

CC16 Ameliorates Inflammatory Responses and Acute Lung Injury in Mice by Modulating NF- κ B/MAPK Signaling

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Background: Acute lung injury (ALI) is a severe inflammatory lung condition characterized by alveolar damage, barrier disruption, and excessive inflammation. Current therapeutic strategies remain limited, highlighting the need for novel therapeutic targets. Dysregulated nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling contributes to ALI progression. Club cell protein 16 (CC16) has emerged as a potential regulator of pulmonary inflammation and tissue protection; however, its role in ALI remains unclear. This study aimed to elucidate the protective effects and underlying mechanisms of CC16 in ALI.

Methods: Lipopolysaccharide (LPS)-induced ALI mouse models were established and treated with varying doses of recombinant human CC16 (rhCC16). The evaluation of lung injury, inflammation, oxidative stress, and NF- κ B/p38/extracellular regulated protein kinases (ERK) MAPK signaling activation was carried out using the lung wet-to-dry weight ratio (W/D), hematoxylin and eosin (H&E) staining, immunohistochemical analysis, enzyme-linked immunosorbent assay (ELISA), and Western blot assays. Meanwhile, an LPS-stimulated A549 cell inflammation model was also established to verify the protective effect of rhCC16 *in vitro*. Additionally, tumor necrosis factor- α (TNF- α) stimulation was performed to further investigate whether inflammatory signaling activation could attenuate the protective effects of rhCC16.

Results: LPS-induced ALI significantly increased pulmonary edema ($p < 0.05$), inflammation, and oxidative stress in mice, accompanied by reduced CC16 expression ($p < 0.05$) and enhanced activation of NF- κ B/p38/ERK MAPK signaling-related molecules. rhCC16 treatment significantly alleviated pulmonary edema and inflammatory damage, increased detectable CC16 levels, and reduced activation of NF- κ B/p38/ERK MAPK signaling. *In vitro*, rhCC16 markedly enhanced cell viability, suppressed apoptosis, lactate dehydrogenase (LDH) release, oxidative stress, and inflammatory cytokine expression in LPS-stimulated A549 cells, while LPS and TNF- α pre-stimulation partially reversed these protective effects of rhCC16.

Conclusion: Restoration of CC16 expression by rhCC16 attenuates inflammatory responses and oxidative damage accompanied by reduced activation of NF- κ B/p38/ERK MAPK signaling, alleviating lung injury in ALI mice and LPS-exposed A549 cells. These results clarify both the protective effects of CC16 in ALI and the molecular pathways involved, highlighting its value as a potential intervention target.

Keywords: CC16; ALI; NF- κ B/p38/ERK MAPK signaling; inflammation; oxidative stress

Introduction

Acute lung injury (ALI) is a severe inflammatory pulmonary disorder characterized by increased alveolar-capillary barrier permeability, excessive inflammation, and alveolar fluid accumulation, representing an important pathological process within the spectrum of acute respiratory distress syndrome (ARDS) [1]. At the cellular and molecular levels, ALI involves complex interactions among epithelial cells, endothelial cells, and immune responses, resulting in uncontrolled inflammatory amplification, oxidative stress, and tissue damage [2]. In severe cases, ALI can progress to ARDS, causing profound hypoxemia

and high mortality [3,4]. Current clinical management primarily focuses on supportive strategies, including lung-protective ventilation, fluid management, and treatment of the underlying causes, while effective therapies targeting ALI progression remain limited [5,6]. Therefore, elucidating the molecular mechanisms involved in ALI development and identifying novel therapeutic targets for ALI are of critical scientific and clinical importance.

In ALI, inflammation is fundamental to disease initiation and subsequent deterioration. During ALI development, excessive activation of immune cells and pulmonary structural cells leads to uncontrolled production of pro-inflammatory mediators, disruption of the alveolar-

capillary barrier, and amplification of local inflammatory cascades, ultimately aggravating lung tissue damage [7,8]. Nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways are critical regulators of inflammatory responses [9]. The classical inflammatory transcription factor NF- κ B induces the transcription and secretion of several cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) [10,11]; while the MAPK pathway, comprising extracellular regulated protein kinases (ERK), jun n-terminal kinase (JNK), and p38 branches, participates in inflammatory cytokine production and apoptosis regulation [12,13]. Extensive evidence indicates that sustained overactivation of NF- κ B and MAPK signaling in ALI models is a key driver of dysregulated inflammatory responses and pulmonary tissue damage [14,15]. Therefore, modulation of the NF- κ B/p38/ERK MAPK signaling pathway could represent a promising therapeutic strategy for mitigating ALI.

Club cell protein 16 (CC16), often referred to as Clara cell protein, is a low molecular weight protein secreted by bronchial Clara cells, exhibiting anti-inflammatory, antioxidant, and immunomodulatory properties [16]. Airway epithelial cells are the primary source of CC16, which can cross the alveolar–capillary barrier and enter the bloodstream [17]. Previous studies have suggested that decreased CC16 levels are associated with the onset of chronic obstructive pulmonary disease, asthma, and ARDS [18,19,20], and that it is commonly used as a biomarker of airway epithelial injury and inflammation [21,22]. Experimental evidence indicates that CC16-based intervention may protect against pulmonary injury by suppressing excessive inflammation, reducing epithelial cell damage, and facilitating tissue repair in inflammatory lung disease models [23]. Nevertheless, the therapeutic effects of exogenous CC16 administration in ALI and the molecular mechanisms underlying its protective actions remain poorly defined, particularly regarding its potential modulation of NF- κ B/p38/ERK MAPK signaling.

In the present study, ALI mouse models were generated via lipopolysaccharide (LPS) administration, and human alveolar epithelial A549 cells were stimulated with LPS to systematically explore the protective effects of exogenously administered CC16 and its regulatory effects on the NF- κ B/p38/ERK MAPK signaling pathway. This study aims to clarify the molecular pathways through which CC16 mitigates inflammatory lung injury, thereby enhancing our understanding of ALI pathophysiology and providing potential targets for clinical intervention.

Materials and Methods

Experimental Animals

Twenty-five male C57BL/6 mice (8–10 weeks old, weighing 25.0 ± 3.0 g) were purchased from Spebfo Biotech Co., Ltd. (Beijing, China; license no. SCXK [Beijing]:

2019-0010). Mice were housed under specific pathogen-free conditions and provided with unrestricted access to food and water. After a 7-day acclimation period, the experimental interventions were carried out.

Mouse Model and Grouping

After acclimatization, mice were randomly allocated into five groups, with five animals in each group: a normal control (Control) group, an ALI model (ALI) group, and three rhCC16 treatment groups receiving low, medium, and high doses (1.5, 3.0, and 5.0 μ g/g, respectively). Prior to modeling, mice were anesthetized with 5% isoflurane (YS1412C, YaJi Biological, Shanghai, China) for approximately 5 min. ALI was established via intratracheal administration of LPS (5 mg/kg; ST1470, Beyotime, Shanghai, China) [24]. One hour after LPS administration, recombinant human Club cell protein 16 (rhCC16, RPA857Hu01, Cloud-Clone Corp., Wuhan, China) was intratracheally administered to the treatment groups at the indicated doses; Control and ALI mice were administered an equal volume of sterile phosphate-buffered saline (PBS; G2156, Servicebio, Wuhan, China). After a 24-hour post-treatment period, mice were deeply anesthetized with isoflurane (5% in room air; flow rate: 0.4 L/min) delivered via the small animal anesthesia system. Subsequently, mice were euthanized by cervical dislocation, and lung tissues were collected for further analysis. All animal experiments were performed in accordance with the ARRIVE Guidelines 2.0 and approved by the the Animal Ethics Committee of South Zhejiang Institute of Radiation Medicine and Nuclear Technology Applications.

