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Cardamine komarovii flower extract reduces lipopolysaccharide-induced acute lung injury by inhibiting MyD88/TRIF signaling pathways

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[ABSTRACT] In the present study, we investigated anti-inflammatory effect of *Cardamine komarovii* flower (CKF) on lipopolysaccharide (LPS)-induced acute lung injury (ALI). We determined the effect of CKF methanolic extracts on LPS-induced pro-inflammatory mediators NO and prostaglandin E2 (PGE2), production of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6), and related protein expression levels of MyD88/TRIF signaling pathways in peritoneal macrophages (PMs). Nuclear translocation of NF-κB-p65 was analyzed by immunofluorescence. For the *in vivo* experiments, an ALI model was established to detect the number of inflammatory cells and inflammatory factors (IL-1β, TNF-α, and IL-6) in bronchoalveolar lavage fluid (BALF) of mice. The pathological damage in lung tissues was evaluated through H&E staining. Our results showed that CKF can decrease the production of inflammatory mediators, such as NO and PGE2, by inhibiting their synthesis-related enzymes iNOS and COX-2 in LPS-induced PMs. In addition, CKF can downregulate the mRNA levels of IL-1β, TNF-α, and IL-6 to inhibit the production of inflammatory factors. Mechanism studies indicated that CKF possesses a fine anti-inflammatory effect by regulating MyD88/TRIF dependent signaling pathways. Immunocytochemistry staining showed that the CKF extract attenuates the LPS-induced translocation of NF-kB p65 subunit in the nucleus from the cytoplasm. *In vivo* experiments revealed that the number of inflammatory cells and IL-1β in BALF of mice decrease after CKF treatment. Histopathological observation of lung tissues showed that CKF can remarkably improve alveolar clearance and infiltration of interstitial and alveolar cells after LPS stimulation. In conclusion, our results suggest that CKF inhibits LPS-induced inflammatory response by inhibiting the MyD88/TRIF signaling pathways, thereby protecting mice from LPS-induced ALI.

[KEY WORDS] *Cardamine komarovii* flower; Inflammation; MyD88; TRIF; Acute lung injury

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Introduction

Inflammation is a basic immune response to infection, irritation, and other injuries [1-2]. However, the excessive production of inflammatory factors may lead to pulmonary fibro-

sis, rheumatoid arthritis and pulmonary atherosclerosis [3-5]. Macrophages release nitric oxide (NO), prostaglandin E2 (PGE2), and other cytokines, such as IL-1β, TNF-α, and IL-6 during lipopolysaccharide (LPS) stimulation [6]. Thus, the production of these inflammatory mediators and cytokines considerably affects the treatment of inflammatory diseases.

Toll-like receptor 4 (TLR4) is a toll-like receptor subtype that can activate endogenous or exogenous immune responses after LPS stimulation [7]. TLR4 can activate two signaling pathways. The first signaling pathway is the MyD88-dependent pathways of mitogen-activated protein kinase (MAPK) and NF-κB that induce the release of IL-1β, TNF-α, and IL-6. The second signaling pathway is the TRLF-dependent pathway that facilitates TLR4 signaling, which leads to the activation

of interferon regulatory factor 3 (IRF3) [8].

Cardamine komarovii is a cruciferous plant of genus Cardamine, and is mainly distributed in Korea and Northeast China. *C. komarovii* flower (CKF) is an edible and medicinal plant. For example, CKF can maintain hemostasis, reduce blood pressure, and induce sedation [9-10]. CKF consumption increases resistance to oxidative stress. However, reports on the anti-inflammatory effect of CKF remain insufficient.

Materials and Methods

Chemical

Enzyme-linked immunosorbent assay (ELISA) kits were acquired from BD Bioscience (SD, USA). SyBr and PrimeScript™ PT reagent kit were obtained from TaKaRa (Shiga, Japan). p-ERK, p-JNK, p-p38 and other antibodies were obtained from Cell Signaling Technology (MA, USA). NF-κB p65 was purchased from Santa Cruz Biotechnology (CA, USA). Alexa Fluor 594-conjugated AffiniPure Goat Anti-mouse IgG was purchased from Jackson ImmunoResearch (PA, USA).

Plant authentication and extraction

CKFs were collected in Helong (GPS Coordinates: N42°31′17.26″ E128°38′47.27″; altitude: 1276 m, June 2015, China) and were authenticated (voucher specimen: YBU-0846) by Prof. LV Hui-Zi, a taxonomist from Yanbian University, China. Dried CKF (50 g) was soaked in 500 mL of ethanol for reflux extraction, and recovery percent was 10.0 %. After freeze-drying, the samples were stored at −20 °C before use.

