV-939 + CLP and ICG-001 + CLP groups were pretreated with daily intraperitoneal injections at fixed times for seven consecutive days, with the dose levels for XAV-939 and ICG-001 being 20 and 13 mg/kg/day, respectively. All mice were fasted for 12 h before CLP but allowed *ad libitum* access to water.

The sepsis model was established by CLP in all mice. Briefly, 1/2 of the cecum was ligated after mice were anesthetized. A 2.5-mL syringe needle was used to perforate the intestinal wall of the ligated segment of the cecum; then, the cecum was inserted back into the abdominal cavity, and the abdominal wall was closed with sutures. For the mice from the sham group, the surgical procedure was identical to that in case of the mice from all other groups, except for the omission of the CLP steps. Postoperatively, the XAV-939 + CLP and ICG-001 + CLP groups received intraperitoneal injections of the respective inhibitors for two consecutive days. All mice were continuously observed for 48 h or until death.

Histology

The fresh left lung of mice of each group was washed with saline to remove blood and immediately fixed in 10% formalin for 48 h. After dehydration and paraffin embedding, each lung was sectioned into 3-μm-thick slices, which were stained with H&E, observed under a microscope, photographed using a digital camera at 200× magnification, and subjected to histological analysis. According to the lung injury pathological scoring standard,[20] lung tissue injury was scored for each group.

IHC

β-catenin expression levels in the lung tissues of mice from each group were measured by IHC. Paraffin-embedded lung tissues were sectioned into 3-μm-thick slices and incubated with an antibody specifically targeting β-catenin in mice. After incubation, the sections were observed and photographed under a microscope, and the obtained images were analyzed using ImageJ software.

ELISA

Forty-eight hours after CLP, serum tumor necrosis factor (TNF)-α, interleukin (IL)-10, and IL-1β levels in the mice from each group were measured using an ELISA kit, following the manufacturer's instructions (BD Biosciences; Franklin Lakes, NJ, USA).

MPO activity detection

The fresh lung tissues of mice from each group were washed with saline to remove blood, blotted dry, weighed, and ground to a homogenate with the addition of homogenization buffer. The samples were then incubated with the MPO assay reaction mix in accordance with the manufacturer's instructions. Measurements of the OD values at 460 nm and a pathlength of 1 cm were made using a microplate reader, and the MPO activity of each sample was calculated from the absorbance readings.

Detection of apoptosis

Apoptosis was detected via TUNEL staining and 4′,6-diamidino-2-phenylindole (DAPI) counterstaining. Briefly, after the tissue sections had been dewaxed and rehydrated, TUNEL staining was performed in accordance with the manufacturer's instructions. Thereafter, the TUNEL-stained sections were washed with phosphate-buffered saline (PBS), counterstained with DAPI at room temperature (25–30°C), incubated in the dark for 15 min, and washed with PBS again. Finally, the sections were mounted on an anti-fade mounting medium for observation and photography under a fluorescence microscope.

Statistical analysis

The experimental data were processed and analyzed using IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA). All data followed a normal distribution and were expressed as the means ± standard deviations. Comparisons of the survival status between groups were performed using the chi-square test, and pairwise comparisons of the remaining data were performed using the *t*-test. Differences with $P < 0.05$ were considered statistically significant. Graphs were plotted using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

Survival study

Forty-eight hours after CLP, the mortality rates of the sham and CLP groups were 0% and 50%, respectively, which were significantly different (Figure 1, $P < 0.01$), indicating the successful construction of the sepsis model. The β-catenin KO + CLP group had a lower mortality rate (35.29%) than that of the CLP group, but the difference was not statistically significant. The mortality rates of the XAV-939 + CLP and ICG-001 + CLP groups were 0% and 27.7%, respectively (Figure 1, $P < 0.05$).

