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LXRs/ABCA1 activation contribute to the anti-inflammatory role of phytosterols on LPS-induced acute lung injury

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ABSTRACT

Phytosterols (PS) showed anti-inflammatory activities in acute lung injury (ALI), but the mechanism needs to be further elucidated. In this study, male C57BL/6J mice were gavaged with PS (75 mg/kg or 150 mg/kg) for 7 consecutive days, mice ALI were induced by tracheal instillation of LPS (50 μL, 1 mg/mL). In vitro, RAW264.7 cells were incubated with LPS in the presence or absence of 50 μM β-sitosterol. Enzyme-linked immunosorbent assay was used to detect levels of inflammatory cytokines. Real-time quantitative PCR and western blot analysis were used to detect changes in nuclear factor-kB (NF-κB) and liver X receptors (LXRs) pathways. The results showed that PS administration activated LXRs/ABCA1 pathway and significantly ameliorated LPS-induced pulmonary inflammation of ALI. The role of phytosterols was verified in LPS-stimulated RAW264.7 cells and the protective effect was weakened by administration of LXR antagonist GSK2033. These findings suggested that PS ameliorates LPS-induced ALI partially by activating the LXRs/ABCA1 pathway.

1. Introduction

Acute lung injury (ALI) and its severe phase, acute respiratory distress syndrome (ARDS) are common complications in acutely critically ill patients, with a mortality rate of 30-40% (Costa & Amato, 2013). It is characterized by respiratory failure, atelectasis, and damage to pulmonary mechanics and gas exchange as a consequence of endothelial cell damage caused by inflammation, apoptosis, and necrosis (de Oliveira et al., 2019). ALI is primarily to be treated by reducing inflammation and inhibiting respiratory failure. Anti-inflammatory drugs such as corticosteroids, aspirin, salbutamol, and ketoconazole are typically used in clinical practice (Ding et al., 2020). With the development of medical research, studies focusing on the etiology and pathophysiological mechanisms of ALI are increasing, but the available effective treatments are very limited, so it is very important to find more effective measures for early intervention and treatment.

Phytosterols (PS) are naturally occurring active substances of plant origin, mainly found in nuts, vegetable oils, seeds, cereals and legumes, and are structurally and functionally similar to vertebrate cholesterol (Zhou et al., 2012). As previously reported (Zaloga, 2015), more than 100 types of PS and 4000 other forms of triterpenoids have been identified. The common PS are β-sitosterol, stigmasterol, campesterol and brassicasterol, which are extremely similar in structure and differ only in the side chains of the molecular backbone (Kritchevsky & Chen, 2005). PS are present in plants in different forms, including free or esterified with fatty acids and glycosides. In recent years, PS have attracted increasing attention for their function of lowering serum cholesterol and cardiovascular disease risk (Makran, Barberá, & Cilla, 2021; Moreau, Whitaker, & Hicks, 2002; Valitova, Sulkarnayeva, & Minibayeva, 2016). In addition, PS have anti-tumor, anti-microbial, weight and blood pressure control, and dementia prevention effects (Garcia-Llatas & Rodriguez-Estrada, 2011; Ghaedi, Foshati, et al., 2019; Ghaedi, Varkaneh, et al., 2019; Kamal-Eldin & Moazzami, 2009; Ramprasath & Awad, 2015; Rocha et al., 2016; Salehi et al., 2020). However, the anti-acute lung injury aspects of PS and their specific mechanisms need to be further elucidated.

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Macrophages are the primary immune cells of the host that fight against invading pathogens and play an important role in the innate immune response (Gordon, 2007; Gordon & Taylor, 2005). Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is one of the most important stimulants for the induction of acute lung injury (Zhao, Zhang, Xu, & Wang, 2017). Treatment with LPS induced inflammatory responses in macrophages has been used as an important tool for developing anti-inflammatory drugs. LPS triggers the TLR4 signaling pathway, which induces NF-κB activation and inflammatory cytokine production (Imai et al., 2008). These inflammatory cytokines sufficiently enhance the inflammatory response and lead to lung injury. Previous studies have suggested that reducing the expression of inflammatory cytokines could significantly reduce the severity of acute lung injury (Seki et al., 2010). Treatments aimed at inhibiting the TLR4/NF-κB signaling pathway may have potential in the treatment of acute lung injury (Qureshi et al., 2006).

Liver X receptors (LXRs), members of the ligand-dependent nuclear receptor superfamily, are sensitive organelles of intracellular cholesterol homeostasis. When activated by endogenous (e.g., metabolites of cholesterol) and exogenous (e.g., T0901317, GW3965) ligands, it can regulate the expression of target genes through transcriptional regulation, reduce cellular cholesterol uptake and synthesis, and increase intracellular cholesterol clearance, thereby playing important regulatory roles in immune response and tumorigenesis (Jakobsson, Treuter, Gustafsson, & Steffensen, 2012). Tontonoz's group found that activation of LXRs pathway inhibited LPS-induced NF-κB-dependent pro-inflammatory genes (Sean B. Joseph et al., 2004). Su et al. established a mouse model of acute lung injury using LPS induction, and after treatment with different concentrations of Chikusetsusepin V (CsV), the acute lung injury abated, and its anti-inflammatory effect may be related to its regulation of NF-κB and LXRα expression (Su, Zhang, Zhang, & Jiang, 2019). Fu et al. also found anti-inflammatory effects of platycodin D (PLD) on LPS-induced inflammation in primary rat microglia, and their results indicated that PLD inhibited LPS-induced inflammatory responses by activating the LXR-ABCA1 signaling pathway and blocking lipid rafts (Fu et al., 2017).

In this study, we evaluated the anti-inflammatory effects of phytosterols in vivo and in vitro, and we mainly found that PS ameliorates LPS-induced ALI partially by activating the LXRs/ABCA1 pathway and affecting the activation of the TLR4/NF-κB pathway.