Bronchoalveolar Lavage Fluid (BALF) Protein and Cell Counts

After euthanasia, the trachea was exposed and cannulated with a sterile catheter. The lungs were gently lavaged with 1 mL of sterile PBS per lavage, repeated three times. For each lavage, PBS was slowly instilled into the lungs and gently withdrawn using a syringe. The recovered BALF was immediately collected, and the lavage fluid from the three sequential washes was pooled for each mouse. The BALF samples were then centrifuged at $500 \times g$ for 10 min at 4 °C to separate the cellular and supernatant fractions.

After collection of BALF samples, total protein content was quantified using a bicinchoninic acid assay (BCA) protein assay kit (E-BC-K318-M, Elabscience, Wuhan, China) as per the manufacturer's guidelines. Cells were reconstituted in PBS and counted using an automated cell counter (C100-Pro, RWD, Shenzhen, China). Differential cell counts, including macrophages and neutrophils, were determined using Giemsa staining (JLC1016, Gelatins, Shanghai, China).

Lung Wet-to-Dry (W/D) Weight Ratio

The extent of pulmonary edema was evaluated using the lung W/D ratio. The left upper lobe was weighed for wet weight (W) and subsequently dried at 60 °C for 24 h to record the dry weight (D).

Hematoxylin–Eosin (H&E) Staining

Lung tissues were treated with 4% paraformaldehyde, embedded in paraffin, and sectioned. Tissue sections were subjected to H&E staining using a kit (60524ES60, YEASEN, Shanghai, China) and examined under a light microscope (Leica DM2000, Leica, Wetzlar, Germany). Lung injury was scored using the Smith scoring method [25].

Myeloperoxidase (MPO) Activity Assay

MPO levels in lung samples were quantified using an MPO assay kit (CB10641-Mu, COLBO BIO, Shanghai, China) as per the manufacturer's guidelines. A microplate reader (Multiskan SkyHigh, Thermo Fisher, Waltham, USA) was used to read absorbance at 460 nm.

Immunohistochemistry (IHC)

Paraffin lung sections (5 µm) were deparaffinized, rehydrated, and subjected to antigen retrieval. Sections were treated with 3% hydrogen peroxide (H103201-s, Yonghua Chemical, Changshu, China) to block endogenous peroxidase activity and were blocked with 5% normal goat serum (36119ES03, YEASEN, Shanghai, China). Sections were exposed to antibodies against F4/80 (1:5000, ab300421, Abcam, Cambridge, MA, USA) overnight at 4 °C, followed by detection using an IHC kit (D601037-0050, Sangon Biotech, Shanghai, China). Images were acquired with an Olympus BX53 microscope, and F4/80-positive cells were counted using ImageJ software (v1.46, NIH, MD, USA).

Cell Culture and Treatment

Human alveolar epithelial cells (A549, JNO-H0022, Jennio Biotech, Guangzhou, China) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (CA0004, SparkJade, Shandong, China) containing 10% fetal bovine serum (FBS; A5670801, Thermo Fisher, Waltham, USA), 10 µg/mL streptomycin (ST487, Beyotime, Shanghai, China), and 10 U/mL penicillin (ST486, Beyotime, Shanghai, China) at 37 °C in a humidified 5% CO₂ incubator. Cells were verified by short tandem repeat analysis and confirmed to be free of mycoplasma contamination. For LPS-stimulated A549 epithelial injury modeling, A549 cells received LPS (1 µg/mL) treatment for 24 h [26]. The experimental groups were designated as Control, LPS, rhCC16, and LPS + TNF-α + rhCC16. To ensure a consistent total experimental duration across groups, all cells were maintained for a total of 48 h according to a two-phase treatment protocol consisting of a first 24-h phase and a sec-

ond 24-h phase. During the first 24 h, the Control group was maintained under standard culture conditions, whereas the LPS and rhCC16 groups were stimulated with LPS (1 µg/mL). The LPS + TNF-α (P4416, FineTest, Wuhan, China) + rhCC16 group was simultaneously stimulated with LPS (1 µg/mL) and TNF-α (40 ng/mL). After the first 24 h, the culture medium was removed and replaced with fresh medium. During the subsequent 24 h, the Control and LPS groups received an equivalent volume of rhCC16 solvent, whereas the rhCC16 and LPS + TNF-α + rhCC16 groups were treated with rhCC16 (2 µg/mL). LPS and TNF-α were not added during the second 24-h phase. Cells and culture supernatants were harvested at the end of the 48-h experimental period for subsequent analyses.

Cell Viability Assay

Cell viability of A549 cells was measured using a Cell Counting Kit-8 (CCK-8) assay (ml095229, mlbio, Shanghai, China). Cells (1 × 10⁴ cells/well) were plated in 96-well plates, treated, and incubated with 10 µL of CCK-8 solution for 1–2 h at 37 °C. Optical density (OD) was recorded at 450 nm (Multiskan SkyHigh, Thermo Fisher, MA, USA).

Cell Death Assay

Lactate dehydrogenase (LDH) release was quantified using an LDH kit (CB11219, COIBO BIO, Shanghai, China) to evaluate cell damage following the manufacturer's instructions.

Apoptosis Detection

A terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay kit (40306ES50, YEASEN, Shanghai, China) was employed to assess apoptosis in A549 cells. Cells on coverslips were fixed with 4% paraformaldehyde (AWI0056, Abiowell, Changsha, China) for 30 min and washed with PBS. Following the manufacturer's protocol, TUNEL staining was applied, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Apoptotic cells were examined under a fluorescence microscope (CX41-32RFL, Olympus, Tokyo, Japan). TUNEL-positive cells were identified based on the co-localization of TUNEL fluorescence signals with DAPI-stained nuclei. The percentage of TUNEL-positive cells was quantified using ImageJ software by calculating the proportion of DAPI-positive nuclei exhibiting TUNEL signals in randomly selected microscopic fields.

Intracellular Reactive Oxygen Species (ROS) Measurement

Intracellular ROS levels were determined using a ROS detection kit (S0034S, Beyotime, Shanghai, China). Cells were incubated with a ROS-sensitive probe and visualized under a fluorescence microscope (CX41-32RFL, Olympus, Tokyo, Japan). Nuclei were counterstained with DAPI, and

ROS fluorescence intensity was quantified using ImageJ software and normalized to the number of DAPI-positive cells in randomly selected fields.

CC16 Measurement

The levels of CC16 in mouse BALF, lung tissues, and A549 cells were determined using enzyme-linked immunosorbent assay (ELISA) kits (CB11005-Mu; CB18820-Hu, COIBO BIO, Shanghai, China) following the manufacturer's instructions.

Inflammatory Cytokine Assays

Concentrations of TNF- α , IL-6, and IL-1 β in mouse BALF and lung tissues were measured using mouse-specific ELISA kits (CB10851-Mu, CB10187-Mu, CB10851-Mu, COIBO BIO, Shanghai, China) according to the manufacturer's protocols. Concentrations of IL-6 and IL-1 β in A549 cells were measured using human-specific ELISA kits (CB10373-Hu, CB10347-Hu, COIBO BIO, Shanghai, China) according to the manufacturer's instructions.

Oxidative Stress Marker Assessment

Contents of malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) in lung tissues and A549 cells were quantified using commercial assay kits (A003-4-1, A006-2-1, A001-3-2; Nanjing Jiancheng, Nanjing, China) following the manufacturer's instructions.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA from lung tissues and A549 cells was extracted with TRIzol (MT0010, BioLab, Beijing, China), converted to cDNA using RT Master Mix for qPCR II (HY-K0511A, MedChemExpress, NJ, USA), and analyzed by quantitative polymerase chain reaction (qPCR) with SYBR Green qPCR Master Mix (HY-K0501A, MedChemExpress, NJ, USA) on an Mx3005P system (Agilent, CA, USA). Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method (see Table 1 for primers).