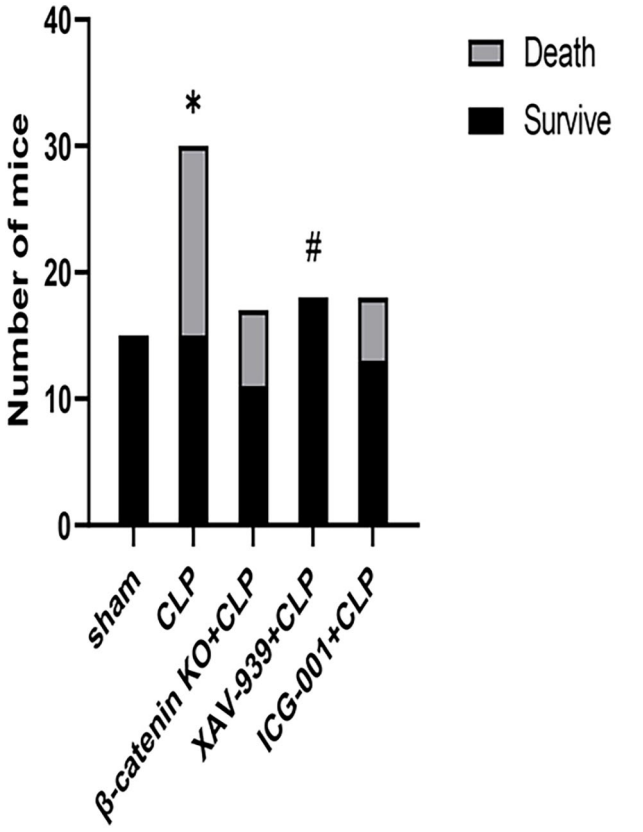

Figure 1. The survival status of mice in each group. Mice were divided into five groups as described in Materials and Methods: (1) sham group; (2) CLP group; (3) β-catenin KO + CLP group; (4) XAV-939 + CLP group; and (5) ICG-001 + CLP group. Mice in each group were observed until 48 h or until death. The mortality of mice in each group was compared by chi-square test. The mortality of the five groups of mice were 0%, 50%, 35.29%, 0%, and 27.7%. *$P < 0.05$ compared with sham group; #$P < 0.05$ compared with CLP group.

Lung histology

Histopathological changes in lung tissue at 48 h post-CLP were observed under a microscope. The lung tissues of the sham group exhibited normal and clear structures without inflammatory cell infiltration. By contrast, the lung tissues of the CLP group showed increased inflammatory cell infiltration, disordered structures in normal lung tissue, edema-induced widening of the alveolar interstitium, alveolar collapse, and neutrophil aggregation in the alveolar lumens and perivascular areas (Figure 2). KO of the β-catenin gene or inhibition of β-catenin expression led to reduced inflammatory cell infiltration and alleviation of pathological injury in lung tissue ($P < 0.05$).

Lung IHC

Microscopic examinations of lung tissue sections and image analysis using ImageJ revealed that β-catenin staining occurred mainly in the cytoplasm of the lung tissues of the mice from the CLP group. By contrast, β-catenin staining in the lung tissues of the other groups was concentrated in the cell membrane, with minimal staining being observed in the cytoplasm (Figure 3, $P < 0.05$). This indicates that β-catenin gene expression was upregulated in sepsis-induced