2. Materials and methods

2.1. Reagents

The phytosterols (PS) were purchased from Xi 'an Hysf Biotechnology Co., Ltd. (Xi 'an, Shanxi, China), and it's a mixture of 44% β-sitosterol, 26% campesterol, and 19% stigmasterol. β-sitosterol (BS, No. S9889), LPS (Escherichia coli 055:B5) and GSK2033 (GSK, No. SML1617) were from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS, No. 04-001-1A) were obtained from Biological Industries (Israel). Trizol reagent and Primescript™ RT reagent kits and SYBR® Premix Ex Taq™ II kits were purchased from Takara Biotechnology Co., Ltd (Takara, Dalian, China). Antibody to NFκB (p65), p-NFκB (p-p65) were purchased from Cell Signaling Technology (Danvers, MA, United States); antibody to TLR4 (ab13556) and ABCA1(ab18180) were from Abcam (Cambridge, UK); antibody to β-actin and the secondary antibody were from Zhongsugi Jinqiao Biotechnology Co., Ltd. (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits, used to determine IL-1β, IL-6 and TNF-α were from Quanzhou Jiubang Biotechnology Co., Ltd. (Fujian, China). Zein (Batch No.c10921803) and Casein sodium salt (NaCas, No. c11105803) were from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). All other chemicals were of reagent grade.

2.2. Animal care

Male C57BL/6J mice (6–8 weeks old) were purchased from the Center of Experimental Animals of Chongqing Medical University (Number of qualitative qualification: 2021008, Chongqing, China). The mice were housed in wood chip-bedded plastic cages at constant room temperature (23 ± 2 °C) under a 12–h light/dark cycle (lights on 07:00–19:00), and fed with water and food available ad libitum. In this study, forty male C57BL/6J mice were randomly divided into four groups: control group, LPS, 75 mg/kg PS + LPS and 150 mg/kg PS + LPS. All studies were approved and agreed by the Animal Care and Research Committee of Chongqing Medical University, China.

2.3. Phytosterols solution preparation and treatment

Due to the insolubility of phytosterols in water, the preparation of phytosterols solution was modified from a published Zein-based nanoparticle method (Zhang et al., 2019). Zein powder and Casein sodium salt (NaCas) powder were mixed with double distilled water to prepare the Zein (1%, w/v) and NaCas (1%, w/v) stock solutions, respectively. 2 mL of Zein solution and 2 mL of NaCas solution were added into a glass vial containing 16 mL of double distilled water with the pH adjusted to 12.0 using 1.0 M NaOH by stirring at 1,000 rpm for 30 min. The resulting mixture was adjusted to a final pH value of 7.0 ± 0.1 using 1.0 M hydrochloric acid (HCl) by stirring at 1,000 rpm for 30 min. PS (150 mg and 300 mg) was then added into the mixture and stirred for 30 min by stirring at 1,000 rpm overnight. The liquid dispersions were sonicated for 10 min to obtain the stock concentrations of 7.5, 15 mg/mL. In PS treated groups, the mice were gavaged with different amount of PS (75 mg/kg BW, 150 mg/kg BW) for 7 consecutive days before LPS challenge.

2.4. LPS-induced acute lung injury model

The acute lung injury model was established as described previously (Rayamajhi et al., 2011). Briefly, one hour after the last treatment of PS or solvent control, the mouse was anesthetized with 0.8% sodium pentobarbital intraperitoneally, and its tongue was gently clamped using forceps and the pharynx was visualized through laryngoscopy. Then LPS solution (50 μL, 1 mg/mL), together with 100 μL of air was pushed into trachea using a 1 mL syringe. If a distinct choking sound was heard, the LPS has entered the lungs accurately and the mice were rotated to make sure the LPS was evenly distributed in both lungs. Mice in the non-LPS treatment group received 50 μL saline. 24 h later, animals were anesthetized for bronchoalveolar lavage and tissue collection.

2.5. Bronchoalveolar lavage fluid (BALF) collection

BALF was collected by intratracheal injection of 1 mL saline with a blunt-tipped sterile needle (1 mL of saline each time, repeated three times). BALF was centrifuged at 2500 rpm for 15 min and the cell pellet was resuspended in saline (600 μL). Total cell count (TCC) was performed using a hemocytometer plate after 10-fold or 20-fold dilution. The cells were subsequently centrifuged onto slides by Thermo Scientific Shandon Cytospin 4 (50 μL, 500 rpm, 5 min) (Waltham, MA, USA) and the approximate distribution of cells in each group was then observed qualitatively according to the protocol of the Diff-Quik Stain kit (Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, China). A portion of the BALF supernatant was collected to measure the total protein level of each group by the BCA protein quantification kit. Another portion of BALF supernatant was collected to check the levels of IL-1β, IL-6 and TNF-α by using ELISA kits.

2.6. Hematoxylin-eosin staining

The isolated lung tissues were fixed using 4% formaldehyde solution,

and the lung tissues were dehydrated, paraffin-embedded and cut into pieces at 4 μm thickness, stained with hematoxylin-eosin (HE), and scanned in full by PANNORAMIC Digital Slide Scanners (3DHISTECH Ltd., Budapest, Magyarország) at a magnification of ×200 to observe histopathological changes in the lung.

2.7. Cell culture and intervention

The murine macrophage RAW264.7 cells were kindly donated by Prof. Xiaoqiu Xiao from the First Hospital of Chongqing Medical University and cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin (HyClone, Utah, USA) at 37 °C in a 5% CO_2 incubator. β-sitosterol (BS) was dissolved in Ethanol: Tween 80 = 8.5:1 and configured to a target stock concentration of 50 mM. It was then diluted to 50 μM with cell culture medium for subsequent experiments. Cell interventions were categorized as follows: (a). After pretreatment with BS (50 μM) for 12 h, RAW264.7 cells were stimulated with LPS (1 μg/mL) for 30 min or 12 h; (b). RAW264.7 cells were pretreated with GSK2033 (LXRs antagonist) along with BS for 12 h and then co-cultured with 1 μg/mL LPS for another 12 h. After intervention, the corresponding samples (e.g., RNA, protein, cell supernatant) were collected and stored at −80 °C for later analysis.