Animals

C57BL/6 mice (20 ± 2 g, 5–6 weeks old) were obtained from Changchun Yisi Experimental Animal Co. (Changchun, China; SPF, SCXK (J) 2017-0001). The mice were fed in an experimental animal room under a constant temperature of 23 ± 3 °C, humidity of (50 ± 3)%, and a light of 12 h and dark of 12 h. Food and drinks were sufficient. All experiments were conducted in accordance with the guidelines of Yanbian University Experimental Animal Center.

High-performance liquid chromatography (HPLC) analysis of CKF extracts

CKF extracts were dissolved in methanol and were filtered through a membrane filter (0.45 μm) prior to chromatographic detection. Flavonoids were determined, as previously described [11].

Cell viability test

Cell viability was determined as previously described [11]. The medium was discarded, and dimethyl sulfoxide was added to each well to solubilize formazan crystals. Optical density value was measured at 540 nm by using a spectrophotometer. The cell viability of the treatment groups was compared with the control group.

Determination of NO and PGE2 production

The effect of CKF on NO concentration was determined as previously described [11]. PGE2 was measured based on the manufacturer's instructions.

ELISA

ELISA was performed as previously described [11]. Different kits were used to measure the levels of IL-1β, TNF-α, and IL-6, in accordance with the manufacturer's instructions. Optical density was measured at 450 nm by using a spectrophotometer.

Quantitative PCR

Cells (2.7×10^6) were seeded in each well plate. Different concentrations of CKF (12.5, 25, and 50 μg·mL^{-1}) with or without LPS were added to the plates. RNA was extracted using TRIzol, reverse-transcribed into cDNA, and subjected to quantitative PCR (qPCR), as previously described [11]. The housekeeping gene, Cyclopilin A, was used to normalize tested genes, and data quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

Immunoblot analysis

Immunoblot analysis was performed as previously described [11]. CKF extracts with different concentrations were added to plates containing peritoneal macrophages (PMs, 2.5×10^6 cells/well). PMs were stimulated with LPS or were untreated. The cells were treated with RIPA, and the supernatant was removed. The protein concentration of each sample was measured, and the same amount of protein was added to 12% SDS-PAGE protein sample buffer to separate the samples and was transferred to PVDF membranes. The membranes were incubated overnight with primary antibodies at 4 °C after overnight blocking with 0.1% Tris-buffered saline, polysorbate 20 (TBS-T), and 5% skim milk. The membranes were incubated with secondary antibodies for 1 h and visualized by using an ECL detection kit (ChemiDoc™ XRS+ System, Bio-Rad, USA).

Immunocytochemistry

Immunocytochemistry analysis was performed as previously described [12]. CKF extracts (50 μg·mL^{-1}) and DEX (0.5 mmol·L^{-1}) was added to plates containing peritoneal macrophages (PMs, 2.5×10^6 cells/well). PMs were stimulated with LPS or were untreated for determining the nuclear translocation of NF-κB.

BALF cytokine and cell population measurement

An LPS-induced ALI model was established as previously described [13]. LPSs were instilled in the intratracheal of mice after administration of CKF extracts (5 and 10 mg·kg^{-1}). Mice were sacrificed to collect BALF by washing with ice PBS. After centrifugation, part of cell-free supernatants was used to detect the levels of inflammatory factors, such as IL-1β, TNF-α, and IL-6 in BALF by using ELISA kits based on the instructions. Cellularity was determined by using a flow cytometer after the suspension of cell precipitation in PBS solution. FITC-labeled anti-Gr1 (neutrophils) were purified from BALF cells by using BD FACSVerse™. Data were analyzed in FlowJo 10.0.
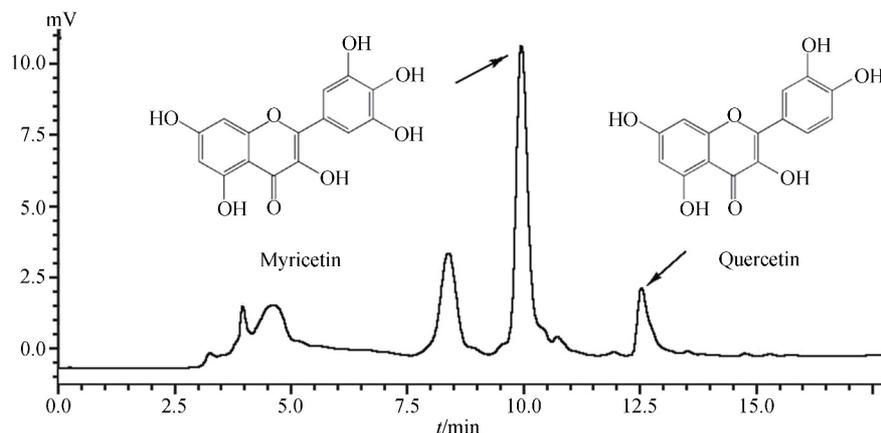
Lung histopathology

The right lung was soaked in formalin and was embedded in paraffin. The paraffin tissue was cut into 5 μm sections and was stained with H&E. The lung histopathology scores as

previously described [14].