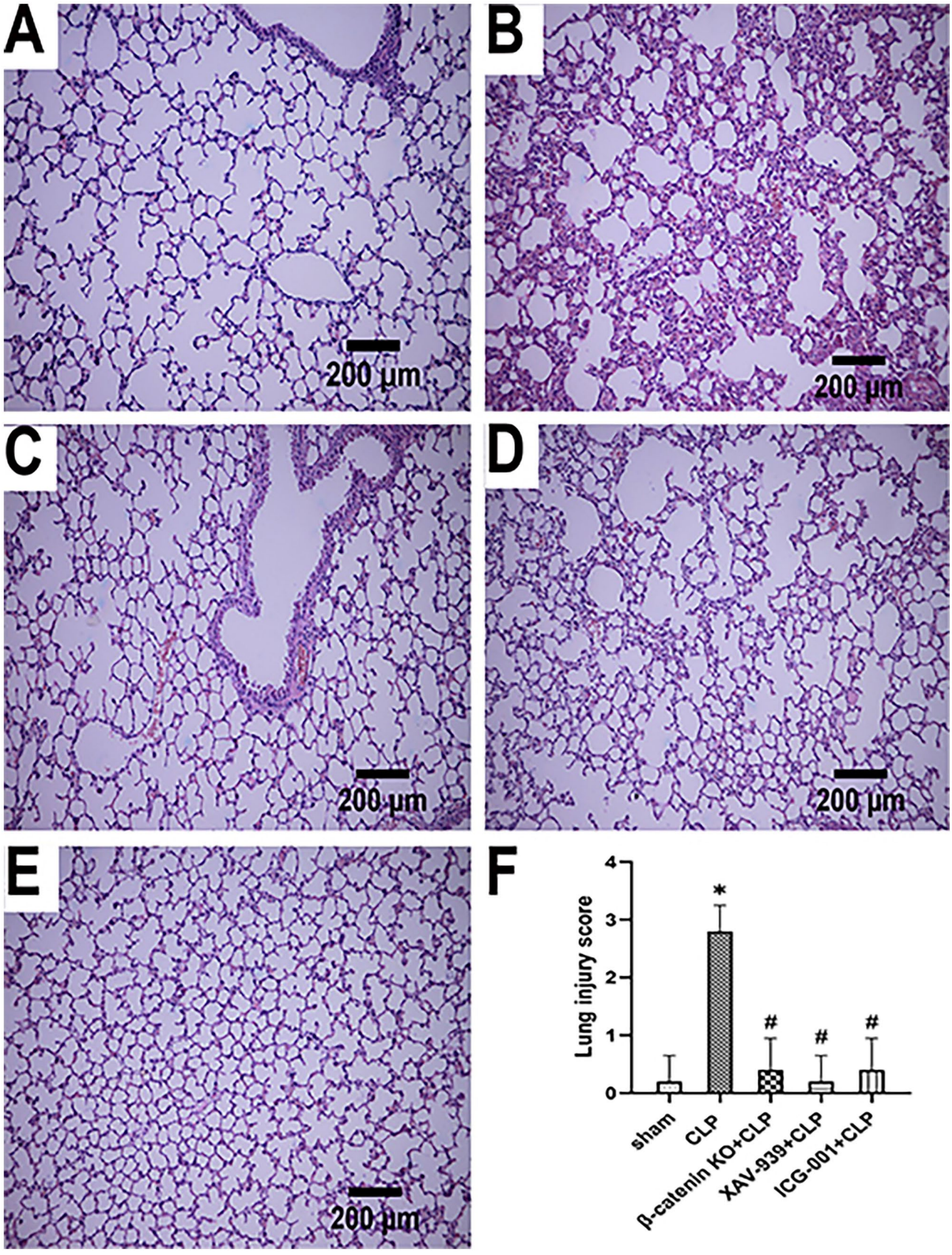

Figure 2. Lung histopathological injury. Lung tissue samples collected 48 h after CLP were analyzed by conventional histological techniques and HE staining for light microscopy. Then score each group of pictures according to lung histopathology scoring standards: (A) sham group; (B) CLP group; (C) β-catenin KO + CLP group; (D) XAV-939 + CLP group; and (E) ICG-001 + CLP group. (F) Histopathological mean lung injury scores.*$P < 0.05$ compared with sham group; #$P < 0.05$ compared with CLP group. Magnification of original images was 200×, Scale bars represent 200 μm. (A color version of this figure is available in the online journal.)

