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ORIGINAL ARTICLE

Esculetin Ameliorates Lipopolysaccharide-Induced Acute Lung Injury in Mice Via Modulation of the AKT/ERK/NF-κB and RORγt/IL-17 Pathways

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Abstract— Esculetin, a coumarin derivative from various natural plants, has an anti-inflammatory property. In the present study, we examined if esculetin has any salutary effects against lipopolysaccharide (LPS)-induced acute lung injury (ALI) in mice. Acute lung injury (ALI) was induced *via* the intratracheal administration of LPS, and esculetin (20 and 40 mg/kg) was given intraperitoneally 30 min before LPS challenge. After 6 h of LPS administration, lung tissues were collected for analysis. Pretreatment with esculetin significantly attenuated histopathological changes, inflammatory cell infiltration, and production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, in the lung tissue. Furthermore, esculetin inhibited the protein kinase B (AKT), extracellular signal-regulated kinase (ERK), and nuclear factor-kappa B (NF-κB) pathways and downregulated the expression of RORγt and IL-17 in LPS-induced ALI. Our results indicated that esculetin possesses anti-inflammatory and protective effects against LPS-induced ALI *via* inhibition of the AKT/ERK/NF-κB and RORγt/IL-17 pathways.

KEY WORDS: esculetin; LPS; lung injury; AKT/ERK/NF-κB; RORγt/IL-17.

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), which is its more severe form, are widespread inflammatory processes in the lung, which could result in pulmonary interstitial edema, deteriorated lung compliance, and subsequent severe hypoxemia [1]. Despite extensive advances in supportive care, including ventilatory and non-ventilatory strategies, the mortality rate of ALI remains quite high at approximately 40% [2]. The pathogenesis of ALI is acute inflammatory response characterized by the accumulation and activation of inflammatory cells, secretion of pro-inflammatory cytokines and chemokines, production of reactive oxygen species (ROS), and subsequent death of pulmonary cells [3–6]. Accordingly, several studies have shown that anti-inflammatory mediators or anti-inflammatory drugs could treat early-

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stage ALI [7–9]. Lipopolysaccharide (LPS), a major component of the outer membrane present in gram-negative bacteria [10], is an extremely strong stimulator of the innate immunity system [11] and has been often used to induce ALI in several animal models [12].

Nuclear factor-kappa B (NF-κB), a key nuclear transcription factor, is an important regulator of inflammatory processes and immune responses [13]. Previous studies have shown that NF-κB plays a vital role in the pathogenesis of ALI/ARDS [14, 15]. The activation of NF-κB induced by LPS is required for the maximal transcription of various inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, in ALI [16]. Excessive production of these pro-inflammatory cytokines is significantly associated with the severity of ALI [17]. Moreover, the mitogen-activated protein kinase (MAPK) signaling pathway, which regulates the intracellular signal transduction to extracellular stimuli, plays an important role in the expression of pro-inflammatory mediators and activation of NF-κB [18]. The MAPK families, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, mediate inflammatory processes and immune responses in LPS-induced ALI [19]. A recent study has shown that the inhibition of MAPK and NF-κB activity could attenuate inflammatory responses during LPS-induced ALI [20]. Furthermore, growing evidence has indicated that the protein kinase B (AKT) pathway, an important cellular signaling that regulates numerous cellular processes, plays a key role in cell survival and inflammatory processes [21]. In addition, another recent study has revealed that AKT could regulate NF-κB activity [22].

IL-17, a signature cytokine of the T help 17 (Th17) cell subset, is a pro-inflammatory cytokine that plays a crucial role in the recruitment and activation of neutrophils [23]. Apart from Th17 cells, IL-17 is also produced by innate immune cells, such as natural killer (NK) cells, NKT cells, and γδ T cells [24]. Moreover, previous study has shown that IL-17 facilitates neutrophil recruitment and accumulation by upregulating the expression of chemokines in pulmonary infection [25]. Moreover, IL-17 has been involved in various inflammatory pulmonary diseases, including autoimmune diseases, asthma, and infection [26–29]. The overexpression of IL-17 could lead to the upregulation of inflammatory cytokines and chemokines, which recruit and activate inflammatory cells in the lung [30]. A recent study has shown that anti-IL-17 can protect against LPS-induced ALI mediated by the decreased expression of inflammatory cytokines [31].