Statistical analysis

All data were expressed as the mean ± standard error of at least three separate experiments conducted in triplicate. Statistical analysis was performed through variance analysis combined with rank–sum test. $P < 0.05$ was considered statistically significant.

Results

Extract standardization through HPLC

Fig. 1 presents the HPLC profiling analysis results of methanol-extract material of CKF. The total flavonoid, myricetin, and quercetin contents of the extracts are 41.13, 2.88, and 0.36 $mg·g^{-1}$, respectively.

Fig. 1   Structures of two flavonoid monomers and HPLC profiles of *C. komarovii* flowers sample solution. Myricetin and quercetin were determined at 366 nm

Effect of CKF extracts on NO and PGE2 production in LPS-induced PMs

The anti-inflammatory effect of CKF was investigated, and MTT assay was conducted to determine the cell viability of CKF. CKF extracts with concentrations of up to 50 $μg·mL^{-1}$ did not exert any toxic effect on PMs. Therefore, we detected the in vitro anti-inflammatory activities of 12.5, 25, and 50 $μg·mL^{-1}$ CKF extracts (Fig. 2). We evaluated the NO, PGE2, and their synthesis-related enzymes iNOS and COX-2 accumulation in PMs to investigate the effect of CKF extracts on LPS-stimulated NO and PGE2 production. PMs were stimulated with or without LPS after treatment with different concentrations of CKF extracts. NO (Fig. 3A) and PGE2
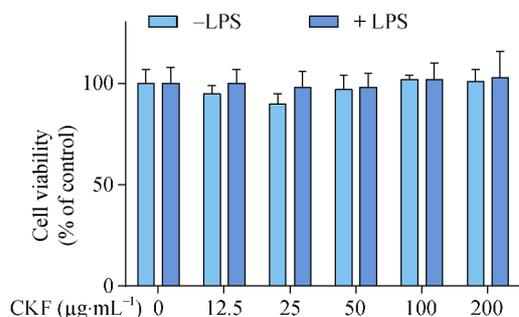
Fig. 2   Effect of CKF extract on cell viability in PMs. PMs were pre-treated with different concentrations of the CKF extract (0, 12.5, 25, 50, 100 and 200 $μg·mL^{-1}$) for 1 h and then with or without LPS for 24 h. Cell viability was measured with an MTT assay. The results are expressed as the mean ± SEM of three independent experiments. *$P < 0.05$ *vs* the LPS-treated cells

(Fig. 3B) levels remarkably decreased with the treatment of CKF extract in a dosage-dependent manner ($P < 0.05$). iNOS and COX-2 are precursors of NO and PGE2. Therefore, we measured NO and PGE2 levels by conducting an immunoblot assay. As shown in Fig. 3C, iNOS and COX-2 expression levels notably decreased in the CKF group, compared with model group ($P < 0.05$).

*Inhibitory effect of CKF on pro-inflammatory cytokine production in LPS-induced PMs*

Pro-inflammatory cytokines, such as TNF-$α$, IL-6, and IL-1$β$, play important roles in various inflammatory diseases [15-16]. CKF treatment can remarkably reduce IL-1$β$ (Fig. 4A), TNF-$α$ (Fig. 4B), and IL-6 (Fig. 4C) secretion in a dosage-dependent manner. In addition, qPCR analysis showed that CKF treatment decreases IL-1$β$ (Fig. 4D), TNF-$α$ (Fig. 4E), and IL-6 (Fig. 4F) mRNA expression levels.

*Effect of CKF extracts on MyD88/TRIF signaling pathways*

We evaluated the effect of CKF extracts on the phosphorylation of MAPKs, such as p38, ERK, and JNK, in LPS-induced PMs to investigate whether they can regulate the MyD88-dependent pathway. As shown in Fig. 5, the phosphorylation levels of ERK, and JNK has no significantly decreased, but the phosphorylation levels of p38, decreased under CKF treatment in a dosage-dependent manner compared with the model group ($P < 0.05$). Immunocytochemistry staining (Fig. 6) showed that CKF extracts attenuate the LPS-induced translocation of NF-kB p65 subunit into the nucleus from the cytoplasm. Therefore, our findings suggested that CKF plays an anti-inflammatory role by regulating the NF-$κ$B signaling pathway. The attenuation of CKF on the production of pro-inflammatory mediators through the

TRIF-dependent signaling pathway was investigated. As shown in Fig. 7, CKF remarkably inhibits p-IRF3, p-STAT1, and p-STAT3 production compared to the model group ($P < 0.05$).