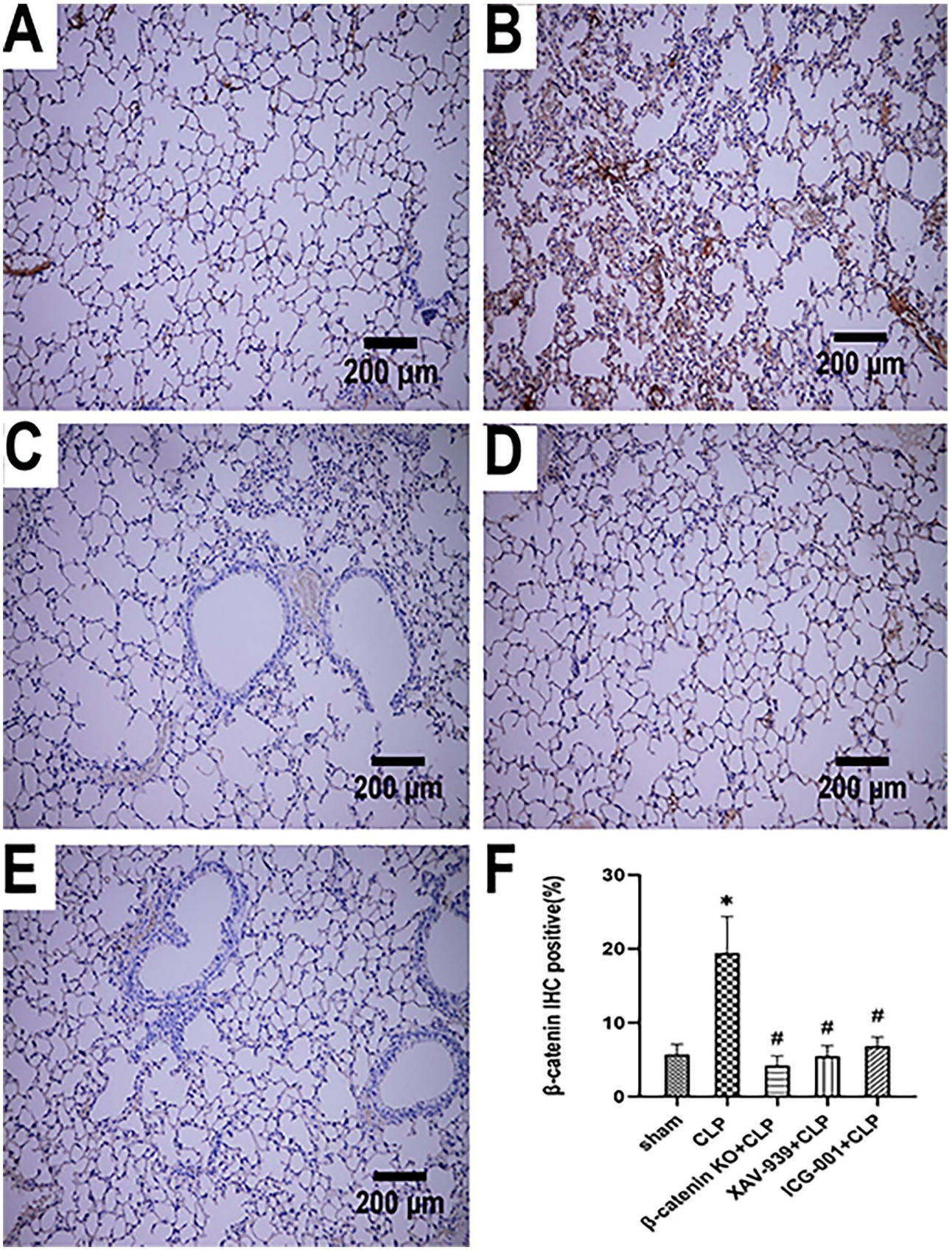

Figure 3. Immunohistochemical results of β-catenin in lung tissue: (A) sham group; (B) CLP group; (C) β-catenin KO + CLP group; (D) XAV-939 + CLP group; and (E) ICG-001 + CLP group. (F) Analysis of the percentage of positive results of β-catenin immunohistochemistry in lung tissue with ImageJ software. β-catenin immunohistochemically positive is brown. Among them, the CLP group is mainly expressed in the cytoplasm, and the other groups are mainly expressed on the cell membrane. The staining intensity reflects the abundance of β-catenin. $^{*}P < 0.05$ compared with sham group; $\#P < 0.05$ compared with CLP group. Magnification of original images was 200×. Scale bars represent 200 μm. (A color version of this figure is available in the online journal.)