Esculetin (6,7-dihydroxycoumarin) is a coumarin derivative found in the products of various natural plants, such as *Artemisia capillaris*, *Ceratostigma willmottianum*, and *Euphorbia lathyris* [32]. Esculetin possesses diverse biological and pharmacological properties, including anti-asthma, anti-inflammatory, anti-nociceptive, antioxidative, antitumor, and antiviral activities [33–35]. Although esculetin has a protective effect against LPS-induced lung injury *via* partly inhibiting the NF-κB pathway, the detailed mechanism about the protective role of esculetin had not been investigated in a previous study [36]. We hypothesized that esculetin protects against LPS-induced ALI through MAPK/NF-κB and IL-17 pathways. Therefore, the present study aimed to investigate the protective role of esculetin against LPS-induced lung injury *via* the modulation of the AKT, MAPK, NF-κB, and IL-17 pathways. Importantly, our study may provide future directions for ALI treatment in humans and animals.

MATERIALS AND METHODS

Animals

Adult male C57BL/6C (B6) mice (20–25 g, 8–10 weeks old) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). All mice were housed in an environmentally controlled facility with a 12-h light and dark cycle and were allowed ad libitum access to sterile food and clean water. All animal experimental procedures were performed in accordance with the guidelines of the *Animal Welfare Act* and *e Guide for Care and Use of Laboratory Animals* from the National Institute of Health. The protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital.

Experimental Model and Treatments

The mice were randomly separated into six groups, each with 5–6 mice: control group (phosphate-buffered saline [PBS]), esculetin alone group, LPS group, dexamethasone (Dex) + LPS group, esculetin 20 mg/kg + LPS group, and esculetin 40 mg/kg + LPS group. Dex (5 mg/kg), esculetin (20 and 40 mg/kg) [36], and equal volume of saline were administered intraperitoneally 30 min before LPS challenge. Moreover, 2.5 μg/g of LPS in 50 μl PBS was injected to the trachea to induce lung injury [37]. After 6 h of LPS treatment, the mice were anesthetized and sacrificed, and the lung tissues were harvested for further analyses.

Histology and Immunohistochemistry

The lungs were excised and fixed in 4% paraformaldehyde and embedded in paraffin. For histological examination, sections of 4-μm thickness were stained using hematoxylin and eosin (H&E). For immunohistochemical staining, the sections were incubated in primary antibody against Ly-6G (neutrophil; 1:500; BD Biosciences Pharmingen, San Diego, CA, USA), Mac-2 (macrophage; 1:500; eBioscience, Inc., San Diego, CA, USA), RORγt (1:500; Thermo Fisher Scientific, Inc. Waltham, MA, USA), or IL-17 (1:500; Abcam, Cambridge, MA, USA). After rinsing with PBS, the sections were incubated in biotin and streptavidin horseradish peroxidase (HRP)-conjugated secondary antibody (IHC Select; Millipore). Finally, after washing with PBS, the immune complex was visualized using 3,3′-diamino-benzidine chromogen reagent (IHC Select; Millipore).

Measurement of TNF-α, IL-1β, IL-6, and IL-23 Levels in the Lung

For evaluating immune response at the site of inflammation, we measured the levels of TNF-α, IL-1β, IL-6, and IL-23. The lung tissues were homogenized in lysis buffer (20 mM HEPES, 1 mM 2-mercaptoethanol [2-ME], 3 mM $MgCl_2$, 150 mM NaCl, 1 M dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.25 μg leupeptin, 0.05 μg pepstatin A, and 0.01 μg aprotinin) on ice. Next, to obtain the supernatants, tissue homogenates were centrifuged at 12000 g for 10 min at 4 °C. Finally, the levels of TNF-α, IL-1β, IL-6, and IL-23 were measured in accordance with the manufacturer's instructions with absorbance at 450 nm.