These findings suggested that CKF has an anti-inflammatory effect through the regulation of the TRIF-dependent signaling pathway.
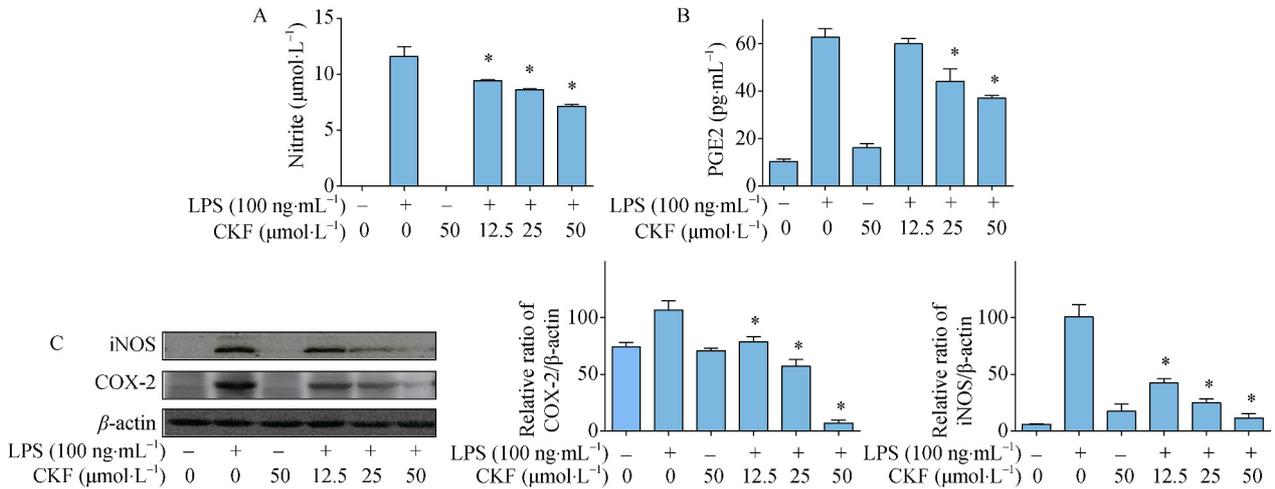
Fig. 3 Effect of CKF extract on nitrite and PGE$_2$ production. PMs were pretreated with CKF extract at the indicated concentration for 1 h before treatment with LPS. The medium was harvested and assayed after 24 h for nitrite production (A) and PGE2 production (B). Immunoblot analysis was performed to detect iNOS and COX-2 expression with antibodies (C). The results are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.05$ *vs* the LPS-treated group
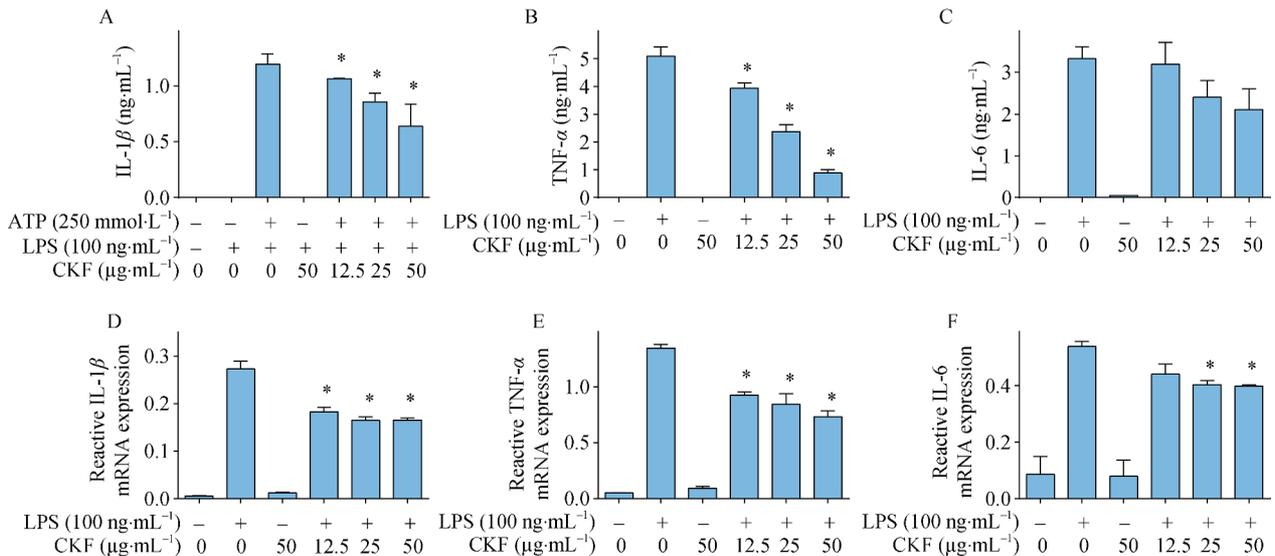
Fig. 4 Effect of CKF extract on LPS-induced production of pro-inflammatory cytokines. The production of the cytokines, IL-1β (A) TNF-α (B)and IL-6 (C) in the medium was assayed by ELISA. The mRNA expression levels of IL-1β (D), TNF-α (E) and IL-6 (F) were measured with qPCR. Cyclophilin A was used as an internal control. The results are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.05$ *vs* the LPS-treated group