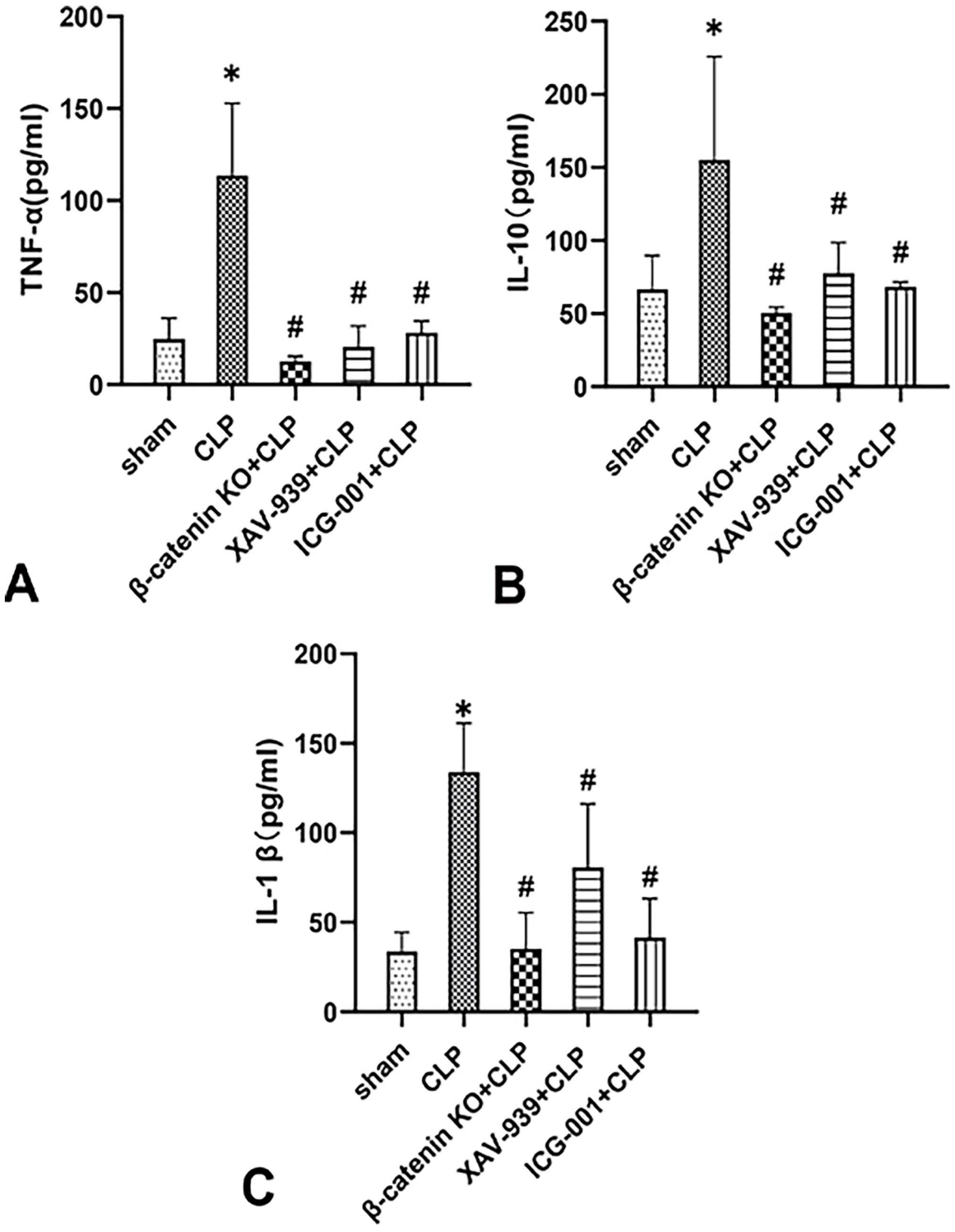

Figure 4. Mouse serum ELISA test results: (A) TNF-α, (B) IL-10, and (C) IL-1β. *$P < 0.05$ compared with sham group; #$P < 0.05$ compared with CLP group.

inflammatory lung injury, whereas KO of the β-catenin gene or injection of β-catenin inhibitors significantly reduced β-catenin expression in lung tissue.

Serum cytokines

To investigate the effects of β-catenin on cytokines in the serum of septic mice, we measured the serum TNF-α, IL-10, and IL-1β levels via ELISA. It was found that the serum TNF-α, IL-10, and IL-1β levels of the CLP group were significantly higher than those of the sham group (Figure 4, $P < 0.05$). Compared with the CLP group, the β-catenin KO + CLP, XAV-939 + CLP, and ICG-001 + CLP groups had significantly lower serum cytokine levels ($P < 0.05$). These results demonstrate that the KO of the β-catenin gene or blocking the downstream signaling pathways of β-catenin significantly

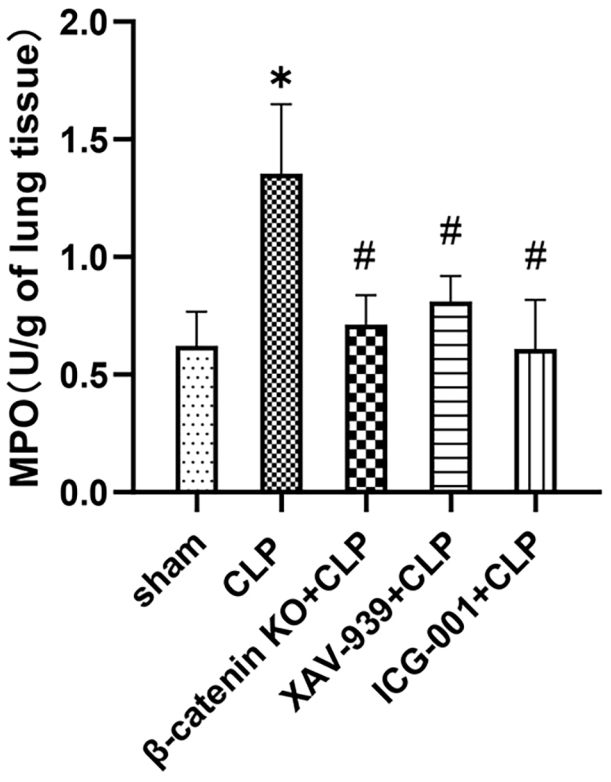

Figure 5. Results of mouse MPO activity assay in fresh lung tissue. $^{*}P < 0.05$ compared with sham group; $^{\#}P < 0.05$ compared with CLP group.

attenuated the release of inflammatory cytokines in the serum after the establishment of the CLP-induced sepsis model.