2.8. Pro-inflammatory cytokine assay

After pretreatment with 50 μM β-sitosterol for 12 h at 37 °C, RAW264.7 cells were inoculated in 6-well cell culture plates at a density of 1×10^6/well and stimulated with LPS (1 μg/mL) for another 12 h at 37 °C. The levels of IL-1β, IL-6 and TNF-α in the cell culture supernatant were measured by Enzyme-linked immunosorbent assay (ELISA).

2.9. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

RAW264.7 cells (1×10^6 cells in 6-well plates) were pretreated with β-sitosterol (50 μM) for 12 h and then co-cultured with LPS (1 μg/mL) for 12 h at 37 °C and 5% CO_2. Intracellular total RNA was extracted using TRIzol® reagent, and Primescript™ RT reagent kit was used to synthesis cDNA. Quantitative real-time PCR was performed using iQ SYBR green Supermix (Bio-Rad Laboratories, Munich, Germany) and the Bio-Rad CFX96 system. PCR conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The relative expression of genes was calculated by the 2(ˆ-ΔΔCt) method, in which Ct represents the threshold cycle. The primer sequences of the mouse genes used are shown in the Table1.

2.10. Western blot analysis

Total protein from treated RAW264.7 cells and lung tissues were extracted using a protein extraction kit (No. BC3710, Solarbio, Beijing, China), and then protein concentration was determined using a BCA protein assay kit (No. CW0014S, CWBIO, Beijing, China). Equal amounts of denatured proteins were separated on 8% or 10% SDS-PAGE gels and transferred to PVDF membranes. After sealing the PVDF membrane with 5% bovine serum albumin for 1 h, the PVDF membrane was incubated with primary antibody for more than 16 h on a 4 °C shaker. The primary antibody dilution ratio used in the study were: p-P65, P65 and β-actin were 1:1,000; TLR4 and ABCA1 were 1:500. After washing with TBST, the membranes were incubated with secondary antibody (1:8,000) for 1 h at 37° shaker and exposed with ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Table 1
Primers used for the qRT-PCR study.

Gene	Sequence (5′ to 3′)
IL-6	F: GCCTTCTTGGGACTGATGCT
	R: TGCCATTGCACAACTCTTTTCT
IL-1β	F: TCGCAGCAGCACATCAACAAGA
	R: AGGTCCACGGGAAAGACACAGG
TNF-α	F: AGGCACTCCCCCAAAAGATG
	R: GCCATTTGGGAACTTCTCAT
ABCA1	F: CGTTTCCGGGAAGTGTCCTA
	R: GCTAGAGATGACAAGGAGGATGGA
LXR α	F: GCCTCAATGCCTGATGTTTC
	R: CTGCATCTTGAGGTTCTGTCTTC
β-actin	F: TGAGCTGCGTTTTACACCCT
	R: GCCTTCACCGTTCCAGTTTT

2.11. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance of data was analyzed using a two-tailed Student's *t*-test (when two groups were compared), ordinary one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc comparison with test multiple groups in GraphPad Prism software. P-values < 0.05 were considered significant. Significance levels are expressed as follows: $*P < 0.05$, $** P < 0.01$, $\# P < 0.05$, $\#\# P < 0.01$.

3. Result

3.1. Effect of phytosterols on histopathological changes of lung in mice with ALI

After LPS or saline control was administered to mice by tracheal drip for 24 h, lung tissue sections were taken and HE staining was performed. The results showed that no histopathological changes were observed in the lung tissues of control mice. However, lung tissues from LPS-treated mice exhibited extensive pathological changes, such as inflammatory cell infiltration, interstitial edema and increased alveolar wall thickness, which initially indicated that the LPS-induced acute lung injury model was successfully constructed (Haute et al., 2020). Pretreatment with phytosterols (75 mg/kg or 150 mg/kg), especially 150 mg/kg, for one week significantly attenuated LPS-induced lung histopathological damage (Fig. 1A).

3.2. Effect of phytosterols on inflammatory cells counts as well as protein content in BALF

In this study, we observed the effect of phytosterols on LPS-induced inflammatory cell infiltration in broncho-alveolar lavage fluid (BALF). As shown in Fig. 1B, the results presented by Diff-Quik Staining showed that the total number of BALF cells in the LPS group was increased compared to the control group. Pretreatment with PS (75 mg/kg or 150 mg/kg) inhibited the LPS-induced increasing of the total cell numbers in BALF which consistent with the results presented by quantitative BALF cell number counting (Fig. 2A). Alveolar edema is the main feature observed during the inflammatory phase of ALI/ARDS, and leads to protein-rich leakage. Therefore, total protein detected in BALF is an indirect indicator of increased alveolar capillary permeability (Donahoe, 2011). The protein content in BALF was significantly increased in the LPS group (1.267 ± 0.4630 mg/mL) compared to the control group (0.3228 ± 0.1266 mg/mL). After administration of phytosterols pretreatment, the protein contents in BALF of both groups (0.7588 ± 0.3149 mg/mL and 0.7513 ± 0.3440 mg/mL) with 75 mg/kg and 150 mg/kg phytosterols were significantly reduced compared to the LPS group (Fig. 2B).