Effect of CKF extracts on in vivo anti-inflammatory activity

This study conducted the anti-inflammation experiment of CKF *in vivo* by using the ALI model. BALF and lung tissues were collected from the sacrificed mice, the number of cell populations, total protein in BALF, and secretion of pro-inflammatory mediators (IL-1β, TNF-α, and IL-6) were measured, and the pathological conditions of lung tissues were observed through H&E staining. The results showed that

CKF treatment can decrease the protein of BALF (Fig. 8A). The number of neutrophils was analyzed to determine the cellular infiltration components in BALF. The results showed that the neutrophils (Fig. 8B) in the LPS group remarkably increase, whereas the neutrophils in the CKF and DEX groups remarkably decrease ($P < 0.05$). The production of IL-1β (Fig. 8C) was remarkably inhibited under 5 and 10 mg·kg^{-1} of CKF extracts ($P < 0.05$). However, CKF showed no obvious de-

pressant action on TNF-*α* or IL-6 (data not shown). The results showed that the lungs have remarkable decrease of alveolar space, interstitial and alveolar cell infiltration after the intratracheal instillation of LPS. CKF treatment remarkably improves the LPS-induced lung histopathological injury ($P < 0.05$; Fig. 8D).
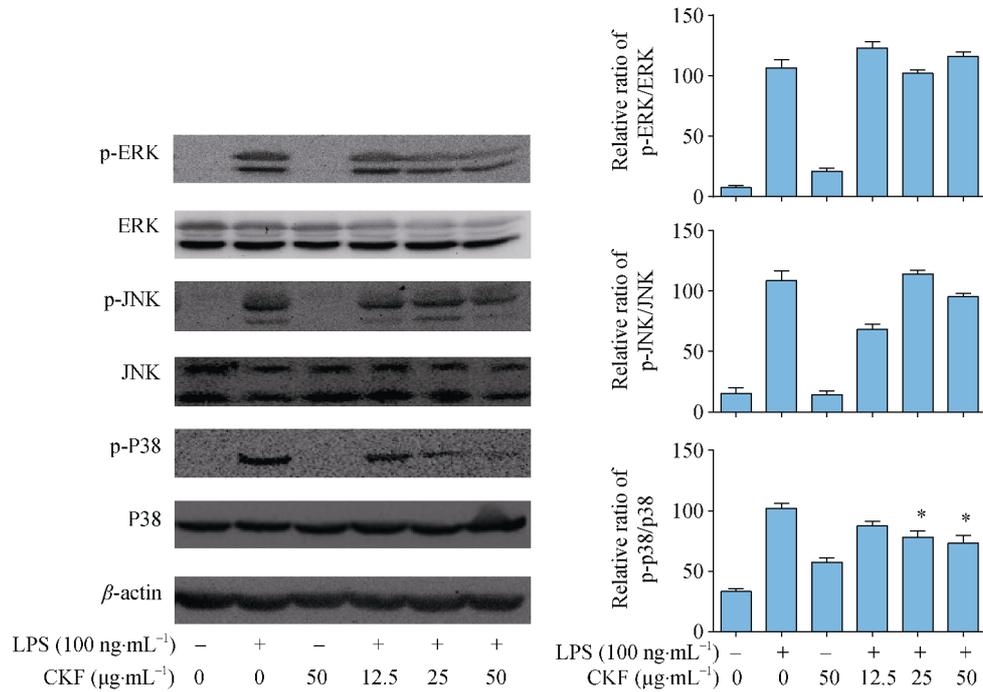