Western Blot (WB) Analysis

Total protein was isolated from lung tissues and A549 cells using radioimmunoprecipitation assay lysis buffer (20101ES60, YEASEN, Shanghai, China). Protein concentrations were measured, and equivalent amounts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk for 1 hour at room temperature (RT) and incubated overnight at 4 °C with primary antibodies against CC16 (1:1000, PAA857Mu01; 1:1000, PAA857Hu01, Cloud-Clone Corp., Wuhan, China), p-NF- κ B p65 (1:1000, 31013ES50, Yeason, Shanghai, China),

Table 1. Primer sequences for qRT-PCR.

Gene name	Species	Sequence (5'-3')
<i>IL-6</i>	Human	F: AGACAGCCACTCACCTCTTCAG R: TTCTGCCAGTGCCTCTTTGCTG
<i>IL-6</i>	Mouse	F: TACCACTTCAACAAGTCGGAGGC R: CTGCAAGTGCATCATCGTTGTTG
<i>TNF-α</i>	Human	F: CTCTTCTGCCTGCTGCACTTTG R: ATGGGCTACAGGCTTGCTCACTC
<i>TNF-α</i>	Mouse	F: AAGCCTGTAGCCACGTCGTA R: GGCACCACTAGTTGGTTGTCTTTG
<i>IL-1β</i>	Human	F: CCCTAAACAGATGAAGTGCTCCTT R: GGTGGTCGGAGATTCGTAGCT
<i>IL-1β</i>	Mouse	F: GCCACCTTTTGACAGTGATGAG R: GTTTGGAAGCAGCCCTTCATC
<i>CC16</i>	Human	F: CAGAGACGGAACCAGAGACG R: CAGATCTCTGCAGAAGCGGA
<i>CC16</i>	Mouse	F: ATGAAGATCGCCATCACAAATCA R: GAATCTTAAATCTTGCTTACACAG
<i>TLR4</i>	Human	F: CCCTGAGGCATTTAGGCAGCTA R: AGGTAGAGAGGTGGCTTAGGCT
<i>CD14</i>	Human	F: CTGGAACAGGTGCCTAAAGGAC R: GTCCAGTGTGCAAGTTATCCACC
<i>NF-κB p65</i>	Human	F: TGAACCGAAACTCTGGCAGCTG R: CATCAGCTTGCGAAAAGGAGCC
<i>NF-κB p65</i>	Mouse	F: CTGGCAAGCGTATCCCAAGA R: AGTCGAACAGCCTCCATTCTG
<i>p38</i>	Human	F: CCTGATGATGAGCCTGTTGC R: GAGAAGGTCTTCCCCTCACA
<i>p38</i>	Mouse	F: CTGACCGACGACCACGTTTC R: CTTCGTTACAGCTAGGTTGC
<i>ERK</i>	Human	F: CCTAAGGAAAAGCTCAAAGA R: AAAGTGGATAAGCCAAGAC
<i>ERK</i>	Mouse	F: CAGGTGTTGACGCTAGGGC R: TCTGGTGCTCAAAAGGACTGA
<i>GAPDH</i>	Human	F: ATGGGGAAGGTGAAGGTCTG R: GGGGTCATTGATGGCAACAATA
<i>GAPDH</i>	Mouse	F: TGCACCACCAACTGCTTAG R: GGATGCAGGGATGATGTTT

qRT-PCR, Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; CC16, Club cell secretory protein 16; TLR4, Toll-like receptor 4; CD14, cluster of differentiation 14; NF- κ B, Nuclear factor kappa B; ERK, extracellular regulated protein kinases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

NF- κ B p65 (1:1000, 773007ES50, Yeason, Shanghai, China), p-p38 MAPK (1:1000, 773045ES50, Yeason, Shanghai, China), p38 MAPK (1:1000, 31048ES50, Yeason, Shanghai, China), p-ERK (1:1000, 773056ES50, Yeason, Shanghai, China), ERK (1:1000, 773038ES50, Yeason, Shanghai, China), and GAPDH (1:1000, GB11002-100, Servicebio, Wuhan, China). Following washes, membranes were incubated with HRP-conjugated goat anti-

rabbit secondary antibodies (1:1000, A0208, Beyotime, Shanghai, China) for 1 h at RT. Bands were visualized using chemiluminescence (Bio-Rad, California, USA) and quantified using ImageJ software. For total protein expression analysis, GAPDH was used as the loading control. The levels of phosphorylated proteins were normalized to their corresponding total protein levels.

Statistical Analysis

Data were analyzed in GraphPad Prism 9.5 (version 9.5, GraphPad Software, CA, USA) and are shown as mean $\pm$ standard deviation (SD). Data distribution was assessed for normality before statistical analysis. For comparisons between two groups, an unpaired Student's *t*-test was applied, whereas one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for comparisons among multiple groups. A *p* value < 0.05 was considered statistically significant.

Results

rhCC16 Alleviated Lung Edema and Inflammation and Improved Histopathological Injury in ALI Mice

Relative to the control group, the lung W/D ratio was notably increased in mice with ALI ($p < 0.05$, Fig. 1A), along with elevated BALF protein concentrations and total cell counts ($p < 0.05$, Fig. 1B,C), indicating pronounced pulmonary edema and increased alveolar-capillary barrier permeability. H&E staining demonstrated disruption of alveolar architecture, interstitial thickening, and widespread infiltration of inflammatory cells, accompanied by a significant increase in Smith scores ($p < 0.05$, Fig. 1D). Furthermore, BALF neutrophil and macrophage counts, as well as lung MPO activity, were markedly increased ($p < 0.05$, Fig. 1E–G), suggesting robust inflammatory activation. rhCC16 treatment significantly mitigated ALI-induced lung injury, significantly reducing the W/D ratio, BALF protein concentration, and cell counts, while improving histopathological scores and decreasing inflammatory cell infiltration and MPO activity ($p < 0.05$, Fig. 1A–G). IHC analysis demonstrated that ALI lungs exhibited a marked elevation in F4/80-positive cells compared with control ($p < 0.05$, Fig. 1H), an effect that was substantially attenuated by rhCC16 ($p < 0.05$, Fig. 1H). Collectively, rhCC16 effectively attenuated pulmonary edema and inflammation in ALI mice, reduced inflammatory cell infiltration, and improved lung structural integrity, demonstrating a pronounced protective effect.

rhCC16 Attenuated Oxidative Stress and Suppressed Inflammation in ALI Mouse Lungs

As shown in Fig. 2, lung tissues from ALI mice displayed significantly elevated MDA levels ($p < 0.05$) and reduced GSH levels and SOD activity ($p < 0.05$), indicating enhanced oxidative stress (Fig. 2A). ELISA results

showed significantly increased TNF- α , IL-6, and IL-1 β levels in BALF and lung tissues of ALI mice compared with Controls ($p < 0.05$, Fig. 2B,C), reflecting marked inflammatory activation. rhCC16 treatment significantly ameliorated these changes, with low-dose rhCC16 significantly reducing oxidative stress and inflammatory responses ($p < 0.05$), while medium- and high-dose treatments also produced pronounced protective effects ($p < 0.001$, Fig. 2A–C). qRT-PCR analysis showed that the mRNA levels of inflammatory cytokines were markedly elevated in ALI lung tissues ($p < 0.05$, Fig. 2D), which were significantly down-regulated following rhCC16 treatment ($p < 0.05$, Fig. 2D). These findings indicate that rhCC16 alleviated ALI lung injury by reducing oxidative stress and suppressing inflammatory cytokine expression at both the protein and gene levels.

