


Chrysofenol D protects mice against LPS-induced acute lung injury by inhibiting oxidative stress, inflammation, and apoptosis via TLR4-MAPKs/NF- κ B signaling pathways

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Abstract

This study investigated the effect and mechanism of chrysofenol D (CD) on LPS-induced acute lung injury in mice. Histological changes in the lungs were measured by hematoxylin-eosin staining. The levels of IL-6, IL-1 β , and TNF- α in the bronchoalveolar lavage fluid were detected by ELISA. The levels of oxidative stress were detected by the cuvette assay. Immune cells in peripheral blood, the levels of reactive oxygen species, and apoptosis of primary lung cells were detected by flow cytometry. The mRNA levels of TLR4, MyD88, IL-1 β , and NLRP3 were measured by quantitative real-time polymerase chain reaction. The levels of proteins in apoptosis and the TLR4-MAPKs/NF- κ B signaling pathways were detected by Western blot. Hematoxylin-eosin staining showed that CD could improve lung injury; decrease the levels of inflammatory factors, oxidative stress, reactive oxygen species, and cell apoptosis; and regulate the immune system. Moreover, CD could down-regulate the mRNA levels of TLR4, MyD88, NLRP3, and IL-1 β in lung, and the protein levels of Keap-1, Cleaved-Caspase-3/Caspase-3, Cleaved-Caspase-9/Caspase-9, TLR4, MyD88, p-ERK/ERK, p-JNK/JNK, p-p38/p38, p-p65/p65, NLRP3, and IL-1 β , and up-regulated the levels of Bcl-2/Bax, p-Nrf2/Nrf2, and HO-1. The results suggested that CD could protect mice against LPS-induced acute lung injury by inhibiting oxidative stress, inflammation, and apoptosis via the TLR4-MAPKs/NF- κ B signaling pathways.

Keywords

chrysofenol D, lipopolysaccharide, acute lung injury, oxidative stress, inflammation, apoptosis

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Introduction

Acute lung injury (ALI), a clinical syndrome with an extremely high morbidity and mortality rate, is a serious and diffuse lung damage triggered by a variety of causes and can develop into acute respiratory distress syndrome (ARDS) in severe cases.^{1,2} It is considered the leading cause of death in patients in intensive care units.³ LPS is a major component derived from the outer membranes of most Gram-negative bacteria. ALI is caused when there is a severe infection in the body or other reasons induce endothelial barrier damage, leading to the recruitment of inflammatory cells into the lung.⁴

Increasing evidence indicates that lung epithelial apoptosis results in the release of pro-inflammatory cytokines (such as TNF- α and TGF- β 1), causing inflammation.⁵ Previous *in vivo* and *in vitro* studies demonstrated that exposure of

animals or lung cells to LPS induced inflammatory factors such as TNF- α , IL-1 β , and IL-6 and the generation of reactive oxygen species (ROS).^{6,7} The generation of ROS and pulmonary cell apoptosis plays a critical role in the pathogenesis of ALI and ARDS.^{8,9} MAPK cascades are key signaling

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pathways that regulate a wide variety of cellular processes, such as proliferation, apoptosis, differentiation, and oxidative stress responses.^{10,11}

Chrysanthemum morifolium Ramat. is an herb produced in the city of Jiaozuo, Wenxian County, Henan Province, and its stems and leaves are widely used as an edible and pharmaceutical food in China.¹² Chrysosplenol D (CD) is a flavonoid isolated from the stems and leaves of *C. morifolium* Ramat. It has been shown that CD has antioxidant,¹³ anti-inflammatory,¹⁴ and antitumor¹⁵ effects. However, the pharmacological activity of CD on LPS-induced ALI remains unclear. Therefore, the aim of this study was to examine the mechanisms and underlying preventive effects of CD on ALI.

Materials and methods

Plant materials and reagents

The stems and leaves of *C. morifolium* Ramat. (Voucher Specimen No. 20181110) were collected in November 2018 from the city of Wenxian, Henan, China. Prof. Dong Chengming of the Henan University of Chinese Medicine identified the collected materials, which were then deposited in the Key Laboratory of Chinese Medicine Resources and Chinese Medicine Chemistry of Henan Province. The CD, extracted from the stems and leaves of *C. morifolium* Ramat., was prepared by our laboratory and identified by Prof. Feng Weisheng of the Henan University of Chinese Medicine. LPS was purchased from Sigma (St. Louis, MO, USA). Dexamethasone was used as a positive control drug (Shanghai Macklin Biochemical Co. Ltd., China).

Animals

All the experiments and procedures carried out as part of this study were approved by the Animal Ethics Committee of the Henan University of Chinese Medicine, Zhengzhou, China. Male Balb/C mice (weighing 18 ± 2 g, $n = 60$) were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. (SCXK2016-0011). The mice were housed in specific pathogen-free (SPF) grade animal laboratories at 18°C to 22°C, alternating day and night, eating and drinking freely. Adaptive feeding was used for the experiment after 1 wk. The mice were randomly divided into 5 groups as follows: control (CON, $n = 12$), LPS (LPS, 10 mg/kg, $n = 12$), DEX (5 mg/kg, $n = 12$), low-dose chrysosplenol D + LPS (CD-L, 12.5 mg/kg, $n = 12$), and high-dose chrysosplenol D + LPS (CD-H, 25 mg/kg, $n = 12$). DEX, CD-L, and CD-H were intraperitoneally administered. The mice in the CON and LPS groups were injected with saline at the same time for 3 d. On the fourth day, all the mice received intranasal LPS (10 mg/kg), except for the mice in the CON group treated with saline. After 6 h, the animals were anesthetized with ketamine hydrochloride,

and serum and lung samples were collected for further analysis. Parts of the lungs were fixed with formalin for histological analysis.