Fig. 5   CKF affected LPS-stimulated PMs through MAPK pathway. PMs were pretreated with CKF at various concentrations (12.5, 25, 50 µg·mL^{-1}) for 1 h and then treated with LPS for 30 min were determined with western blot analysis. $^{*}P < 0.05$ *vs* the LPS-treated group
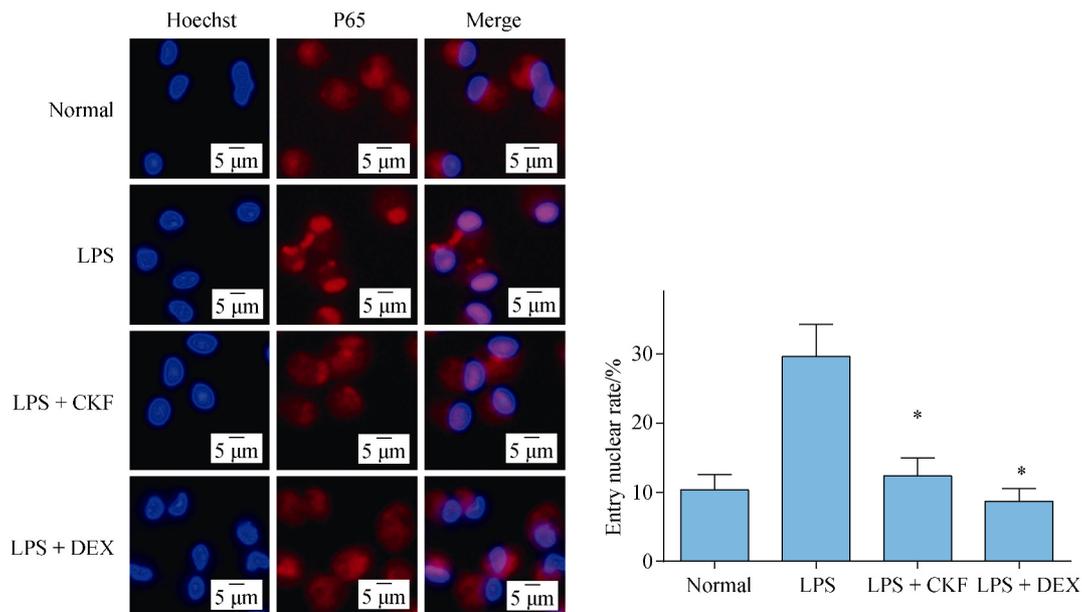
Fig. 6   CKF affected LPS-stimulated PMs through NF-κB pathway. PMs were pretreated with CKF (50 µg·mL^{-1}) and DEX (0.5 mmol·L^{-1}) for 1 h and then treated with LPS for 30 min. P65 translocation into nucleus was measured by immunocytochemistry. $^{*}P < 0.05$ *vs* the LPS-treated group
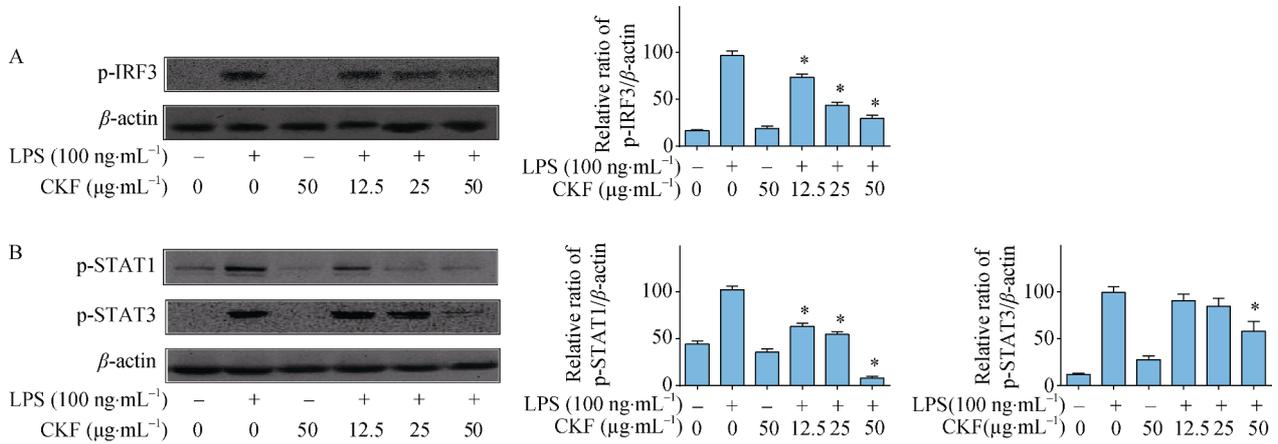
Fig. 7   CKF affected LPS-stimulated PMs through TRIF signal mediators. PMs were pretreated with CKF at various concentrations (12.5, 25, 50 μg·mL⁻¹) for 1 h and then treated with LPS for various times. The