3.3. Effect of phytosterols on the protein levels of IL-1β, IL-6 and TNF-α in lung tissues of mice

To further evaluate the anti-inflammatory effect of phytosterols, ELISA kits were used to detect the protein levels of pro-inflammatory

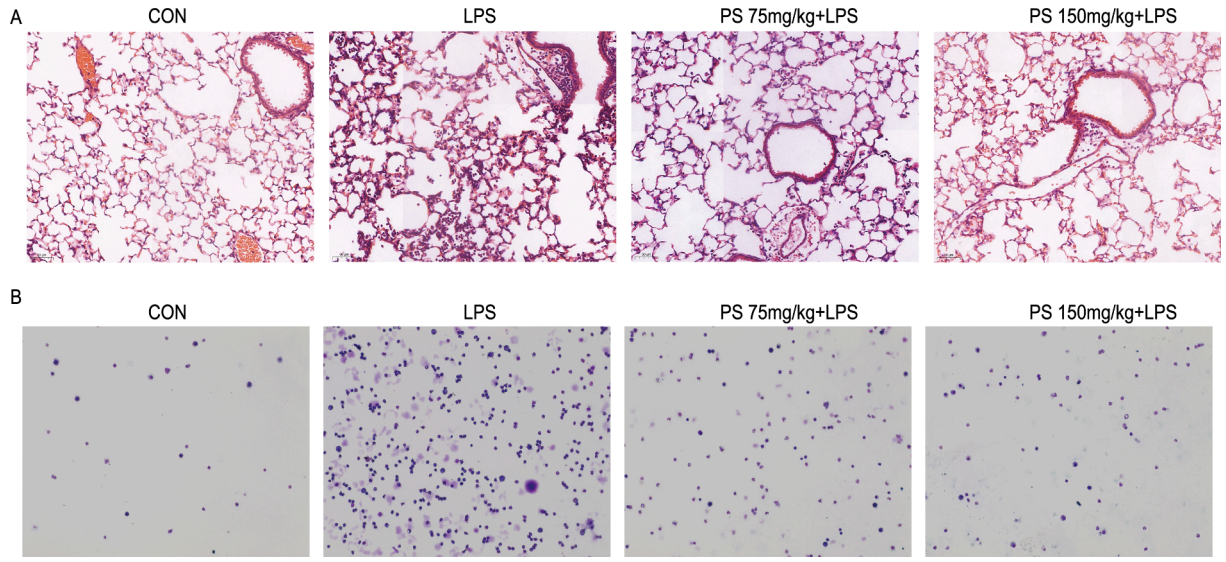

Fig. 1. Phytosterols (PS) exerts protective effects against LPS-induced acute lung injury in C57BL/6J mice. A. H&E staining of histopathological changes in lung tissues from different groups (Control, LPS, PS (75 mg/kg) + LPS, PS (150 mg/kg) + LPS) (magnification 200×, n = 4). B. Diff- Quik Staining of cell precipitation from BALF of each group. (Magnification 200×, n = 4).

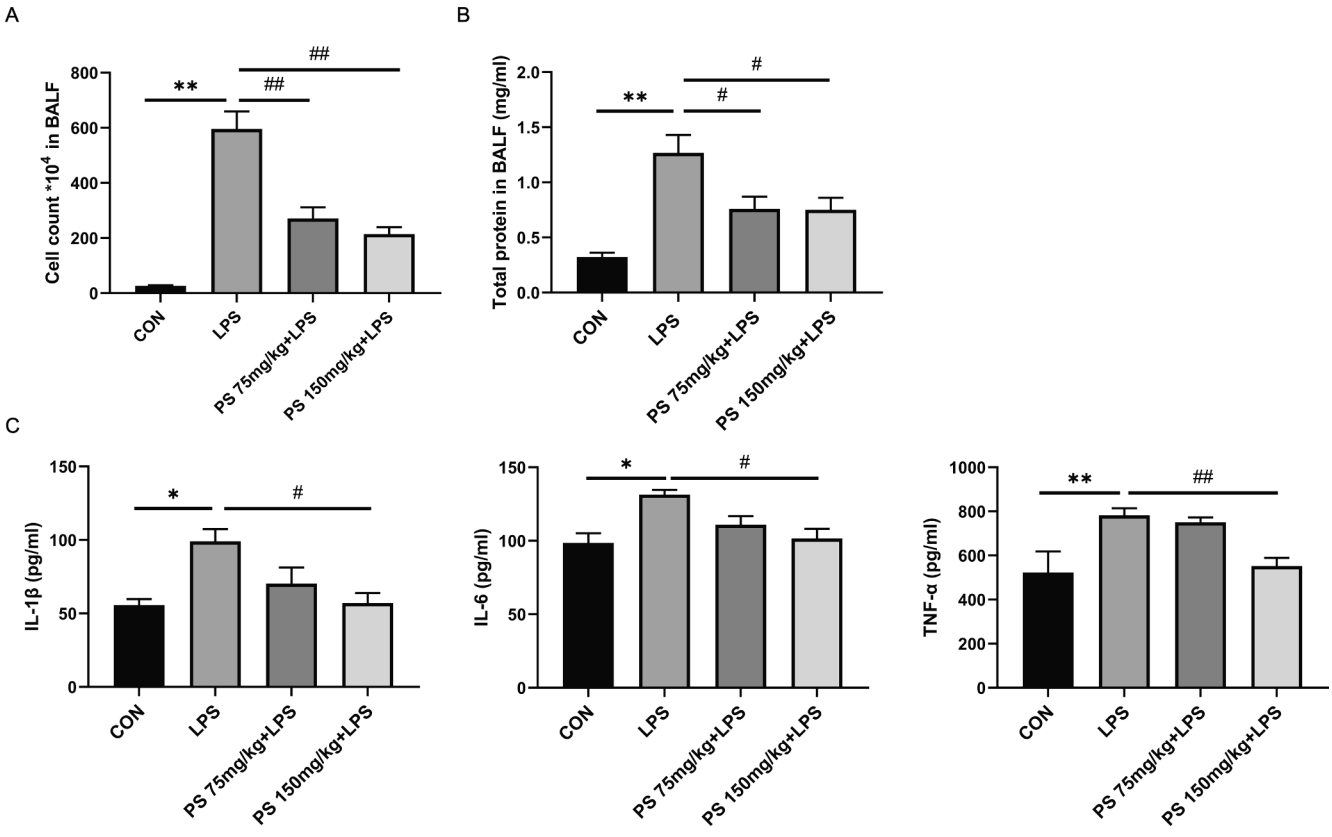

Fig. 2. Phytosterols (PS) inhibits inflammatory response in LPS-induced ALI in mice. A. Total number of inflammatory cells in the BALF of mice from different groups (Control, LPS, PS (75 mg/kg) + LPS, PS (150 mg/kg) + LPS). B. Total protein content in the BALF of different groups. C. ELISA measurements of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in BALF. Data are expressed as mean ± SEM (n = 4, * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus LPS group).