Histomorphological examination

The lungs were fixed in 4% paraformaldehyde and embedded in paraffin, whereupon sections were made and stained with hematoxylin and eosin (H&E). The images were viewed under a microscope (Nikon, Shinagawa, Tokyo, Japan).

ELISA analysis

Bronchoalveolar lavage fluid (BALF) was collected and used to detect the levels of IL-6 (E-EL-M0044c, Elabscience Biotechnology Co. Ltd., Wuhan, China), TNF- α (E-EL-M0049c, Elabscience Biotechnology Co. Ltd.), and IL-1 β (E-EL-M0037c, Elabscience Biotechnology Co. Ltd.) according to the respective manufacturer's instructions.

Assessments of biochemical parameters

The levels of GSH-Px (A005-1-2, Nanjing Jiancheng Bioengineering Institute), SOD (A001-3-1, Nanjing Jiancheng Bioengineering Institute), and MDA (A003-1-1, Nanjing Jiancheng Bioengineering Institute) were detected using specific kits following the manufacturer's protocols.

Primary lung cells

Fresh lung tissue (10 mg) was taken, chopped to a size of about 1 mm³, and washed twice with phosphate-buffered saline (PBS). It was resuspended in 1% trypsin for digestion. After that, FBS was added to stop the digestion process, and this was followed by filtration with a 70- μ m cell sieve to obtain primary lung cells. Briefly, the cells with ROS were collected and incubated with 10 μ M 2',7'-dichlorofluorescein (DCFH-DA) for 20 min at 37°C in the dark. At the end of the incubation, the cells were washed with PBS 3 times to remove the free DCFH-DA molecules. The cells with apoptosis were incubated with 5 μ l 7-AAD and PE for 15 min at room temperature. The resulting cells were used to detect cell apoptosis and ROS by flow cytometry (FCM; FACS Aria III, USA).

FCM Analysis of Peripheral Blood Immune Cells

Whole blood (100 μ l) anticoagulated with heparin sodium was conjugated with the corresponding Ab at room temperature for 30 min. Th cells and cytotoxic (Tc cells) were detected by Abs against CD3/4/8. Dendritic cells (DCs) were examined by anti-CD86 Ab and anti-CD11c Ab. NK cells were tested by anti-CD49b Ab and anti-CD3e Ab. Regulatory T cells (Tregs) were detected by anti-CD4 Ab, anti-CD25 Ab, and anti-Foxp3 Ab. All Abs

Table 1. Sequences of the primers for quantitative real-time PCR.

Gene	Primer Sequences (5'-3')
Mouse TLR4	F: TGAGGACTGGGTGAGAAATGAGC R: CTGCCATGTTTGAGCAATCTCAT
Mouse MyD88	F: CGATGCCTTTATCTGCTACTGC R: GCTTCTGTTGGACACCTGGAG
Mouse NLRP3	F: TAAGAAGTGCATAGGGTCAAACG R: GTCTGGAAGAACAGGCAACATG
Mouse IL-1 β	F: GCATCCAGCTTCAAATCTCGC R: TGTTTCATCTCGGAGCCTGTAGTG
Mouse GAPDH	F: CCTCGTCCCCTAGACAAAATG R: TGAGGTCAATGAAGGGGTCGT

were from ThermoFisher (USA). Finally, the supernatant was discarded again, and 500- μ l PBS was added, followed by FCM.

Detection of TLR4, MyD88, NLRP3, and IL-1 β in lung by real-time PCR (RT-PCR)

Lung tissue (10 mg) was taken, and RNA was extracted with a total RNA extraction kit (R1200, Solarbio, China). After the extracted samples were quantified by the Nano

Drop One ultraviolet-spectrophotometer (Thermo Scientific, China), the mRNA was reverse-transcribed into c-DNA by the BeyoRT III First Strand cDNA Synthesis Kit (D7178 M, Beyotime, China). Finally, fluorescence quantitative PCR detection was performed using the QuantiNova SYBR Green PCR Kit (Qiagen, Germany). The primers used for the qRT-PCR amplification are listed in Table 1.

Western blot analysis

Proteins from the lung tissue were extracted with a mammalian protein extraction kit (Beijing Com Win Biotech Co. Ltd., Beijing, China) and quantified using a BCA protein assay kit (PC0020, Beijing Solarbio Science & Technology Co. Ltd., China). A protein amount of 60 μ g from each sample was loaded and separated using the SDS-PAGE gel. Then, BSA was used to block the membrane for 1.5 h, followed by the addition of the primary Abs for 2.5 h: Bcl-2 (ab59348, Abcam), Bax (ab32503, Abcam), Cleaved-caspase-3 (ab2302, Abcam), Cleaved-caspase-9 (ab2324, Abcam), p-Nrf2 (ab76026, Abcam), Nrf2 (ab137550, Abcam), HO-1 (A19062, ABclonal), Keap-1 (ab139729, Abcam), Caspase-3 (ab13847, Abcam), Caspase-9 (ab32539, Abcam), TLR4