phosphorylation levels of IRF3 (LPS for 60 min) (A) and STATs (LPS for 120 min) (B) were determined with western blot analysis. *$P < 0.05$ *vs* the LPS-treated group
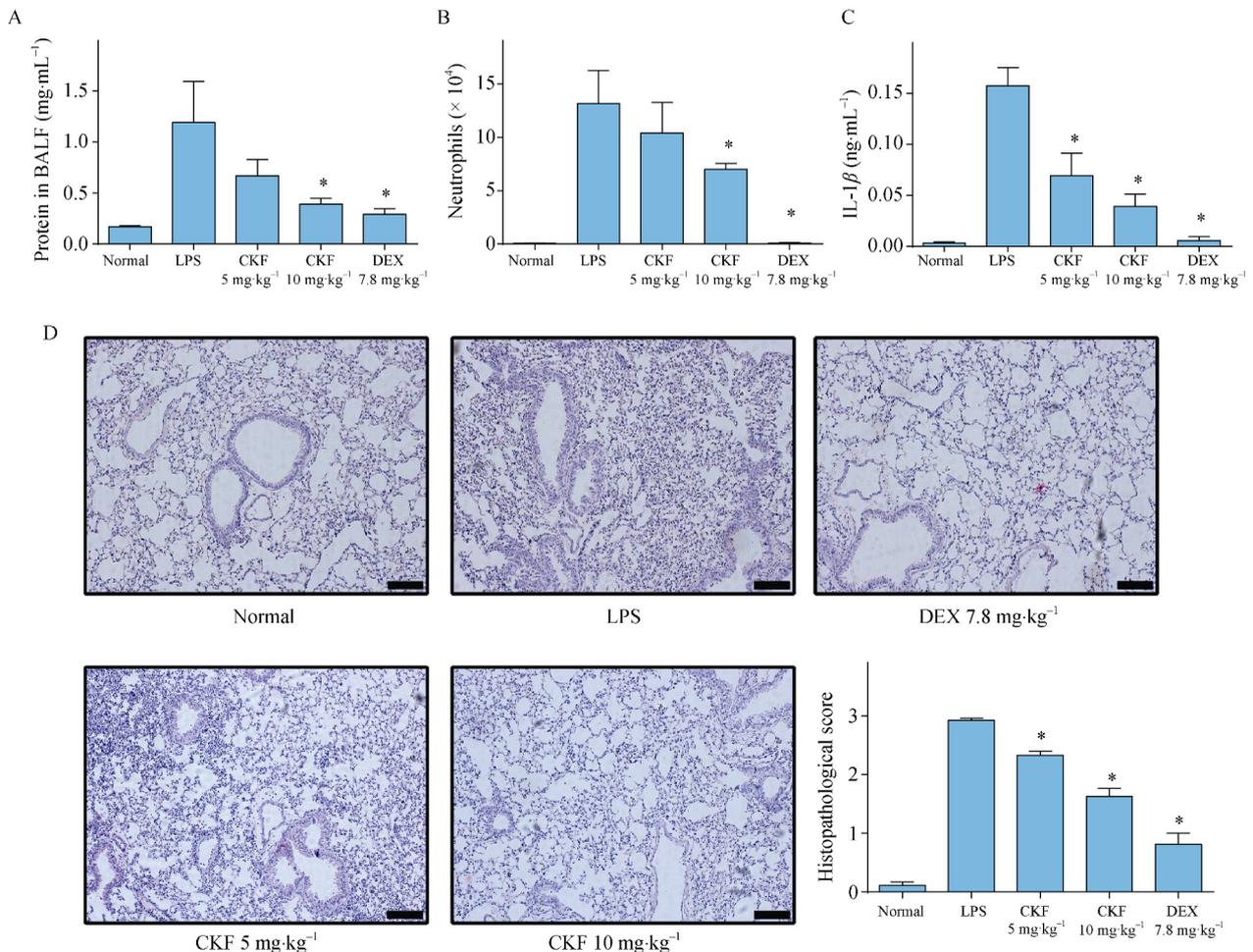
Fig. 8   Effect of CKF on LPS-induced ALI model. Mice were administered saline or extract for 7 days and DEX for 2 days before intratracheal instillation of LPS (5 mg·kg⁻¹). After 24 h of LPS stimulated, BALF and lung tissue were collected in sacrificed mice. The proteins in BALF (A) were determined (A). Effects of CKF on the number of neutrophils (B) in the BALF in LPS-induced ALI mice. The levels of IL-1β (C) was determined. The lung tissue was harvested for H&E staining and viewed under a light microscope (scale bar = 100 um) (D). The results are expressed as the mean ± SEM of six independent experiments. *$P < 0.05$ *vs* the LPS-treated group