rhCC16 Increased Detectable CC16 Levels and Inhibited NF- κ B/p38/ERK MAPK Pathway Activation in ALI Mouse Lungs

As shown in Fig. 3, CC16 levels in both BALF and lung tissues were markedly reduced in ALI mice relative to the control group ($p < 0.05$, Fig. 3A), indicating suppressed detectable CC16 levels during ALI. Western blot analysis revealed decreased CC16 protein levels and increased p-NF- κ B p65, p-p38, and p-ERK levels in ALI lungs ($p < 0.05$), while total NF- κ B p65, p38, and ERK levels remained unchanged ($p > 0.05$, Fig. 3B,C), suggesting activation of the NF- κ B/p38/ERK MAPK pathways. rhCC16 treatment significantly increased detectable CC16 levels and suppressed the activation of NF- κ B/p38/ERK MAPK signaling proteins (Fig. 3A–C). qRT-PCR analysis further revealed corresponding transcriptional changes in genes related to the NF- κ B/p38/ERK signaling axis (Fig. 3D). These observations suggest that rhCC16 protected against lung inflammation in ALI mice by increasing detectable CC16 levels, which was accompanied by reduced activation of NF- κ B/p38/ERK MAPK signaling and attenuation of inflammatory responses.

rhCC16 Ameliorated LPS-Induced A549 Cell Injury and Apoptosis

A549 cell viability was evaluated following rhCC16 treatment using CCK-8 assays. Within the concentration range of 0–3 μ g/mL, rhCC16 did not exhibit significant cytotoxic effects ($p > 0.05$). In contrast, treatment with 5 μ g/mL significantly reduced cell viability ($p < 0.05$), indicating potential cytotoxicity at higher concentrations. Therefore, rhCC16 concentrations ranging from 0–3 μ g/mL were selected for subsequent experiments. When various concentrations of rhCC16 (0.5, 1, 2, and 3 μ g/mL) were applied following LPS stimulation, cell viability was significantly restored ($p < 0.05$). Notably, the protective effects of 2 μ g/mL and 3 μ g/mL rhCC16 were comparable ($p > 0.05$); therefore, 2 μ g/mL rhCC16 was selected for subsequent cell experiments (Fig. 4A).

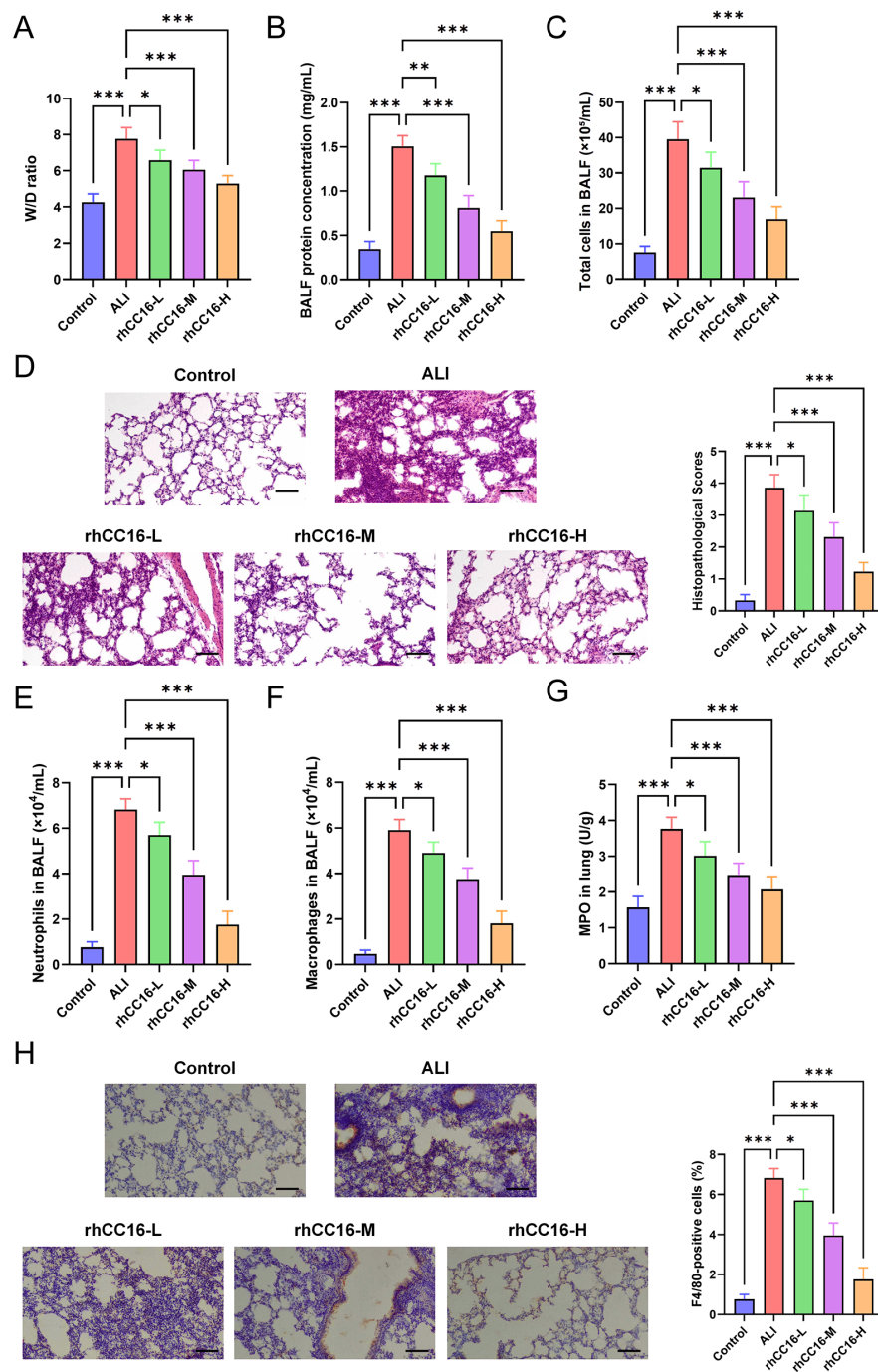


Fig. 1. Effect of rhCC16 on lung edema and inflammation in ALI mice. (A) Lung W/D ratio. (B,C) Protein concentration and total cellularity in BALF were quantified. (D) H&E staining and Smith score (100 $\times$, 100 μ m). (E,F) BALF neutrophil and macrophage counts. (G) Lung MPO activity. (H) Lung IHC (100 $\times$, 100 μ m). $n = 5$; $*p < 0.05$; $**p < 0.01$, $***p < 0.001$. rhCC16, recombinant human CC16; ALI, acute lung injury; W/D, Wet-to-Dry; BALF, Bronchoalveolar Lavage Fluid; H&E, hematoxylin–eosin; MPO, myeloperoxidase; IHC, immunohistochemistry.