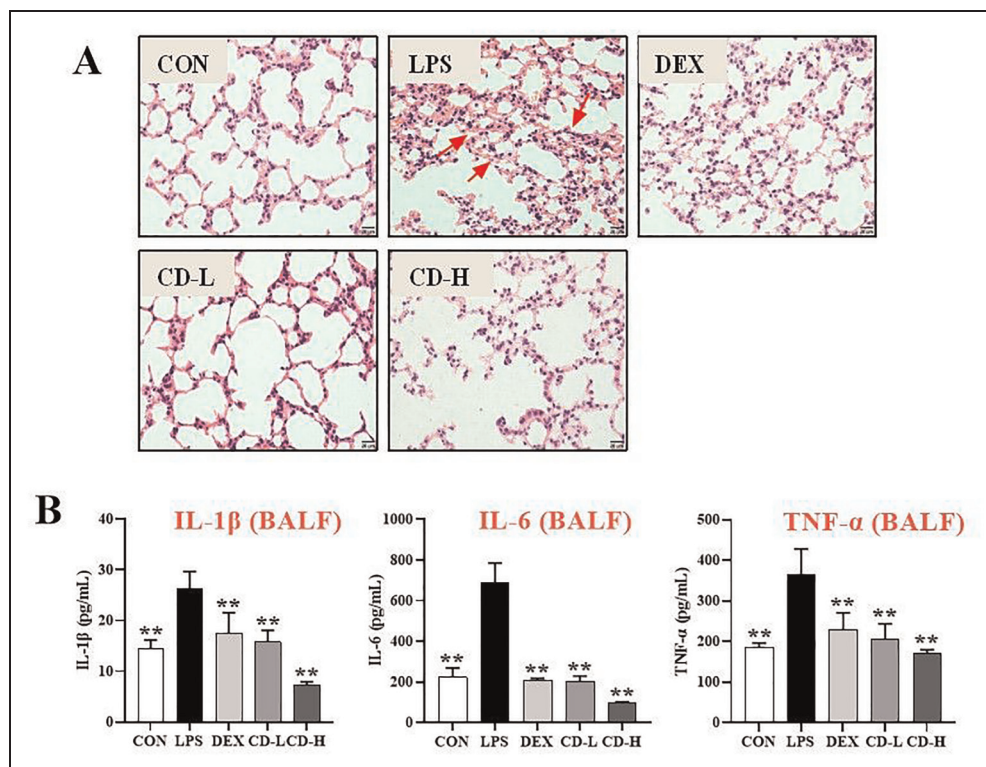


Figure 1. Effects of CD on lung function and inflammation in mice with ALI. CD was administered before intranasal LPS (10 mg/kg). (A) H&E staining of lung tissue (40 \times magnification). (B) The levels of inflammatory factors in lung tissue. $n = 3$ or 8 mice per group; * $P < 0.05$, ** $P < 0.01$, compared with model group.

(ab22048, Abcam), NF- κ B p65 (ab16502, Abcam), NF- κ B p-p65 (ab86299, Abcam), NLRP3 (ab4207, Abcam), IL-1 β (ab9722, Abcam), MyD88 (ab135693, Abcam), ERK (ab54320, Abcam), p-ERK (ab201015, Abcam), JNK (ab208035, Abcam), p-JNK (ab124956, Abcam), p38 (ab31828, Abcam), p-p38 (ab4822, Abcam), and β -actin (Ac026, ABclonal). Then, the protein was washed 4 times with PBST for 5 min each time. After that, secondary Ab (goat anti-rabbit 925-68071, goat anti-mouse 925-32210, Li-COR, USA) was added and incubated for 1 h in the dark and washed 4 times with PBST for 5 min each time and finally with PBS. The protein levels were quantified using Image Studio version 5.2.

Statistical analysis

Data were analyzed using SPSS 20.0 (IBM, New York, NY, USA). Statistical significance was assessed in comparison with the respective control for each experiment using one-way analysis of variance (ANOVA). A *P* value less than 0.05 indicated a statistically significant difference.

Results

CD Improved lung function and inhibited inflammation in mice with ALI

CD improved the lung function and inhibited inflammation in mice with ALI. As shown in Figure 1A, lung pathological damage, including airway wall thickening and inflammatory cell infiltration of lung parenchyma, was greater in the model group. However, the changes in LPS-induced pathology were attenuated by CD. The levels of TNF- α , IL-6, and IL-1 β in the BALF showed that LPS elevated the levels of pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β , which were reduced by CD (Figure 1B).

CD Inhibited Oxidative Stress in LPS-Induced ALI

The levels of ROS were detected by FCM (Figure 2A), which indicated that the levels of ROS decreased in lung primary cells of mice treated by CD. Moreover, the levels of GSH-Px, SOD, p-Nrf2/Nrf2, and HO-1 decreased, and MDA and Keap-1 increased in mice with ALI, which could be reversed by CD (Figure 2B and C).

CD Increased percentage of immune cells in mice blood

Research demonstrates that innate immune cells play a vital role in regulating the immune response. FCM analyzed the effects of CD on the number of immune cells. Effects of CD on the levels of Th, Tc, DC, NK, and Treg in mice

peripheral blood. CD significantly increased the percentage of Th cells, Tc cells, DCs, and NK cells in a dose-dependent manner, while it decreased the percentage of Treg in mice blood compared with the model mice (Figure 3).