cytokines (IL-1β, IL-6 and TNF-α) in BALF. IL-1β, IL-6 and TNF-α protein levels in BALF were significantly higher in the LPS-treated 24 h compared with the saline group ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively). Pretreatment of PS 150 mg/kg, but not PS 75 mg/kg, significantly reduced the LPS-induced elevation of IL-1β, IL-6 and TNF-α protein levels in BALF ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively) (Fig. 2C). Although the PS 75 mg/kg intervention group showed significant differences in the inflammatory phenotypes, such as total cell counts in BALF as well as protein levels compared to the LPS group, the upregulation of pro-inflammatory factors induced by LPS was only

reversed in the PS 150 mg/kg treatment group as the ELISA assay results presented. In summary, we will pay more attention to the PS 150 mg/kg intervention group in the following experiments.

3.4. Effect of β-sitosterol on the secretion of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and their mRNA expression in RAW264.7 cells

Reference to dissolution methods in the literature with some adjustments (Liao et al., 2018), we tried various solvents to dissolve β-sitosterol (BS) such as DMSO, Ethanol, Chloroform, DMSO: Tween 80 = 8.5:1 and Ethanol: Tween 80 = 8.5:1. The results showed that chloroform and ethanol plus tween 80 dissolve better after the sonication (Supplementary Fig. 1). Considering the toxicity of the solvents, β-sitosterol (BS) was finally decided to be dissolved in Ethanol: Tween 80 = 8.5:1 and configured to a target stock concentration of 50 mM. Cell Counting Kit-8 study showed no viability damage to RAW264.7 cells with Ethanol: Tween 80 = 8.5:1 (v/v, 0.1%) or 50 μM β-sitosterol treatment (Supplementary Fig. S2). The real time RT-PCR results showed that β-sitosterol significantly suppressed the mRNA levels of IL-1β, IL-6 and TNF-α elevated by LPS challenge ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) (Fig. 3A). Meanwhile, the secreted protein levels of pro-inflammatory factors showed similar trends after β-sitosterol pre-treatment (Fig. 3B).

3.5. Effect of β-sitosterol on TLR4/NF-κB signaling pathway in LPS-stimulated RAW 264.7 cells

The TLR4 signaling pathway is involved in the inflammatory response of the body, and the MyD88-dependent TLR4 pathway promotes the production of inflammatory factors through NF-κB activation (Scheiermann & Klinman, 2016). NF-κB is considered to be a central mediator of inflammation and immune response (DiDonato, Mercurio, &