LPS stimulation markedly decreased A549 cell viability ($p < 0.05$) and increased LDH release ($p < 0.05$), confirming LPS-induced cellular injury (Fig. 4B,C). rhCC16 treatment significantly restored cell viability and reduced LDH release ($p < 0.05$, Fig. 4B,C). Compared with LPS-stimulated cells treated with rhCC16, LPS and TNF- α co-

stimulated cells subsequently treated with rhCC16 exhibited partially reduced cell viability and elevated LDH release ($p < 0.05$), suggesting that modulation of inflammatory signaling pathways may contribute to the protective effects of rhCC16 (Fig. 4B,C). To further confirm the responsiveness of A549 cells to LPS stimulation, the ex-

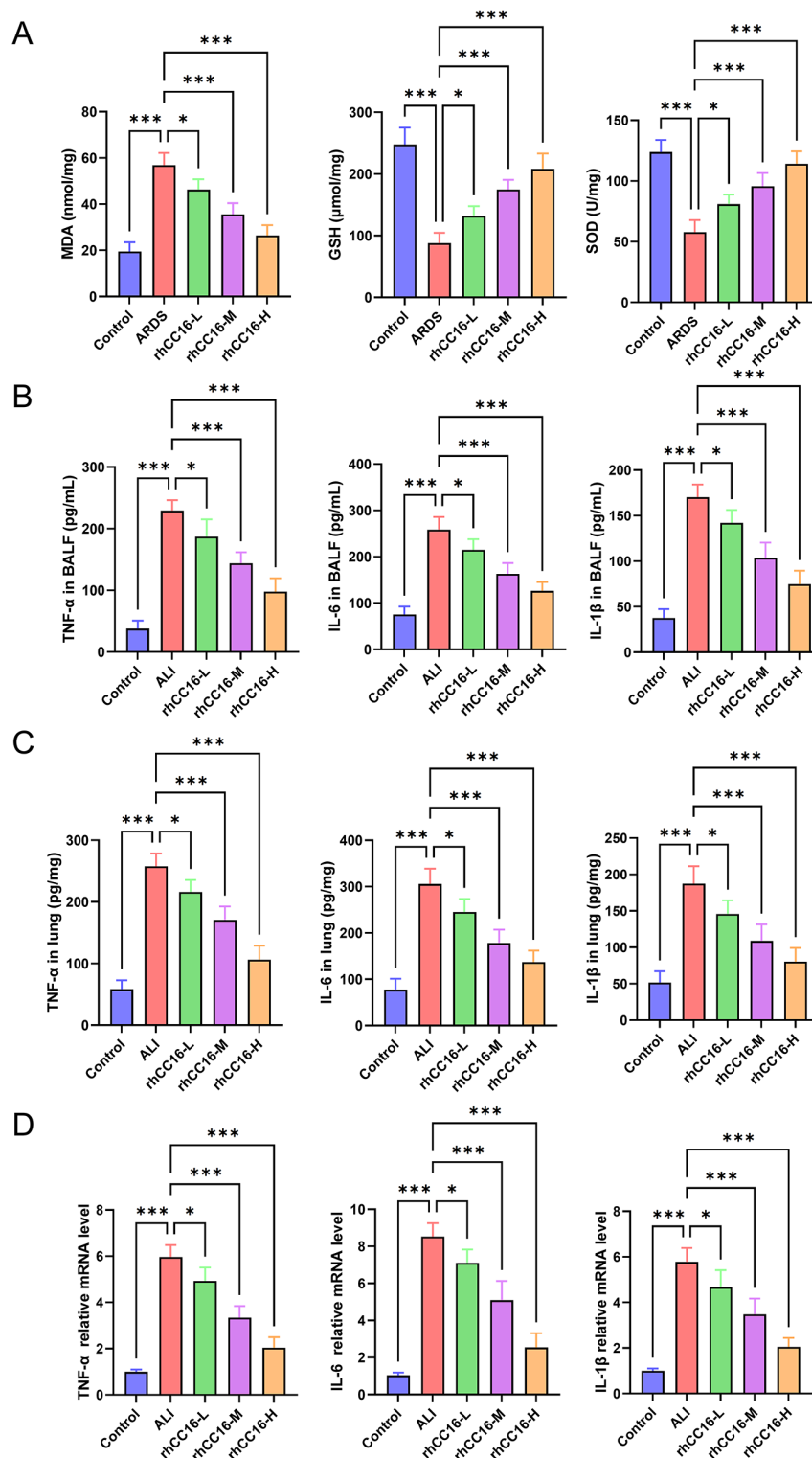


Fig. 2. Effects of rhCC16 on oxidative stress and inflammatory responses in ALI mouse lungs. (A) Lung MDA, GSH, and SOD levels. (B,C) TNF-α, IL-6, and IL-1β levels in BALF and lung tissues. (D) Expression of inflammatory cytokine mRNAs in lung tissues. $n = 5$; $*p < 0.05$, $***p < 0.001$. MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase.

pression levels of LPS recognition-related molecules were evaluated by qRT-PCR. Compared with the control group, LPS stimulation significantly increased the mRNA levels

of TLR4 and CD14 in A549 cells ($p < 0.05$), indicating that A549 cells exhibited molecular responses to LPS challenge (Fig. 4D). TUNEL staining further revealed that

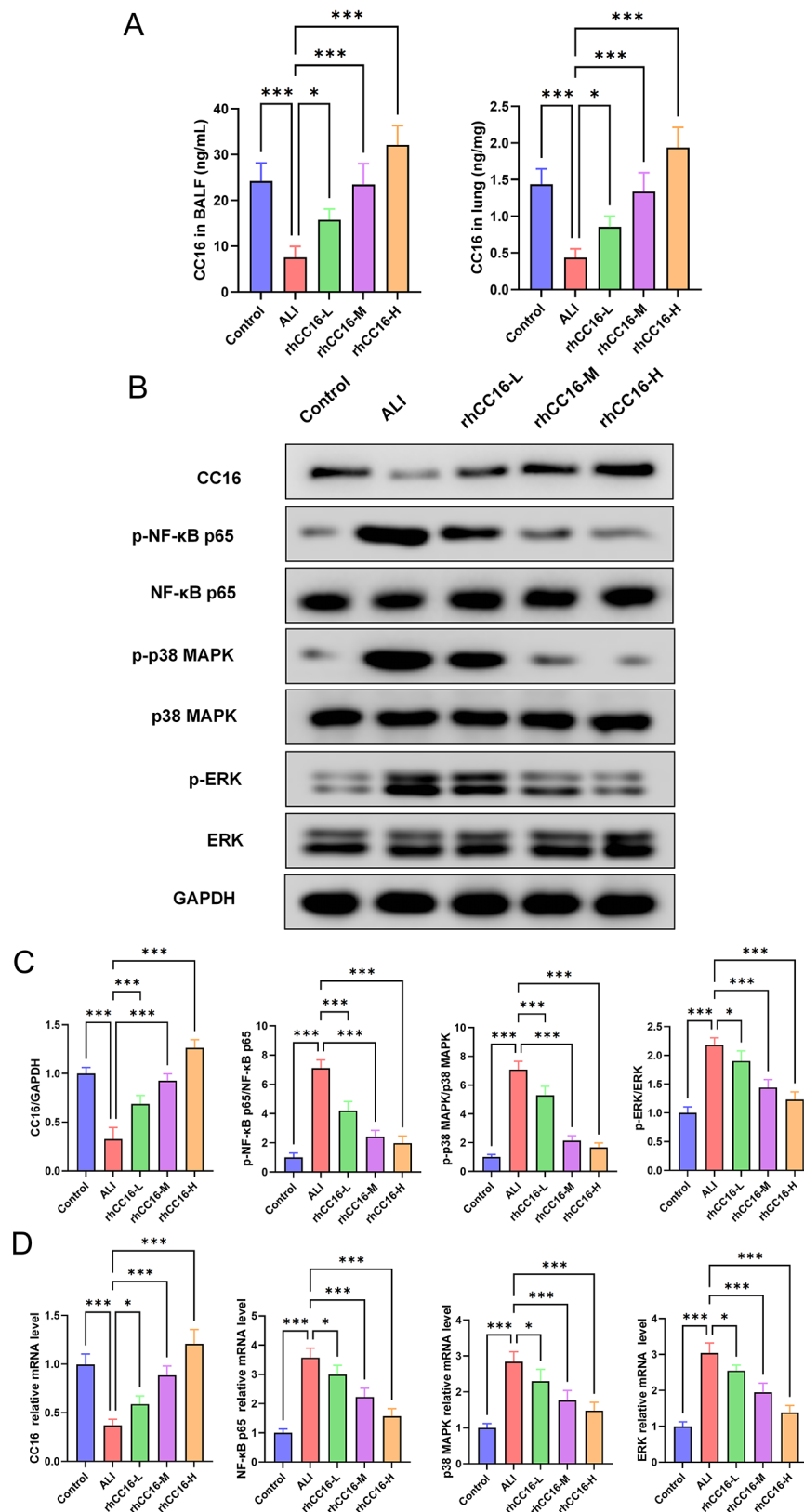


Fig. 3. Effect of rhCC16 on CC16 expression and NF-κB/p38/ERK MAPK signaling in ALI mouse lungs. (A) CC16 amounts in BALF and lung tissues. (B,C) WB of CC16 and NF-κB/p38/ERK MAPK pathway proteins. (D) mRNA expression of CC16 and NF-κB/p38/ERK MAPK-related genes. $n = 5$; $*p < 0.05$, $***p < 0.001$. WB, western blot.