CD Inhibited apoptosis in LPS-Induced ALI

The levels of cell apoptosis were detected by FCM. The results, shown in Figure 4A, indicate that the rate of apoptosis decreased in lung primary cells treated by CD. The levels of Bax, Caspase-3, Caspase-9, and Bcl-2 were detected to understand the mechanism underlying the inhibitory effects of CD on LPS-induced apoptosis in lung tissue. Figure 4B illustrates that, in ALI mice, the increased levels of Bax, Cleaved-Caspase-3/Caspase-3, and Cleaved-caspase-9/Caspase-9, along with the decreased level of Bcl-2, could be reversed by CD.

CD Modulated mRNA levels of TLR4 signaling pathway in mice with ALI

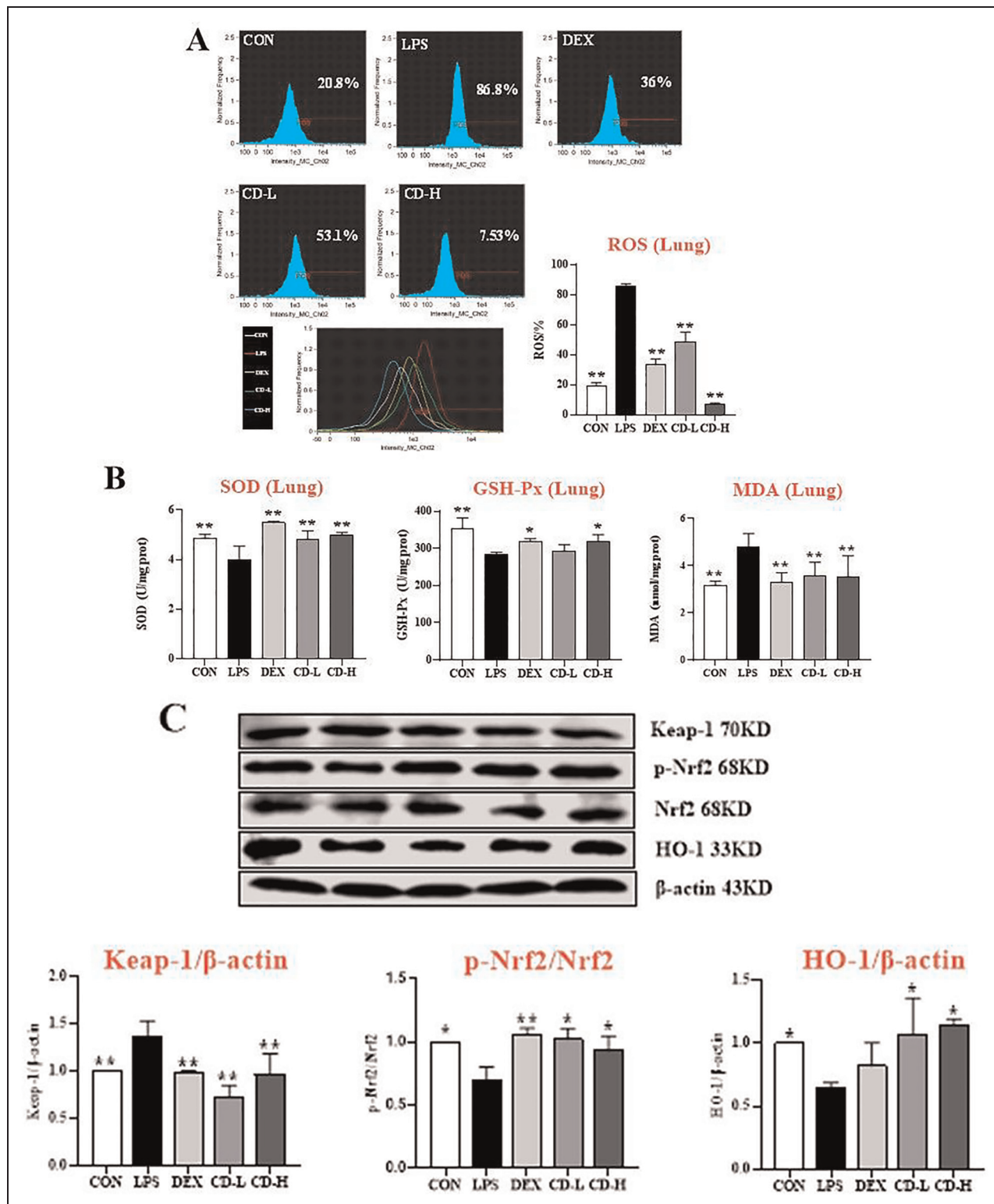
The mRNA levels of lung tissue were detected by qRT-PCR, the results of which showed that, compared with the CON group, the levels of inflammatory factors in the LPS group were boosted, and CD could inhibit the inflammation (Figure 5A).

CD Modulated TLR4-MAPKs/NF- κ B pathway in mice with ALI

The levels of TLR4, MyD88, p-ERK, ERK, p-JNK, JNK, p-p38, p38, p65, p-p65, NLRP3, and IL-1 β in lung tissue on LPS-induced ALI were detected by Western blotting (Figure 6) in order to evaluate the possible role of the TLR4-MAPKs/NF- κ B pathways. The results showed that CD could ameliorate the abnormal expression of proteins related to the TLR4-MAPKs/NF- κ B pathways (Figure 6A).