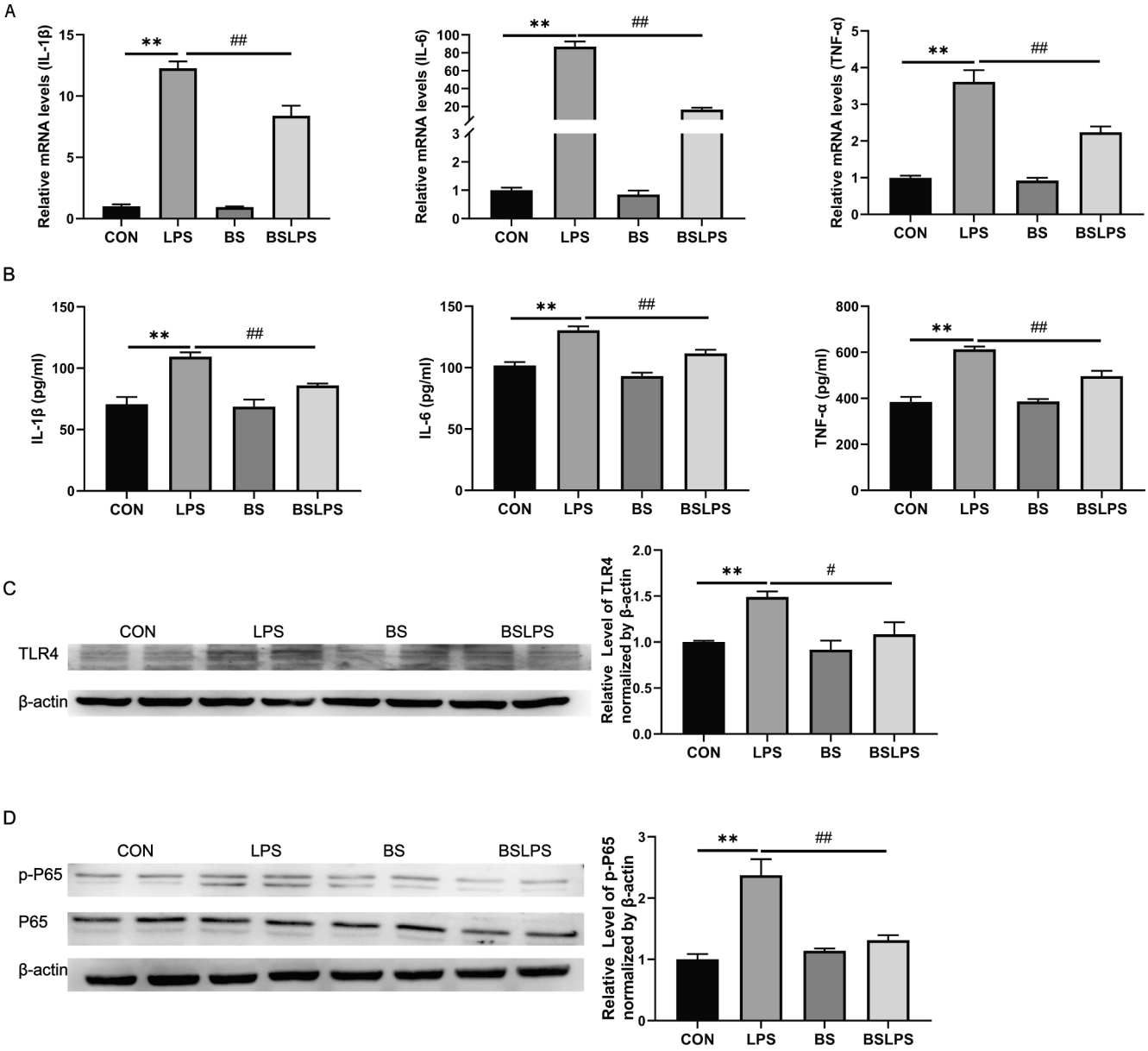

Fig. 3. Effect of β-sitosterol (BS) on the pro-inflammatory cytokines and the protein expression of TLR4 and p-P65/P65 in RAW264.7 cells. RAW264.7 cells were pre-treated with BS (50 μM) for 12 h and treated with LPS (1 μg/mL) for another 12 h. A. mRNA expression of pro-inflammatory cytokines, including IL-1β, and IL-6, TNF-α. B. ELISA measurements of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the cell culture supernatants. C-D. Western blot analysis of TLR4 (C), p-P65/p65 (D) proteins in LPS-induced RAW264.7 cells. Their quantitative densitometric analysis normalized against β-actin were shown on the right, respectively. Data are expressed as mean ± SEM (n = 3, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus LPS group).

Karin, 2012). Fig. 3C and 3D showed the results of Western blot analysis, where β-sitosterol was pretreated with 50 μM for 24 h and then stimulated with 1 μg/mL LPS for 30 min, and protein was used to analyze p-P65/P65; or β-sitosterol was pretreated with 50 μM for 12 h and then co-incubated with 1 μg/mL LPS for 12 h, and protein was used to analyze TLR4. The results showed that TLR4 expression was elevated and p65 was phosphorylated after LPS stimulation compared to the control group. This indicates that the TLR4/NF-κB signaling pathway was activated in RAW264.7 cells after LPS treatment. Pretreatment of cells with β-sitosterol inhibited these activation events.

3.6. *Effect of β-sitosterol on LXRs signaling pathway in LPS-stimulated RAW264.7 cells and LPS-induced ALI mouse model*

Recently, many evidences have indicated that LXRα is an important anti-inflammatory transcription factor that involved in the development of inflammatory diseases (Myhre et al., 2008; Zelcer & Tontonoz, 2006). In this study, RAW264.7 cells were pretreated with 10 μM GSK2033 (LXRs antagonist) together with BS for 12 h, and then co-cultured with 1 μg/mL LPS for another 12 h. Even though the mRNA expression of LXRα changed not much (Fig. 4A), the mRNA expression of ABCA1 increased significantly after 50 μM β-sitosterol treatment of RAW264.7 cells (Fig. 4B). The results of Western blot analysis indicated that a significant increase of ABCA1 protein expression after β-sitosterol intervention compared with the LPS group (Fig. 4C). The protein expression of ABCA1 was significantly inhibited after the combined intervention of BS and GSK2033, which indicated the ligand dependent activation of LXRs/ABCA1 pathway was involved. After the combined intervention of BS and GSK2033, the mRNA expressions of LXRα and ABCA1 were significantly inhibited, and the mRNA and protein secretion levels of corresponding inflammatory factors (IL-6 and TNF-α) were significantly reversed (Fig. 4D, E). In the lung tissue, the mRNA levels of LXRα and ABCA1 were significantly upregulated in the PS 150 mg/kg intervention group compared with the LPS group (Fig. 5A, B), as well as the ABCA1 protein levels were significantly increased (Fig. 5C). These results suggested that phytosterols intervention can exert its anti-inflammatory effect partly by activating LXRs/ABCA1 pathway.

4. Discussion

In this study, we evaluated the anti-inflammatory effects of phytosterols in a mouse model of LPS-induced ALI in vivo. We demonstrated