MPO activity assay

MPO is a hemoprotein present in high levels in neutrophils. Thus, the level of MPO activity serves as a marker of neutrophil aggregation and can be used to evaluate the neutrophil count in tissues. Our results show that MPO activity increased in the CLP group compared with the sham group, whereas the β-catenin KO + CLP, XAV-939 + CLP, and ICG-001 + CLP groups had significantly lower MPO activities compared with that of the CLP group (Figure 5, $P < 0.05$).

Lung tissue cell apoptosis

Excessive tissue inflammation leads to an increase in the number of apoptotic cells, which appear as round spots of green fluorescence under a fluorescent microscope (Figure 6). In the present study, the level of apoptosis in lung tissue was measured using the TUNEL assay. Our results indicate that the number of TUNEL-positive cells was low in the sham group but significantly increased in the CLP group. However, compared with the CLP group, the β-catenin KO + CLP, XAV-939 + CLP, and ICG-001 + CLP groups showed a significant reduction in the number of TUNEL-positive cells (Figure 7, $P < 0.05$).

Discussion

The gastrointestinal tract is the largest bacterial reservoir in the body. When sepsis is induced by CLP, due to the destruction of the intestinal mucosal barrier function, bacteria and endotoxins translocate and enter the lymphatic system of the intestinal wall tissue, and then enter the blood circulation, eventually causing systemic inflammatory response syndrome (SIRS) and organ dysfunction.[21–23] During the early stage of sepsis, the immune system becomes overactive in response to infection,[24] leading to the uncontrolled release of vast numbers of inflammatory cytokines and inflammatory mediators. This causes damage to tissues and organs, with the lungs being among the first organs to suffer severe damage. Sepsis-induced inflammatory lung injury is characterized by damage to the alveolar epithelial cells and pulmonary vascular endothelium[19,25] and substantial inflammatory cell infiltration.[26] β-catenin participates in these processes,[27] owing to its upregulated expression during lung inflammation and involvement in regulatory signaling pathways.[28]

β-catenin, a key molecule of the Wnt pathway, is a multifunctional protein that regulates transcription and affects the regulation of downstream signals for vital cellular activities such as cell differentiation, proliferation, and development.[29–31] β-catenin participates in signal transduction through cascades in various types of diseases such as tumors, cancers, autoimmune diseases, diabetes mellitus, and inflammatory diseases.[32–34] Studies have shown that during inflammation, β-catenin is activated, its expression is upregulated, and it modulates inflammatory responses by regulating the transcription of downstream target genes. For example, it stimulates tissue damage by regulating the expression of target genes that encode mediators such as matrix metalloproteinases.[19] Moreover, β-catenin disinhibits nuclear factor kappa B (NF-κB) by degrading IκB and upregulates p65 protein expression,[35] thereby promoting inflammation, as NF-κB and p65 function together and jointly participate in regulating inflammatory signals.[36] Furthermore, *in vitro* cellular experiments have demonstrated that the inhibition of β-catenin expression leads to the attenuation of inflammatory responses.[11] Recently, it reported an interaction between β-catenin and NLRP3 inflammasome, which may increase the production of pro-inflammatory cytokines.[37] However, the specific mechanisms through which β-catenin participates in regulating inflammatory responses in sepsis have not yet been fully elucidated.

To investigate the effects of β-catenin on sepsis-induced inflammatory lung injury, we constructed a sepsis model by subjecting β-catenin KO C57BL/6 and WT mice from the same litter to CLP. Forty-eight hours after CLP, the β-catenin KO mice exhibited decreased mortality, a lower degree of inflammatory cell infiltration in lung tissues, and reduced serum TNF-α, IL-10, and IL-1β levels. Inhibiting the release of inflammatory factors is crucial to improving the prognosis of sepsis. For example, intravenous injection of IL-1 inhibitors can inhibit the activation of macrophages, thereby inhibiting the development of sepsis inflammation.[38] In addition, measurements of the MPO activity, which reflects the neutrophil count in lung tissues, revealed markedly lower MPO activity in the lung tissues of β-catenin KO mice than that in the lung tissues of mice from the CLP group. These results show that β-catenin gene KO alleviated inflammatory responses and pathological lung injury in septic mice. Furthermore, TUNEL assay results showed a decrease in the number of apoptotic cells in the β-catenin KO group, indicating that β-catenin gene KO enabled the attenuation of apoptosis in lung tissue caused by excessive inflammation.