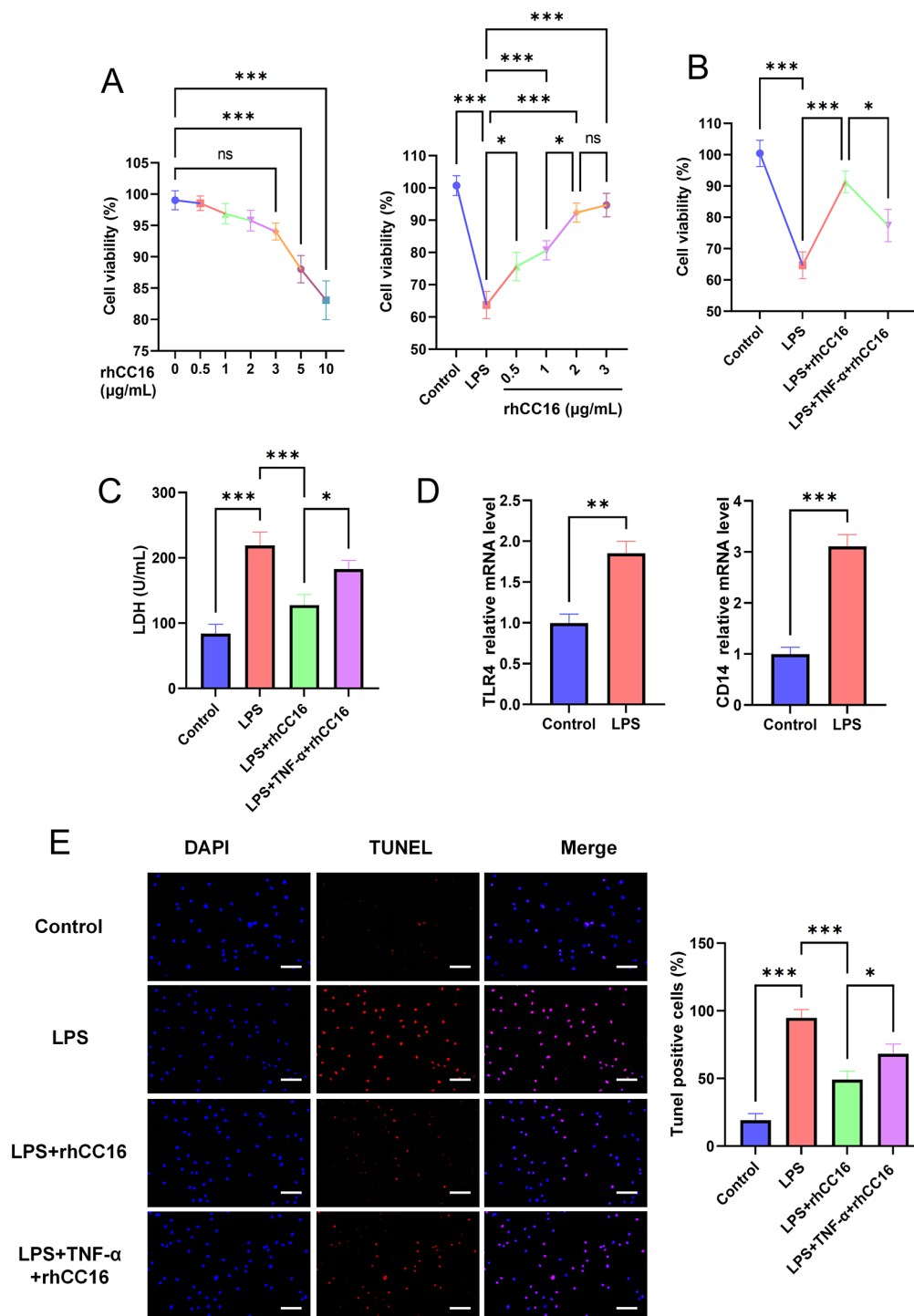


Fig. 4. Protective role of rhCC16 against LPS-induced injury and apoptosis in A549 cells. (A) Effects of different rhCC16 concentrations on A549 cell viability (CCK-8 assay). (B) Cell viability under various treatments. (C) LDH release levels. (D) Relative mRNA expression levels of TLR4 and CD14 in A549 cells following LPS stimulation. (E) TUNEL staining (200 $\times$, 50 μ m). $n = 3$; ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. LPS, lipopolysaccharide; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; CD14, cluster of differentiation 14.

LPS stimulation significantly increased TUNEL positivity ($p < 0.05$), whereas rhCC16 treatment markedly reduced the proportion of TUNEL-positive cells ($p < 0.05$). Com-

pared with LPS-stimulated cells treated with rhCC16, cells pre-stimulated with LPS and TNF- α followed by rhCC16 treatment showed increased TUNEL positivity ($p < 0.05$,