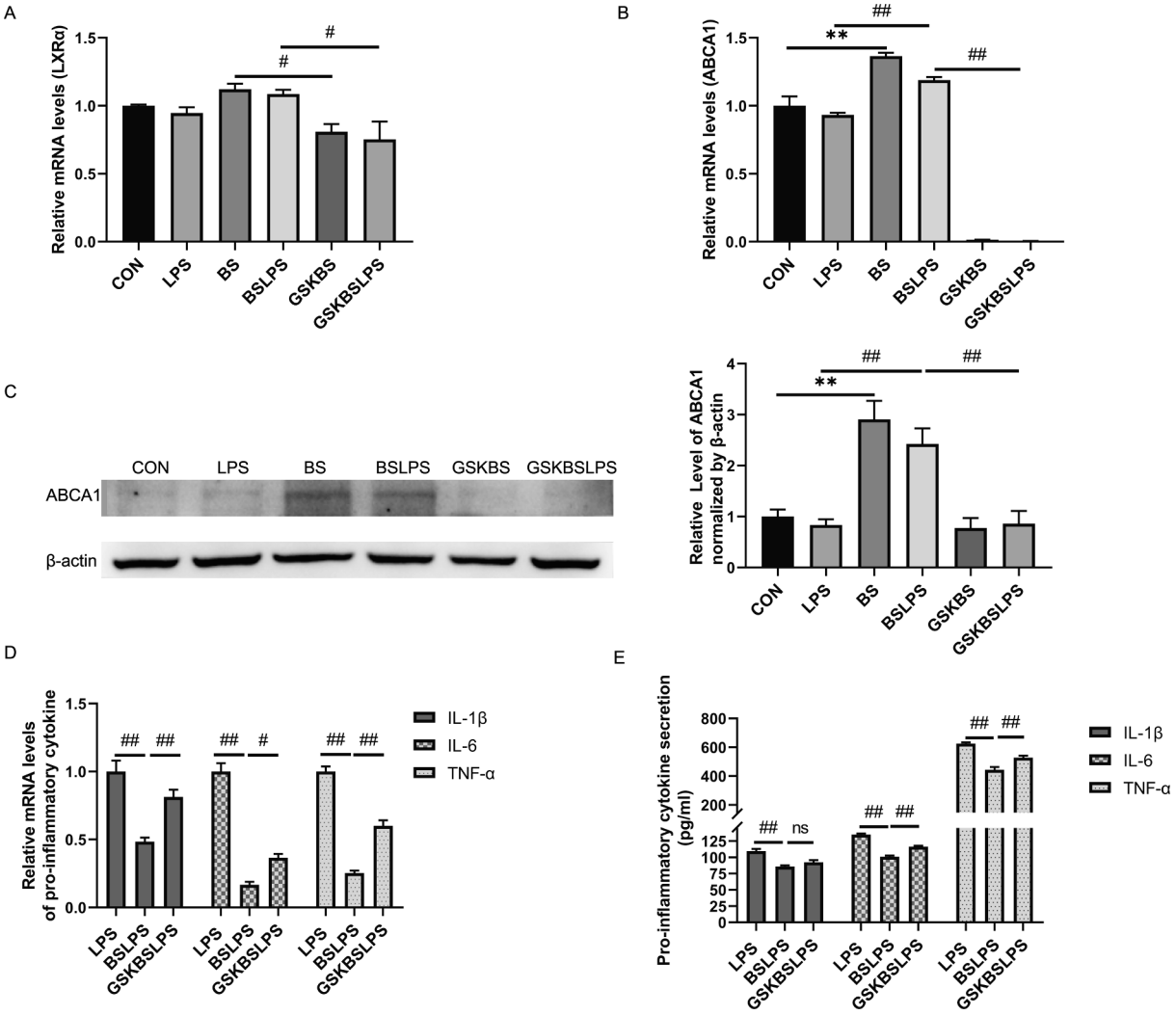

Fig. 4. Effect of LXR antagonist GSK2033 treatment on the mRNA / protein expression of LXRα, ABCA1, IL-1β, IL-6 and TNF-α. RAW264.7 cells were pre-treated with 50 μM β- sitosterol alone or combine with LXR antagonist GSK2033 (10 μM) for 12 h and then treated with LPS (1 μg/mL) for another 12 h. A-B. RT-PCR analysis of LXRα (A), ABCA1 (B) mRNA expression in RAW264.7 cells. C. Western blot analysis of ABCA1 proteins in RAW264.7 cells. The quantitative densitometric analysis normalized against β-actin were shown on the right. D. RT-PCR analysis of pro-inflammatory cytokines IL-1β, and IL-6, TNF-α mRNA expression in RAW264.7 cells. E. ELISA measurements of pro-inflammatory cytokines IL-1β, and IL-6, TNF-α protein expression in RAW264.7 cells. Data are expressed as mean ± SEM (n = 3, * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus LPS group). GSK: GSK2033.

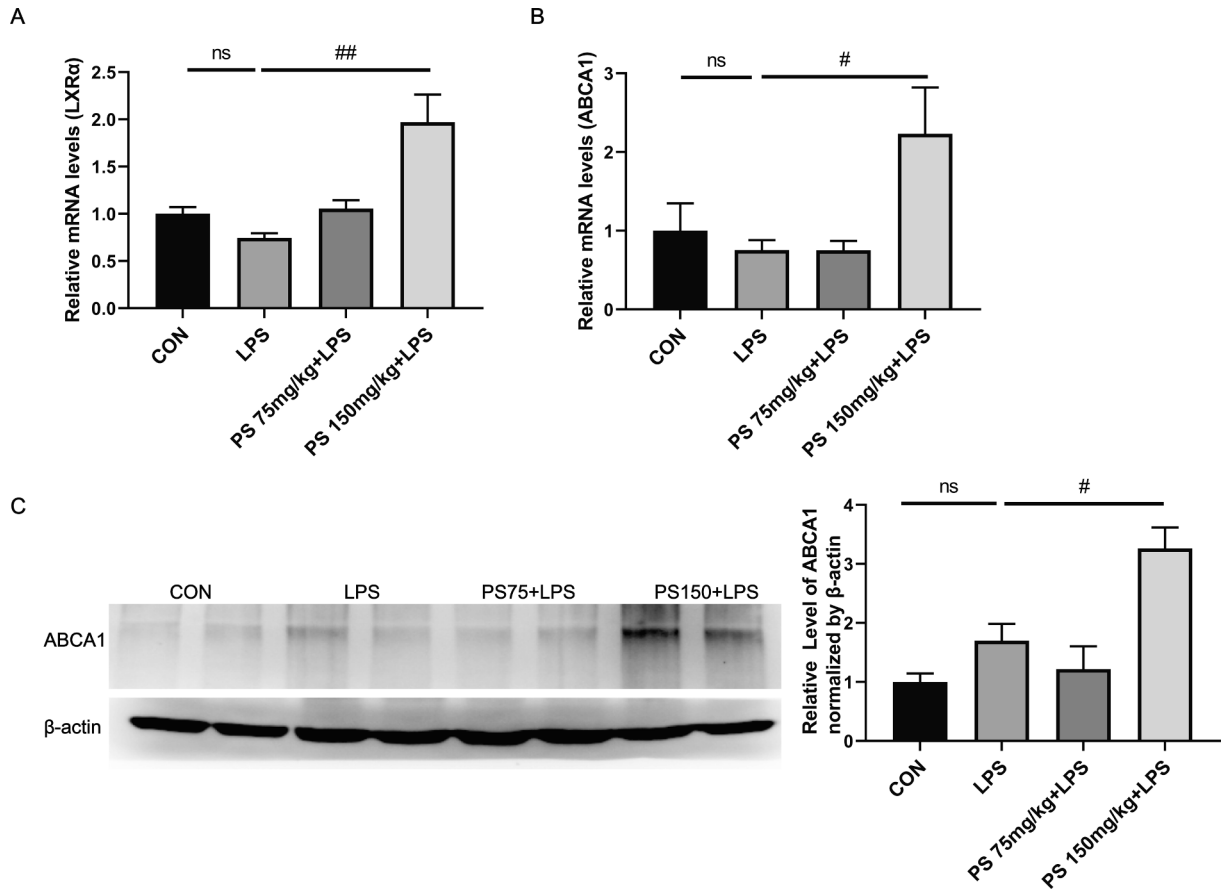

Fig. 5. Effect of phytosterols (PS) on the mRNA or/and protein expression of LXRα, ABCA1 in C57BL/6J mice. A-B: RT-PCR analysis of LXRα (A), ABCA1 (B) mRNA in the lung tissue of LPS-induced ALI mice. C. Western blot analysis of ABCA1 proteins in the lung tissue of LPS-induced ALI mice. Their quantitative densitometric analysis normalized against β-actin were shown on the right. Data are expressed as mean ± SEM (n = 3, * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus LPS group). PS 75: PS 75 mg/kg; PS 150: PS 150 mg/kg.