Western Blotting

The lung tissues were homogenized in lysis buffer (20 mM HEPES, 1 mM 2-ME, 3 mM $MgCl_2$, 150 mM NaCl, 1 M DTT, 0.1 mM PMSF, 0.25 μg leupeptin, 0.05 μg pepstatin A, and 0.01 μg aprotinin). Then, the homogenized tissues were sonicated for 15 s and centrifuged at 12000 g for 10 min at 4 °C. Moreover, 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to separate the protein, followed by electroblotting onto polyvinylidene fluoride membrane. After blocking in 5% fat-free milk solution for 1 h and rinsing thrice with Tris-buffer (1% Tween-20), the membrane was incubated with specific primary antibody against ERK, JNK, p38, AKT, NF-κB, RORγt, phospho-ERK, phospho-JNK, phosphon-p38, phospho-AKT, and phospho-NF-κB (1:1000; Cell Signaling Technology, MA, USA) overnight at 4 °C. After washing and incubation with HRP-conjugated secondary antibody (Cell Signaling Technology, MA, USA) for 1 h at room temperature, the final immune complexes were visualized using the enhanced chemiluminescence system. The antibody specific to β-actin (Proteintech Group, Inc., Chicago, IL, USA) was used to confirm the loading accuracy.

Statistical Analysis

All data were presented as mean ± standard error of mean (each group with 5–6 mice). One-way analysis of variance and Tukey's multiple comparison tests were used to analyze the results. The Prism 6.0 Software (GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analyses. A p value < 0.05 was considered statistically significant.

RESULTS

Effects of Esculetin on Histological Changes in the Lung

To evaluate the effect of esculetin on ALI, the lungs of mice were harvested 6 h after the administration of LPS. Then, the lung tissues were fixed and stained with H&E. The LPS group showed extensive histopathological changes, such as alveolar wall thickening, interstitial edema, and pulmonary congestion, compared to the control group (Fig. 1). These histopathological findings were ameliorated by pretreatment with Dex (5 mg/kg) or esculetin (20 and 40 mg/kg) 30 min before LPS challenge (Fig. 1). These results indicated that esculetin could ameliorate LPS-induced lung injury.

Effects of Esculetin on the Infiltration of Neutrophil in the Lung

To investigate the infiltration and accumulation of neutrophils in LPS-induced ALI, lung sections were immunohistochemically stained with Ly6G antibody, a specific marker for granulocyte. The LPS group presented with dominant neutrophil infiltration in the lung tissue compared with the control group. The Dex (5 mg/kg) and esculetin (20 and 40 mg/kg) pretreated groups had significantly reduced neutrophil accumulation in the lung tissue compared to the LPS group (Fig. 2).