Discussion

Medicinal plants play a vital role in the primary healthcare system. However, few studies have been reported on the anti-inflammatory effect of CKF. The results of this study confirmed that CKF suppresses the inflammatory responses in PMs through MyD88/TRIF pathways. In this study, we found that the total flavonoid content of CKF is 41.13 mg·g^{-1}, and two flavonoid compounds (myricetin and quercetin) are determined through HPLC. These results confirmed that the anti-inflammatory activity of CKF is closely related to high flavonoid content [17].

LPS stimulation can induce macrophages to produce inflammatory cytokines. NO levels are affected by iNOS expression, which induce the continuous production of NO [18]. The aberrant expression of COX-2 causes the massive release of PGE2, which leads to rheumatoid arthritis and inflammatory liver injury [19-20]. We found that CKF can inhibit NO and PGE2 expression levels by down-regulating iNOS and COX-2 levels in LPS-induced PMs. In addition, the overproduction of pro-inflammatory cytokines may accelerate the pathological changes in inflamed tissues. These changes result in shock, tissue necrosis, and endothelial dysfunction [21]. In this study, we detected the mRNA expression of IL-1β, TNF-α and IL-6, and we found that CKF inhibits the expression levels of IL-1β, TNF-α and IL-6 in a dosage-dependent manner.

TLR4 triggers the activation of the MyD88- and/or TRIF-dependent signaling pathway. Ligation of LPS to TLR4 triggers its association with MyD88 signaling pathways, such as the MAPK and NF-κB pathways. The phosphorylation level of ERK, JNK and p38 in MAPK can regulate the production of NO and pro-inflammatory cytokines [22-23]. IκB degradation can lead to the transcription of NF-κB into the nucleus that can regulate COX-2 expression. Our results indicate that CKF inhibits the phosphorylation levels of p38 and attenuate NF-kB into the nucleus from the cytoplasm that inhibits the production of inflammatory cytokines and mediators.

The TRIF-dependent pathway lead to IFN-β expression and IRF3 phosphorylation activates the transcription and protein expression of IFN-β [24]. The extracellular IFN-β protein binds to the IFN receptor and induces the phosphorylation of STAT1 and STAT3 [25]. Subsequently, the down-regulation of STAT1 phosphorylation decreases iNOS expression and NO production. In addition, the induction of IFN-β expression by IRF3 phosphorylation promotes STAT3 phosphorylation, which leads to TNF-α and IL-6 production. Our data indicate that the anti-inflammatory effect of CKF is closely related to the down-regulation of the phosphorylation of vital factors (IRF3 and STAT1/3) that constitutes the TRIF-dependent signaling pathway.

We investigated the protection of CKF on mice from LPS-induced ALI to demonstrate the *in vivo* anti-inflammatory properties of CKF. Previous studies have shown that LPS-induced ALI is characterized by alveolar inflammatory cell infiltration, which is mainly manifested as neutrophil infiltration, and extensive release of inflammatory medium in lung tissues was observed [26]. BALF concentration is a characteristic of alveolar capillary permeability. BALF concentration in the LPS group is higher than that in other groups. Meanwhile, CKF can decrease the BALF concentration and present certain dose dependence. Our study found that CKF can remarkably reduce the excessive production of IL-1β from the BALF in ALI. In the present study, extensive inflammatory cells, including neutrophils, entered the lung tissues after the LPS stimulation on mice. However, CKF treatment can decrease the number of inflammatory cells in the lungs. The result revealed that CKF has a good anti-inflammatory effect. Histopathological observation of lung tissues showed that CKF can remarkably improve alveolar clearance and infiltration of interstitial and alveolar cells after LPS stimulation. *In vivo* experiments demonstrated the anti-inflammatory effect of CKF.

In conclusion, CKF can inhibit the expression of inflammatory factors in LPS-induced PMs by regulating the MyD88 and TRIF signaling pathways. CKF can protect mice from LPS-induced ALI. *In vitro* and *in vivo* studies demonstrated the anti-inflammatory effect of CKF and provided a theoretical basis for subsequent studies on CKF.

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