Discussion

ALI is a life-threatening disorder that is clinically characterized by severe hypoxemia and pulmonary bilateral infiltrates. The pathogenesis of ALI is complex and remains unclear. In recent years, an increasing number of studies have demonstrated that the development of ALI is related to inflammation, oxidative stress, apoptosis, and cellular immunity.^{16–19} However, mortality due to ALI continues to be high. There are many published reports on the activity of natural products to treat ALI. In our study, CD was isolated from the stems and leaves of *C. morifolium* Ramat., which has antioxidant,¹³ anti-inflammatory,¹⁴ and antitumor¹⁵ properties. The present study demonstrated that CD



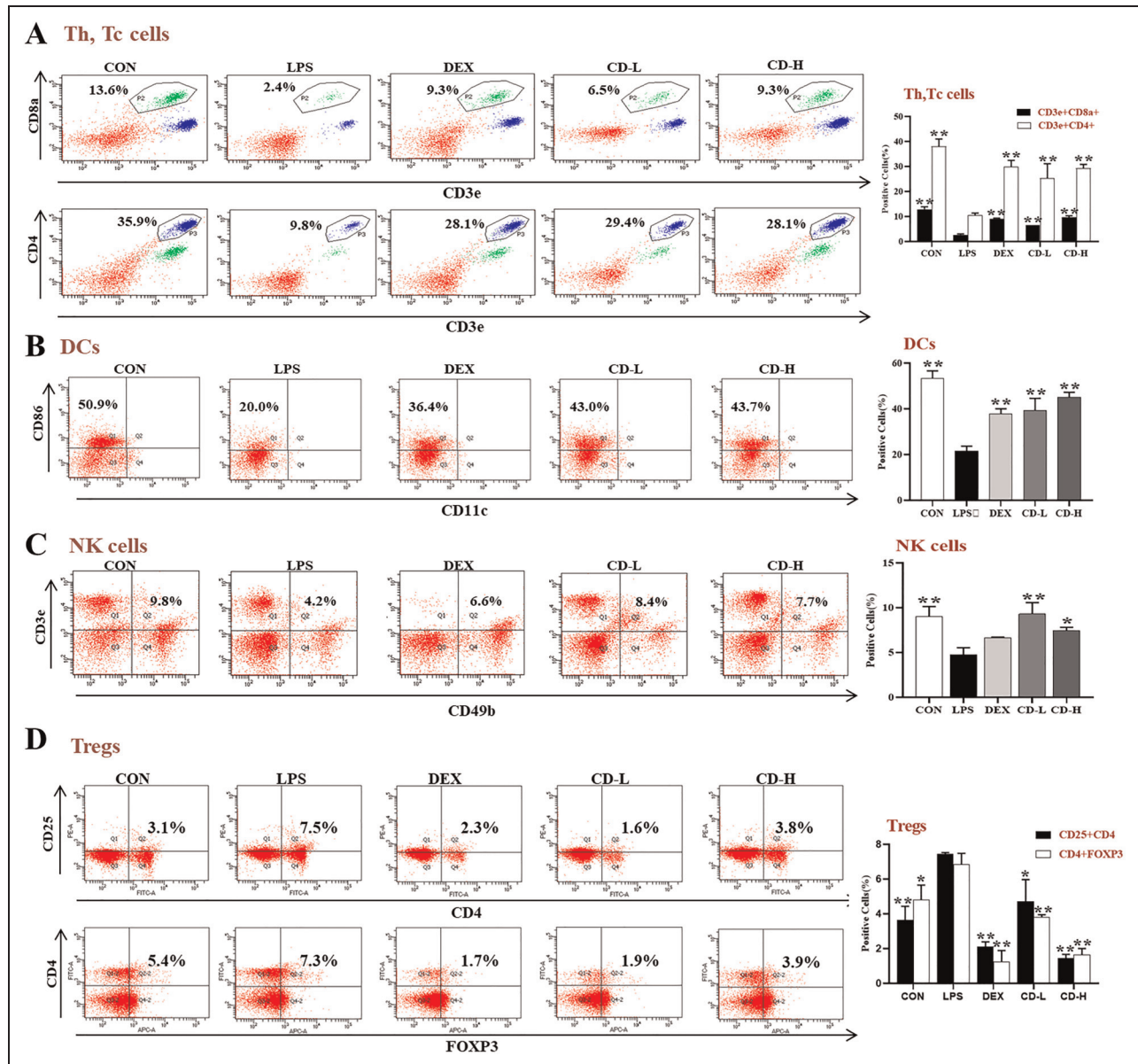


Figure 3. CD altered the percentage of immune cells in mice blood. CD was administered before intranasal LPS (10 mg/kg). (A) Th and Tc cells were analyzed by FCM. (B) DCs in the blood were analyzed by FCM. (C) NK cells in the blood were analyzed by FCM. (D) Treg cells in the blood were analyzed by FCM. * $P < 0.05$, ** $P < 0.01$, compared with the LPS group ($n = 3$ mice per group).

could improve lung function in mice with ALI through apoptosis, oxidative stress, and inflammation.

In recent years, LPS-induced animal models have been widely used in the study of therapeutic drugs and of the mechanism of action of ALI.²⁰ The most common feature of LPS-induced animal models is the sharp rise in the level of inflammation.²¹ When the body is stimulated, neutrophils arrive quickly at the injured site and release a large number of pro-inflammatory cytokines such as IL-6 and TNF- α .⁷ In our study, the levels of lung injury-related factors in mice, such as IL-6, TNF- α , and IL-1 β ,

significantly increased compared with the CON group. At the same time, the results of H&E staining showed there was obvious inflammatory cell infiltration in the pathological section of lung tissue in mice with ALI, which exemplified the successful establishment of the ALI model. According to the present study, CD could reduce the levels of the pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β in BALF, and improve the pathological changes in lung tissue in mice with ALI. These results suggest that CD could inhibit the inflammatory response in mice with ALI.