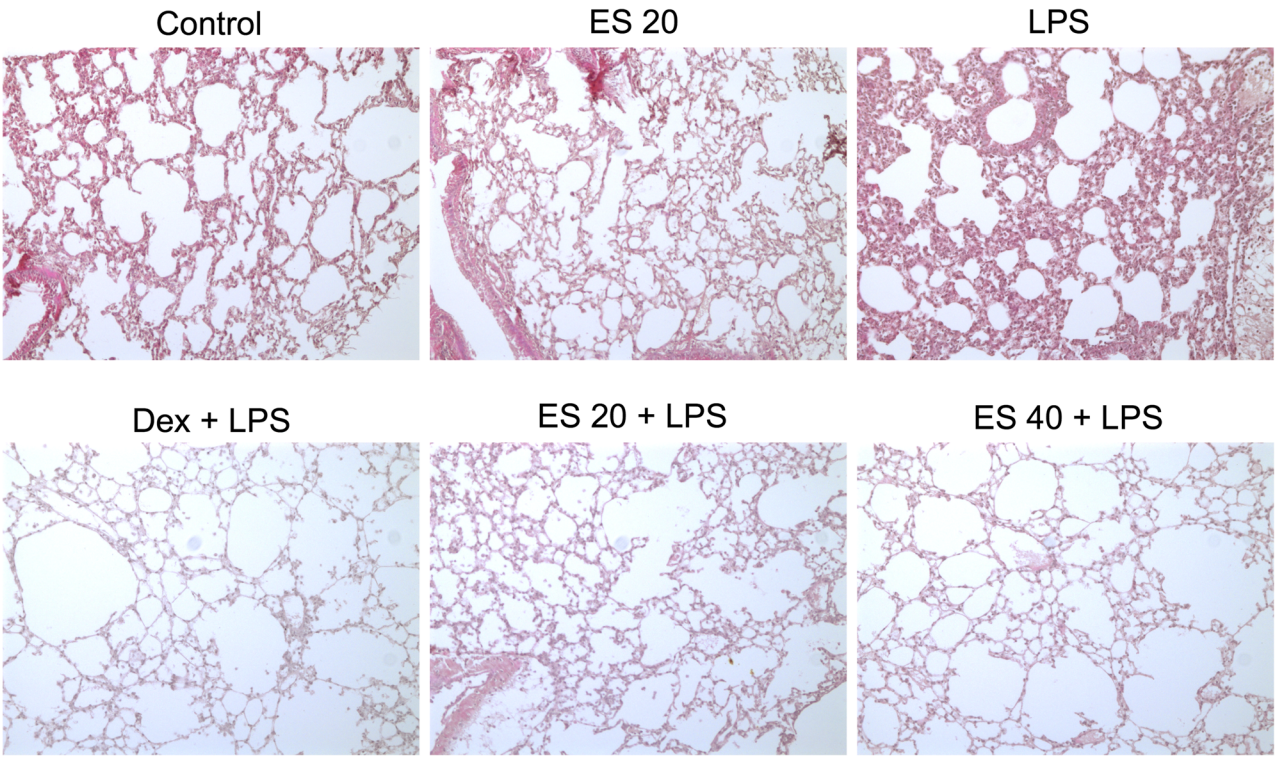

Fig. 1. Effects of esculetin on histological changes in the lung induced by LPS. Dex (5 mg/kg), esculetin (ES, 20 and 40 mg/kg), or equal volume of saline was administered intraperitoneally 30 min before intratracheal LPS challenge. Mice were sacrificed after 6 h of LPS challenge, and lungs were harvested for H&E staining. Representative histological changes of the lung tissues obtained from six groups. (100 × magnifications are shown).

Effects of Esculetin on the Infiltration of Macrophages in the Lung

To investigate the infiltration and accumulation of macrophages in LPS-induced ALI, lung sections were immunohistochemically stained with Mac-2 antibody, a specific marker for macrophage. The LPS group presented with obvious macrophage infiltration in the lung tissue compared with the control group. The Dex (5 mg/kg) and esculetin (20 and 40 mg/kg) pretreated groups had significantly reduced macrophage accumulation in the lung tissue compared with the LPS group (Fig. 3).

Effects of Esculetin on Pneumonic TNF-α, IL-1β, IL-6, and IL-23 Levels

To evaluate the effects of esculetin on the expression of pro-inflammatory cytokine in LPS-induced lung injury, we measured the levels of TNF-α, IL-1β, IL-6, and IL-23 in the lung tissues using ELISA. The pulmonary levels of TNF-α, IL-1β, IL-6, and IL-23 were significantly elevated in the LPS group than in the control group. No differences were observed in the levels of these pro-inflammatory cytokines between the control and esculetin alone groups. About 30 min prior to the administration of LPS, pretreatment with Dex (5 mg/kg) significantly decreased the pulmonary TNF-α, IL-1β, IL-6, and IL-23 levels ($p < 0.005$, 0.005, 0.005, and 0.05, respectively) than LPS group. Moreover, pretreatment with esculetin (20 mg/kg) attenuated pulmonary TNF-α, IL-1β, and IL-6 levels ($p < 0.01$, $p < 0.01$, $p < 0.005$, respectively) than LPS group. Furthermore, pretreatment with a higher dose of esculetin (40 mg/kg) significantly attenuated pulmonary TNF-α, IL-1β, IL-6, and IL-23 levels ($p < 0.01$, 0.005, 0.005, and 0.05, respectively) than LPS group (Fig. 4). These results indicated that pretreatment with esculetin could reduce the production and expression of pro-inflammatory cytokines in LPS-induced lung injury.