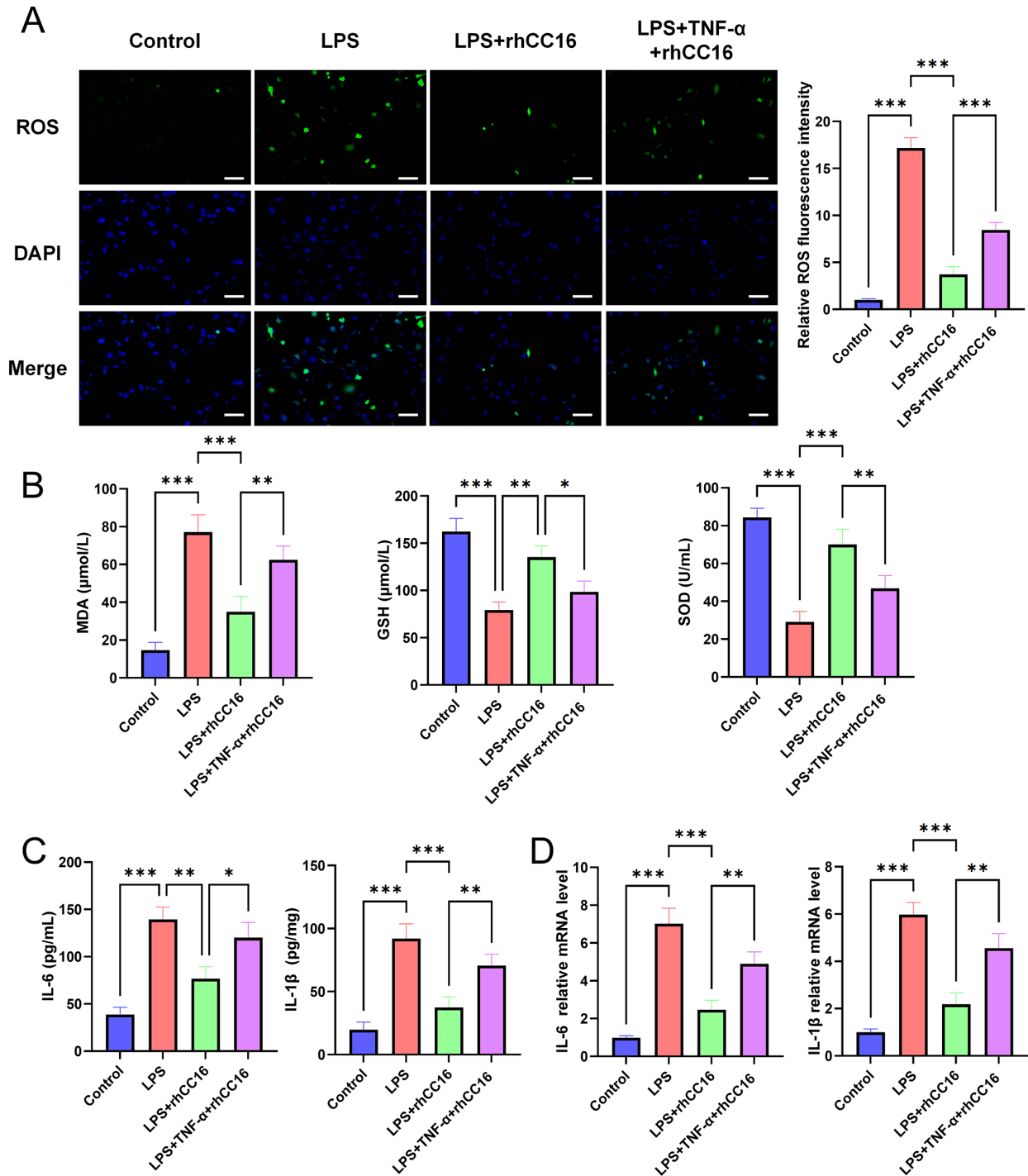


Fig. 5. Impact of rhCC16 on oxidative stress and inflammatory reactions in LPS-stimulated A549 cells. (A) ROS levels (200 $\times$, 50 μm). (B) Cellular MDA, GSH, and SOD measurements. (C) IL-6 and IL-1 β levels. (D) Inflammatory cytokine mRNA expression in cells. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ROS, Reactive Oxygen Species.

Fig. 4E), indicating that enhanced inflammatory signaling responses may attenuate the protective effects of rhCC16. In summary, rhCC16 effectively attenuated LPS-induced injury and apoptosis in A549 cells.

rhCC16 Ameliorated LPS-Induced A549 Cell Injury by Alleviating Oxidative Stress and Suppressing Inflammatory Responses

ROS analysis revealed that LPS stimulation markedly elevated ROS levels in A549 cells relative to the control

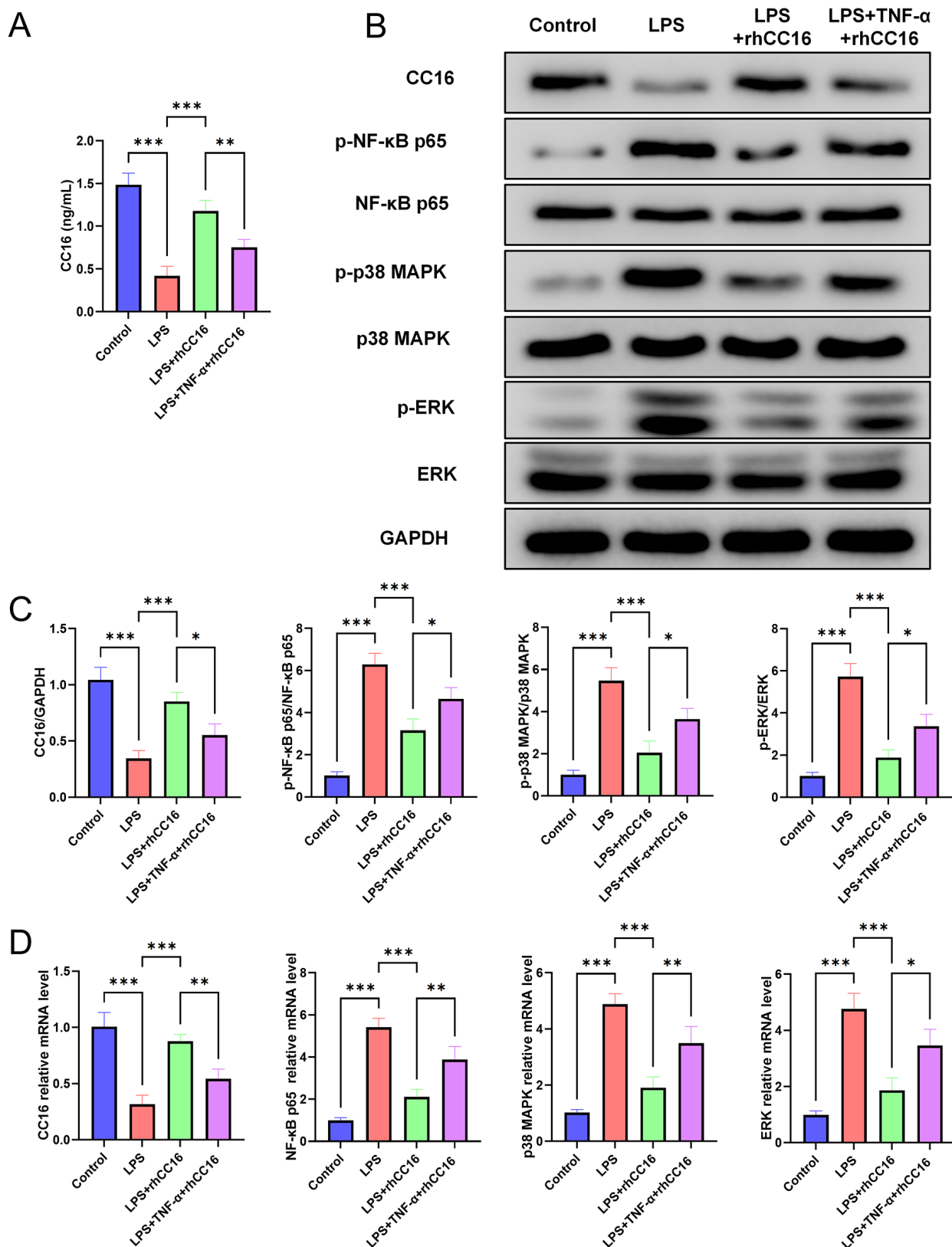


Fig. 6. Effects of rhCC16 on CC16 expression and NF-κB/p38/ERK MAPK signaling in LPS-stimulated A549 cells. (A) CC16 levels in cells. (B,C) WB analysis of CC16 and NF-κB/p38/ERK MAPK pathway proteins. (D) The mRNA levels of CC16 and NF-κB/p38/ERK MAPK-related genes. *n* = 3. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. MAPK, mitogen-activated protein kinase.