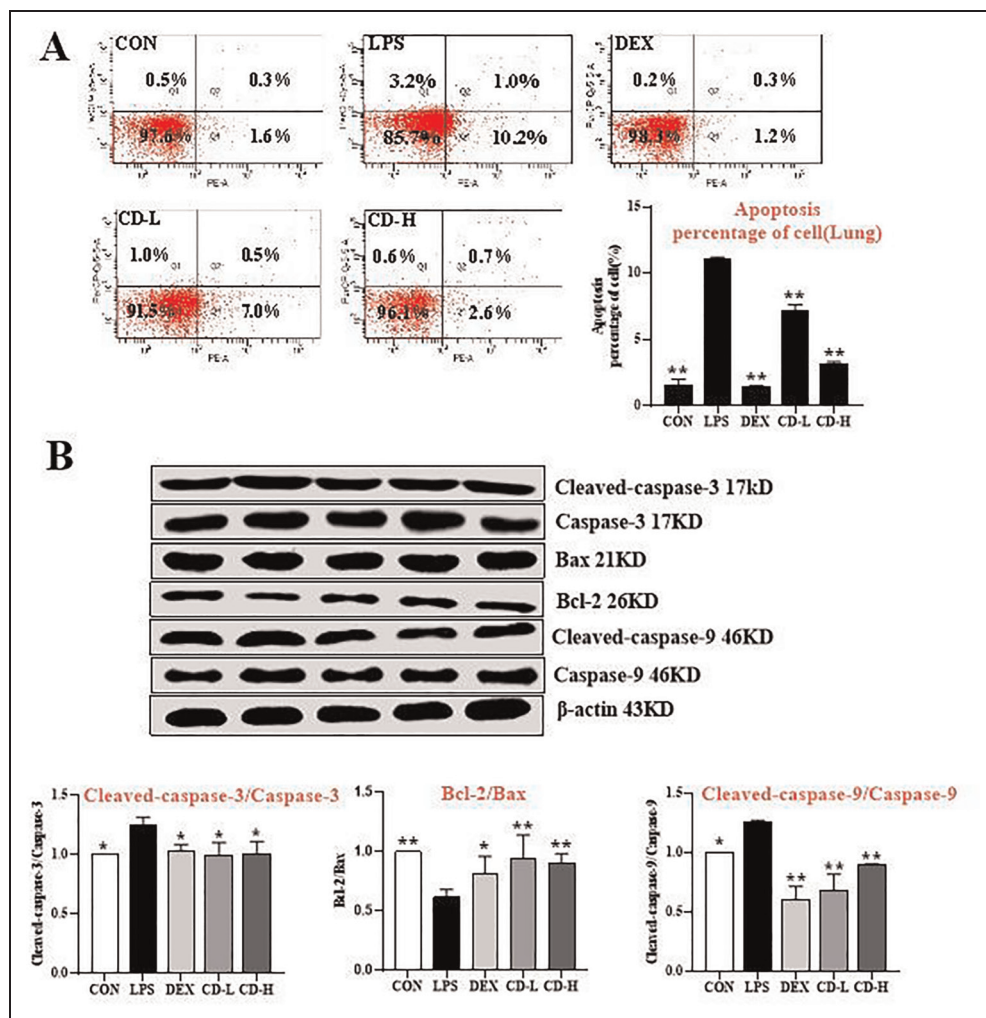


Figure 4. Effects of CD on apoptosis in mice with ALI. CD was administered before intranasal LPS (10 mg/kg). (A) Effects of CD on the level of apoptosis in mice with ALI. (B) Effects of CD on apoptosis signaling pathway in mice with ALI; * $P < 0.05$, ** $P < 0.01$, compared with the LPS group ($n = 3$ mice per group).

The related results demonstrated that the inhibition of oxidative stress has a certain improvement effect on LPS-induced ALI.²² In the event of ALI, the body produces a large number of ROS, which exceeds the antioxidant capacity of the body and causes oxidative stress, thus leading to lung tissue injury. SOD and GSH-PX can effectively remove ROS and terminate free radical reactions, and MDA can reflect the degree of peroxidation damage.^{8,23} Nuclear factor erythroid-2-associated factor 2 (Nrf2) plays a key role in up-regulating several cellular antioxidant,²⁴ and is present in the cytoplasm as an inactive complex along with Keap1. In the nucleus, Nrf2 activates the expression of HO-1 gene, thus reducing inflammation and oxidative stress.²⁵ In addition, studies have shown that Nrf2 is decoupled from Keap1 when the body is affected by ROS, interacts with antioxidant response element (ARE), regulates the activity of ARE-dependent antioxidant genes (such as

SOD and GSH-Px), and maintains the homeostasis of redox.²⁶ Our experimental results clearly confirmed that CD could reduce the level of oxidative stress in mice with LPS-induced ALI. Furthermore, LPS-induced inflammation is regulated by a variety of immune cells. Our results showed that CD significantly increased the percentage of Th cells, Tc cells, DCs, and NK cells in a dose-dependent manner, and decreased the percentage of Treg in mice blood compared with the model group (FCM), suggesting that CD has excellent immunoregulatory efficacy.