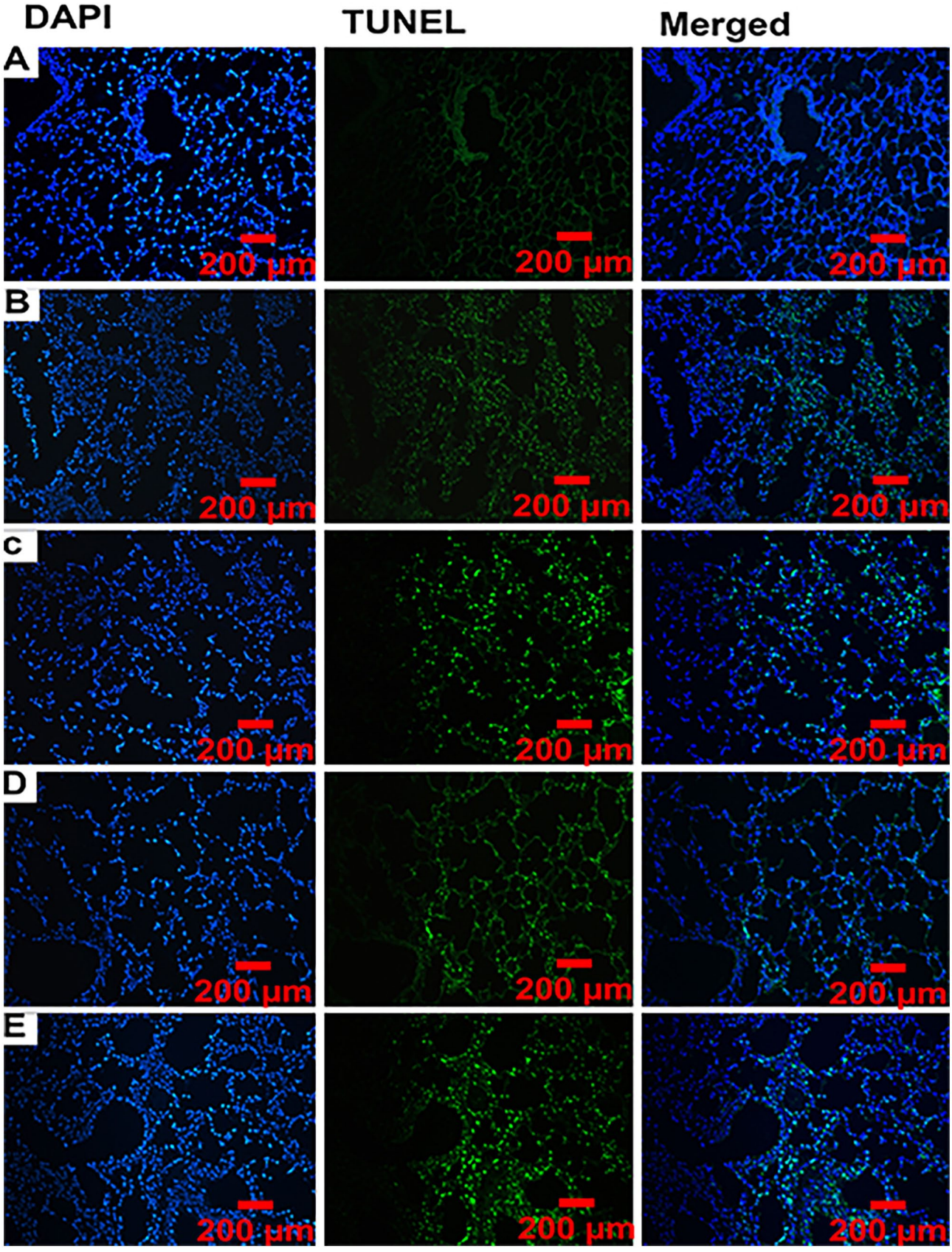

Figure 6. Apoptosis in lung tissue was assessed by TUNEL staining: (A) sham group; (B) CLP group; (C) β-catenin KO + CLP group; (D) XAV-939 + CLP group; and (E) ICG-001 + CLP group. Under the microscope, the nuclei of all cells were stained blue by DAPI, while TUNEL-positive cells showed green dotted fluorescence. Images were obtained using fluorescence microscopy. Magnification of original images was 200×. Scale bars represent 200 μm. (A color version of this figure is available in the online journal.)