group ($p < 0.05$), whereas rhCC16 treatment markedly reduced ROS accumulation. Compared with LPS-stimulated cells treated with rhCC16, cells pre-stimulated with LPS and TNF- α followed by rhCC16 treatment exhibited partially increased ROS levels ($p < 0.05$), indicating that enhanced inflammatory signaling responses may reverse the antioxidant effect of rhCC16 (Fig. 5A). Assessment of oxidative stress markers revealed that LPS stimulation significantly increased cellular MDA levels ($p < 0.05$) and reduced GSH content and SOD activity ($p < 0.05$, Fig. 5B). Intervention with rhCC16 significantly reduced MDA levels ($p < 0.05$) and restored GSH content and SOD activity ($p < 0.05$) in LPS-stimulated cells, whereas cells pre-stimulated with LPS and TNF- α followed by rhCC16 treatment exhibited a weaker antioxidant response (Fig. 5B). Analysis of inflammatory cytokines showed that LPS stimulation increased IL-6 and IL-1 β levels in A549 cells ($p < 0.05$). rhCC16 intervention significantly suppressed these inflammatory mediators ($p < 0.05$) in LPS-stimulated cells, whereas cells pre-stimulated with LPS and TNF- α followed by rhCC16 treatment exhibited a partially reversed inhibitory effect (Fig. 5C). qRT-PCR results were consistent with the observed protein expression patterns (Fig. 5D). Collectively, the results indicate that rhCC16 protected A549 cells against LPS-induced damage by reducing oxidative stress and inhibiting the expression of inflammatory cytokines.

rhCC16 Increased Detectable CC16 Levels and Suppressed NF- κ B/p38/ERK MAPK Pathway Activation in LPS-Treated A549 Cells

As illustrated in Fig. 6, LPS markedly reduced CC16 levels in A549 cells relative to the control group ($p < 0.05$), indicating that inflammatory stimulation suppresses CC16 expression (Fig. 6A). Western blot analysis revealed that LPS markedly reduced CC16 protein expression ($p < 0.05$) while significantly increasing p-NF- κ B p65, p-p38, and p-ERK levels ($p < 0.05$). Total NF- κ B p65, p38, and ERK protein levels remained unchanged ($p > 0.05$), suggesting activation of the NF- κ B/p38/ERK MAPK signaling pathway (Fig. 6B,C). Treatment with rhCC16 significantly increased detectable CC16 levels and inhibited the upregulation of these pathway proteins in LPS-stimulated cells. Compared with LPS-stimulated cells treated with rhCC16, cells pre-stimulated with LPS and TNF- α showed a partial reversal of the inhibitory effect of rhCC16 (Fig. 6A–C). qRT-PCR analysis further revealed changes in the mRNA levels of NF- κ B p65, p38, and ERK (Fig. 6D). Collectively, these results demonstrate that rhCC16 increased detectable CC16 levels in LPS-stimulated A549 cells and was associated with reduced NF- κ B/p38/ERK MAPK pathway activation, indicating that this signaling pathway may contribute to the anti-inflammatory effects of rhCC16.

Discussion

ALI represents a critical inflammatory lung condition characterized by disruption of pulmonary barrier integrity, excessive inflammatory activation, and oxidative imbalance, which contribute to progressive tissue damage and impaired lung function [27]. Despite advances in supportive therapies, including mechanical ventilation and pharmacological management, effective targeted treatments for ALI remain limited, highlighting the need to identify novel therapeutic strategies that modulate key pathological processes. In this study, rhCC16 markedly alleviated pulmonary edema, inflammatory injury, and oxidative stress in ALI mice by increasing detectable CC16 levels, which was accompanied by reduced activation of NF- κ B/p38/ERK MAPK signaling and attenuation of inflammatory responses. Its protective effects were further validated in an LPS-induced A549 cell model, suggesting that rhCC16 may represent a potential therapeutic target for ALI treatment.

Oxidative stress and inflammatory responses are key pathological factors driving ALI. Elevated ROS levels can directly injure alveolar epithelial and endothelial cells while simultaneously activating inflammatory pathways, including NF- κ B and MAPK [28]. Inflammatory responses further promote neutrophil and macrophage infiltration into lung tissue, leading to the release of various pro-inflammatory mediators that exacerbate lung injury and disrupt barrier function [29]. Oxidative stress and inflammation form a positive feedback loop that accelerates ALI progression, resulting in alveolar–capillary barrier disruption and pulmonary edema, which are hallmark pathological features of the disease [9]. During this process, cell death pathways, including apoptosis, autophagy, and pyroptosis, are also involved [30]. In the present study, CC16 expression was reduced under ALI conditions, whereas supplementation with rhCC16 mitigated oxidative stress and inflammatory responses, thereby protecting lung tissue. These findings suggest that CC16 plays an important regulatory role in the development of ALI.

CC16 expression is decreased in multiple respiratory disorders and correlates with disease progression and adverse prognosis. For instance, CC16 levels in airway mucosa are markedly reduced in asthmatic patients compared with healthy individuals, suggesting a protective role of CC16 [31]. In chronic obstructive pulmonary disease (COPD), reduced CC16 may impair airway epithelial function and contribute to disease development [18,32]. Moreover, decreased CC16 levels in serum or airway secretions are associated with impaired lung function and may serve as a potential biomarker [33,34]. Our study further demonstrates that rhCC16 intervention attenuated LPS-induced inflammatory responses and pulmonary injury, highlighting its protective role in ALI.

Previous studies indicate that NF- κ B and MAPK pathways are key regulators of inflammation and oxidative stress, serving as key drivers of ALI pathogenesis [35,36]. Excessive activation of NF- κ B enhances the release of pro-inflammatory mediators, including TNF- α and IL-1 β , thereby aggravating lung injury [37]. Similarly, MAPK family members, including p38 and ERK, regulate inflammatory cytokine transcription and oxidative stress responses [38,39]. In our study, ALI mice exhibited NF- κ B/p38/ERK MAPK pathway activation alongside elevated pro-inflammatory cytokine levels. rhCC16 treatment significantly suppressed the over-phosphorylation of pathway proteins and reduced inflammatory mediator expression. This anti-inflammatory effect was further corroborated *in vitro*, as cells pre-stimulated with LPS and TNF- α followed by rhCC16 treatment exhibited partially reversed protection, suggesting that NF- κ B/p38/ERK MAPK signaling may be involved in CC16-mediated anti-inflammatory and antioxidative effects.

In summary, this study demonstrates the therapeutic potential of CC16 in ALI using both *in vivo* and *in vitro* models. However, several limitations should be acknowledged. First, the absence of an rhCC16-alone treatment group *in vivo* limits the assessment of the independent effects of exogenous rhCC16 under physiological conditions. Second, the efficacy of rhCC16 requires validation in different animal strains and genetic backgrounds to improve generalizability. Third, the lack of highly specific genetic or pharmacological approaches limits definitive confirmation of CC16-related signaling mechanisms; therefore, pathway interpretations based on TNF- α -related interventions may not fully establish causality. Future studies incorporating genetic modulation or more selective inhibitors are needed to further clarify the underlying mechanisms. In addition, the relatively limited sample size warrants validation in larger cohorts.

Conclusion

Our findings demonstrate that CC16 expression is markedly reduced in lung tissues of ALI mice and LPS-stimulated A549 cells. Subsequent experiments demonstrate that rhCC16 mitigates LPS-stimulated inflammatory responses and oxidative stress, which is accompanied by reduced activation of NF- κ B/p38/ERK MAPK signaling-related molecules, thereby contributing to the preservation of lung tissue integrity and cellular function. These findings provide new experimental evidence regarding the molecular mechanisms of CC16 in ALI and highlight its potential as a therapeutic target.

Availability of Data and Materials

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

Author Contributions

ZW and AW designed the study and contributed to conceptualization and methodology. ZW and QXX conducted data collection, investigation, data curation, and validation. ZW performed formal analysis. AW supervised the study, secured funding, and contributed to project administration. ZW drafted the original manuscript. AW contributed to the review and editing of the manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content. All authors have read and approved the final manuscript and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Animal Ethics Committee of South Zhejiang Institute of Radiation Medicine and Nuclear Technology Applications (Approval No. ZFY20251274) and were conducted in accordance with institutional guidelines and the ARRIVE Guidelines 2.0.

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Conflict of Interest

The authors declare no conflict of interest.

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