In addition, apoptosis is one of the key mechanisms of ALI.^{27,28} In particular, the mitochondrial pathway is very important in apoptosis. After LPS stimulates the production of a large number of ROS, the complex of Bax and Bcl-2 proteins enter the mitochondria and increase the permeability of mitochondrial membrane, leading to a decrease in the membrane potential, and this, in turn,

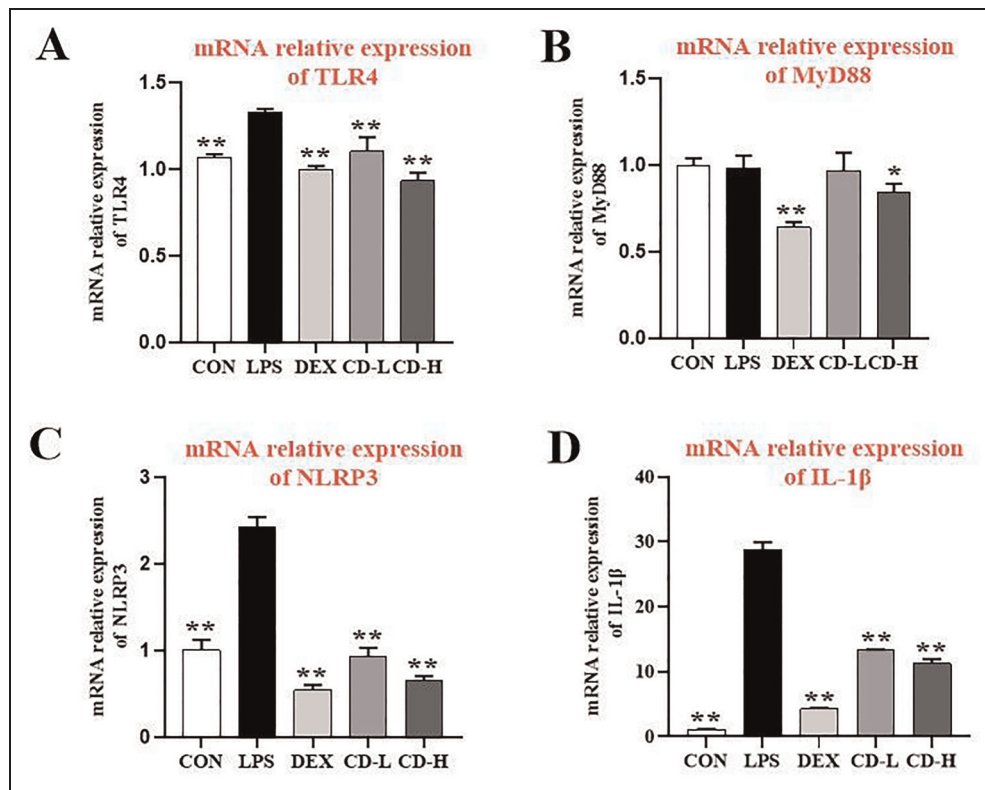


Figure 5. Effects of CD on related mRNA in mice with ALI. CD was administered before intranasal LPS (10 mg/kg). (A) Effects of CD on the mRNA level of TLR4 in mice with ALI. (B) Effects of CD on the mRNA level of MyD88 in mice with ALI. (C) Effects of CD on the mRNA level of NLRP3 in mice with ALI. (D) Effects of CD on the mRNA level of IL-1 β in mice with ALI. * $P < 0.05$, ** $P < 0.01$, compared with the LPS group ($n = 3$ mice per group).

activates Cleaved-Caspase-9/Caspase-9 and Cleaved-caspase-3/Caspase-3, leading to cell apoptosis.²⁹ In this study, CD inhibited the apoptosis of primary lung cells and the expression of apoptosis marker protein in the lung tissue of mice with ALI, suggesting that CD may improve LPS-induced ALI by inhibiting apoptosis mediated by the mitochondrial pathway.

LPS is one of the main triggers of inflammatory reaction in ALI, and TLR4 plays an important role in the activation of innate immune response.³⁰ LPS can bind to TLR4 to activate the TLR4 pathway and prompt downstream MyD88 to send signals, thus activating MAPK and NF- κ B³¹ signaling pathways. MAPK is an evolutionarily conserved signaling protein that plays an important role in signaling pathways, mediating and responding to extracellular stimuli (such as LPS) to cell membrane and nucleus.³² The MAPK family mainly comprises c-jun-N-terminal kinase (JNK), p38 kinase, and ERK,³³ which has a corresponding activation mode, and the pathways interact to regulate cell life activities (e.g., proliferation, growth, survival, migration, gene expression, cell cycle control, and apoptosis).³⁴ Moreover, the ERK pathway is mainly related to growth, and the JNK and p38 pathways are mainly related to inflammation.³⁵ In

our study, CD could down-regulate the levels of p-ERK/ERK, p-JNK/JNK, and p-p38/p38, suggesting that CD improved the LPS-induced ALI-mediated TLR4-MAPK signaling pathway. Additionally, NLRP3 is an important downstream effector in the TLR4-mediated inflammatory pathway.³⁶ The activation of NLRP3 inflammatory bodies can lead to the production of a large quantity of the inflammatory cytokine IL-1 β , which promotes neutrophil infiltration and aggravates inflammation. In the present study, the decrease in the expression of NLRP3 and the phosphorylation level of NF- κ B p65 in mice with ALI could be reversed by CD. The results indicated that CD might suppress LPS-induced ALI by regulating the TLR4-MAPKs/NF- κ B signaling pathways.