that pre-intervention of phytosterols attenuated lung inflammatory injury after LPS stimulation, as evidenced by a decrease in BALF total cell count, total protein content, and a reduction in lung histological damage (Fig. 2A, 2B, 1A). In addition, we found that PS pre-treatment significantly inhibited the release of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the LPS-induced ALI mouse model (Fig. 2C).

According to previous literature, the anti-inflammatory activity of phytosterols is associated with the MAPK pathway and ERK phosphorylation (Chentouh, Fitting, & Cavaillon, 2018; Yuan, Zhang, Shen, Jia, & Xie, 2019). Ergosterol has been demonstrated by reducing pro-inflammatory cytokines (TNF-α, IL-6) to attenuate the LPS-induced inflammatory response (Yuan et al., 2019). β-Sitosterol has been shown to repress the STAT1 pathway and NF-κB translocation, two pro-inflammatory signaling pathways that are supposed to be mediated by the activation of the tyrosine phosphatase SHP-1 (Valerio & Awad, 2011). Stigmasterol has been described to have anti-osteoarthritic properties, inhibiting pro-inflammatory and matrix degradation mediators involved in cartilage degradation, partially through the NF-κB pathway (Gabay et al., 2010). Stigmasterol has also been reported to reduce the fever response, pulmonary inflammation in LPS-induced innate immune responses in mice (Antwi, Obiri, Osafo, Forkuo, & Essel, 2017).

Although there have been some studies on the anti-inflammatory aspects of phytosterols, their specific anti-inflammatory effect could not be fully explained by mechanisms afore mentioned and needs to be further elucidated. LXRs are nuclear receptors which are involved in the regulation of lipid metabolism. As an important regulator of macrophage cholesterol efflux, activation of LXRs leads to improved plasma lipoprotein status by mobilizing reverse cholesterol transport (Repa et al., 2000; Venkateswaran et al., 2000). LXRs also appear to be important regulators of anti-inflammatory responses. Joseph and others demonstrated that activation of LXRs inhibited the induction of pro-inflammatory gene expression following LPS stimulation or bacterial infection (S. B. Joseph, Castrillo, Laffitte, Mangelsdorf, & Tontonoz, 2003). LXRs can suppress inflammatory cytokines, including iNOS, COX2, IL-6 and IL-1β, and MMP-9 etc. (Xue et al., 2014).

Phytosterols were reported to activate LXRs, and the LXR-activating potential of the phytosterols correlated positively with ABCA1 mRNA expression, which is one of the known LXR target genes (Naik et al., 2006). In this study, we found that pre-treatment of β-sitosterol could reverse the pro-inflammatory cytokine levels in LPS-stimulated RAW264.7 cells partially by activating the LXRs/ABCA1 pathway and affecting the activation of the TLR4/NF-κB pathway (Figs. 3, 4). When RAW264.7 cells were treated with LXRs inhibitor GSK2033, the expression of ABCA1 decreased and the pro-inflammatory factor-lowering effect of β-sitosterol was tampered. It is thus clear that β-sitosterol exerts anti-inflammatory effects at least partially through activating LXRs/ABCA1 signaling pathway. The anti-inflammatory effect of phytosterols mixture was also verified in the LPS-stimulated RAW264.7 cells (Supplementary Fig. S3).

The involvement of LXRs/ABCA1 pathway in the anti-inflammatory of phytosterols was also clarified in vivo. The mRNA levels of LXRα and ABCA1 in lung tissue were significantly upregulated in the PS 150 mg/kg group compared to the LPS group, as well as the ABCA1 protein levels were significantly increased (Fig. 5), which consistent with the results from in vitro experiments that pretreatment of RAW264.7 cells with

β-sitosterol.

Phytosterols are a kind of natural plant active substance, mainly found in nuts, vegetable oil, seeds, cereals and legumes. The dietary phytosterols intake is around 0.2–0.5 g/day in general population (Andersson et al., 2004), and the recommended daily intake dose of phytosterols proposed by the Chinese Nutrition Society is 0.9 g/d (Society, 2014). Meanwhile, the European Commission regulation recommends a daily intake of 1.5–3 g of plant sterols/stanols to obtain cholesterol-lowering effects (Yang et al., 2019). In the present study, the supplementation of 150 mg/kg in mice imitate the recommended daily intake of 0.9 g phytosterol in human beings, which activated LXRs/ABCA1 pathway and showed much stronger anti-inflammatory effects compared to the dosage of 75 mg/kg (Fig. 2C, Fig. 5).

5. Conclusions

This study examined the ameliorative effects of phytosterols on LPS-induced ALI in mice, and the results suggested that phytosterols exert anti-inflammatory effects at least partially through activating LXRs/ABCA1 signaling pathway. Further studies focusing on the therapeutic effect of phytosterols in ALI treatment is promising.

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Data Availability Statement

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Ethics Statement

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Center of Experimental Animals of Chongqing Medical University (protocol code: 2021008).

CRediT authorship contribution statement

Daxue He: Methodology, Data curation, Writing – original draft, Validation. **Shengquan Wang:** Methodology, Validation. **Gaofeng Fang:** Data curation. **Qian Zhu:** Methodology, Data curation. **Jingjing Wu:** Methodology. **Jianling Li:** Methodology. **Dan Shi:** Supervision. **Xuemei Lian:** Conceptualization, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.104966.

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