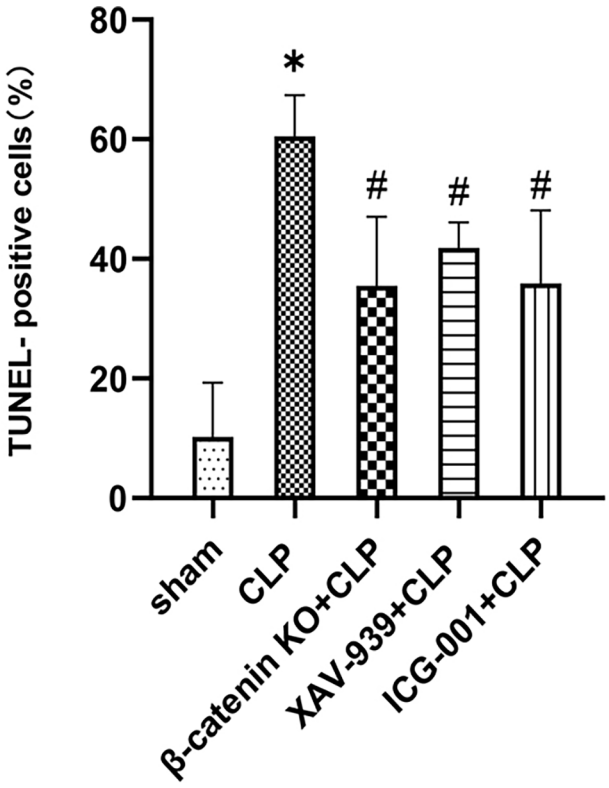

Figure 7. Histograms show the TUNEL-positive rate of lung tissue in different groups. Under ImageJ software analysis, calculate the ratio of the number of green fluorescent cells to the number of blue fluorescent cells, that is, the positive rate of TUNEL. *$P < 0.05$ compared with sham group; #$P < 0.05$ compared with CLP group.

In the present study, we also adopted inhibitor treatment to achieve a notable reduction in the β-catenin level over a short period of time. When the mortality rates of septic mice were compared, it was found that the effects of XAV-939 in reducing mortality were superior to those of ICG-001. This may be caused by the differences between the mode of action of the two inhibitor types; XAV-939 stimulates β-catenin degradation by stabilizing axin, whereas ICG-001 inhibits β-catenin/CBP interactions by antagonizing β-catenin/TCF-mediated transcription and binding specifically to CBP. Thus, unlike XAV-939, ICG-001 merely blocks signal transduction in one of the downstream signaling pathways of β-catenin. However, compared with the CLP group, both the XAV-939 + CLP and ICG-001 + CLP groups exhibited lower mortality rates, downregulation of β-catenin expression in lung tissue, alleviation of pathological injury, decreased MPO activity, and a smaller number of TUNEL-positive cells.

CBP is a transcriptional coactivator and a functional homologue of p300; it possesses histone acetyltransferase activity,[39] which enables it to acetylate histones, non-histones, and various transcription factors. In addition, CBP can regulate the expression of target genes through interactions with transcription factors; thus, it participates in biological processes such as cell differentiation and embryonic development.[40,41] The role of CBP in cancer, tissue fibrosis, and stem cell differentiation has attracted widespread research interest,[42–46] and studies have demonstrated the involvement of CBP in regulating inflammation.[39] In the present study, ICG-001 injections effectively blocked the binding of β-catenin to CBP and decreased β-catenin activity, notably reducing the inflammation levels and alleviating inflammation-induced pathological lung injury in septic mice. Therefore, it can be deduced that the β-catenin/CBP signaling axis participates in regulating sepsis-induced inflammatory lung injury.

Conclusions

The findings of the current study elucidated the effect of β-catenin on the inflammatory response in septic inflammatory lung injury and demonstrated that the β-catenin/CBP signal axis is involved in regulating septic inflammatory lung injury. Inhibiting the interaction of β-catenin and CBP can reduce the level of inflammation, providing a theoretical basis for sepsis research.

AUTHORS' CONTRIBUTIONS

HW contributed to conception and design. XC, DL, YN, and RC contributed to development of methodology. XC contributed to analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis). XC and HW contributed to writing and/or revision of the manuscript. DL, XR, and YZ contributed to technical support. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID ID

Xia Cheng https://orcid.org/0000-0002-2558-2567

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