Conclusion

Chrysosplenol D, isolated from the stems and leaves of *C. morifolium* Ramat., may protect against LPS-induced ALI in mice by inhibiting inflammation, apoptosis, oxidative stress, and regulate the immune system via the TLR4-MAPKs/NF- κ B signaling pathways, thus providing a molecular basis for a novel medical treatment of ALI.

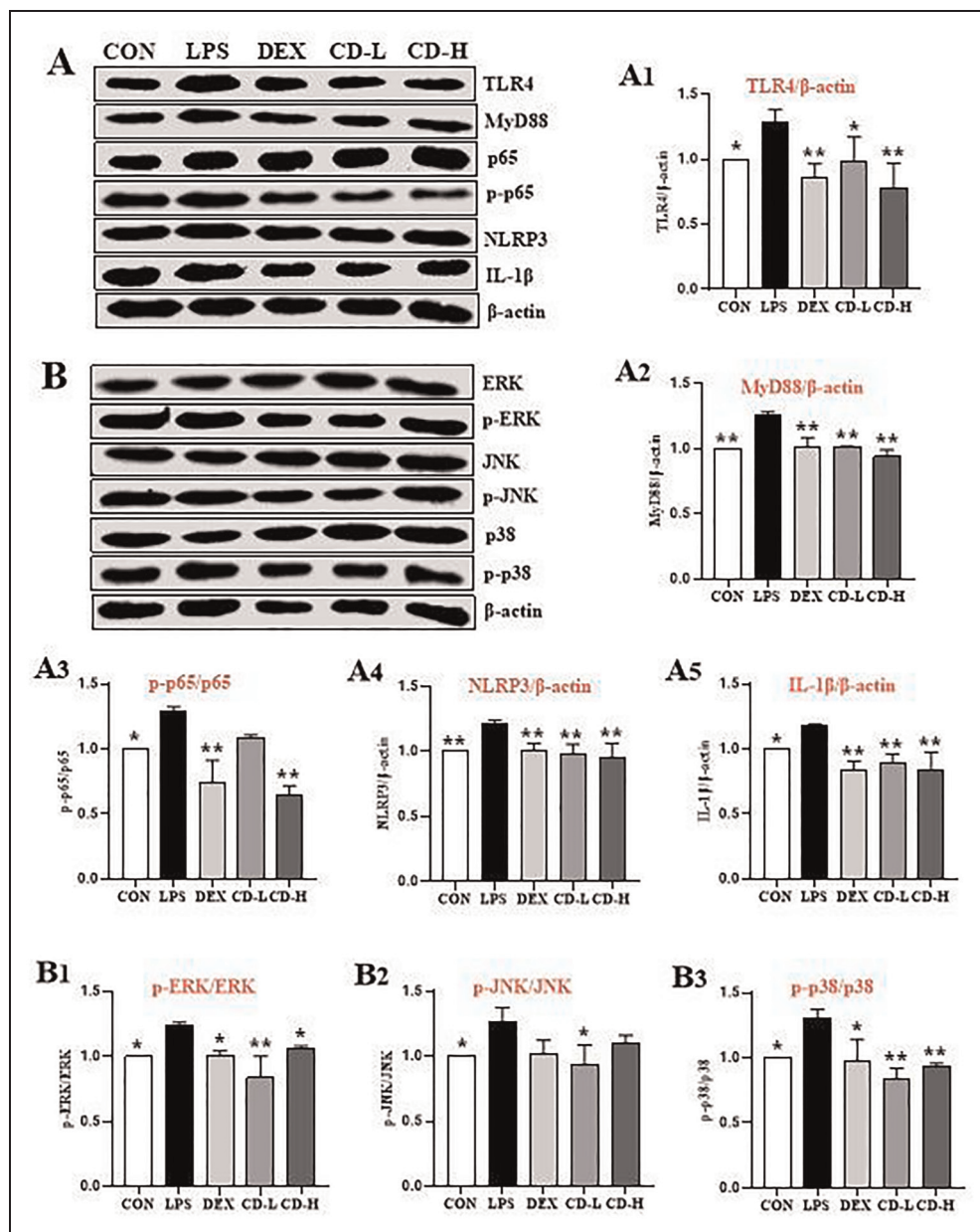


Figure 6. Effects of CD on TLR4-MAPKs/NF- κ B signaling pathways in mice with ALI. CD was administered before intranasal LPS (10 mg/kg). (A) Effects of CD on the level of the TLR4-NF- κ B signaling pathway in mice with ALI. (A1) Effects of CD on the level of TLR4 in lung tissue in mice with ALI. (A2) Effects of CD on the level of MyD88 in lung tissue in mice with ALI. (A3) Effects of CD on the level of p-p65/p65 in lung tissues in mice with ALI. (A4) Effects of CD on the level of NLRP3 in lung tissues in mice with ALI. (A5) Effects of CD on the level of IL-1 β in lung tissues in mice with ALI. (B) Effects of CD on the level of the TLR4-MAPKs signaling pathway in lung tissues in mice with ALI. (B1) Effects of CD on the level of p-ERK/ERK in lung tissues in mice with ALI. (B2) Effects of CD on the level of p-JNK/JNK in lung tissues in mice with ALI. (B3) Effects of CD on the level of p-p38/p38 in lung tissues in mice with ALI; * $P < 0.05$, ** $P < 0.01$, compared with the LPS group ($n = 3$ mice per group).

Declaration of Conflicting Interests

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