Effects of Esculetin on ERK, JNK, p38, AKT, and NF-κB Expression and Phosphorylation in the Lung

We further investigated the AKT, MAPK family protein, and NF-κB expression and phosphorylation in the lung tissues induced by LPS. No significant differences

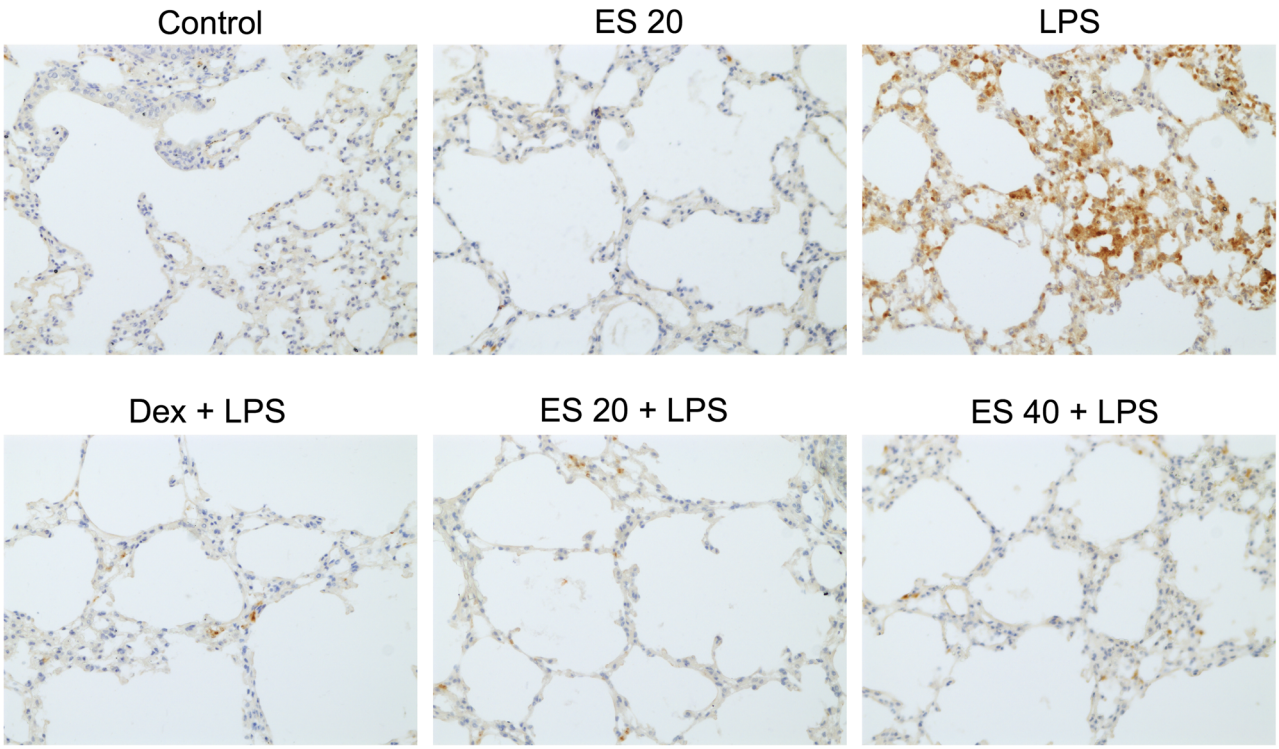

Fig. 2. Effects of esculetin on neutrophil infiltration in the lung induced by LPS. Dex (5 mg/kg), esculetin (ES, 20 and 40 mg/kg), or equal volume of saline was administered intraperitoneally 30 min before intratracheal LPS challenge. Mice were sacrificed after 6 h of LPS challenge and lungs were harvested. The lung sections were immunostained with Ly6G antibody (brown). Representative immunohistochemical staining of the lung tissues obtained from six groups. (200 × magnifications are shown).

were observed in pulmonary AKT, ERK, JNK, p38, and NF-κB protein expressions between the control and esculetin alone groups. The AKT, ERK, and NF-κB activity, as determined by its phosphorylation, increased in the LPS group compared with the control group. Moreover, pretreatment with Dex (5 mg/kg) or esculetin (20 and 40 mg/kg) 30 min before the administration of LPS significantly decreased the phosphorylated AKT, ERK, and NF-κB expressions compared with the LPS group (Fig. 5). However, no significant differences were observed in the pulmonary JNK and p38 protein expressions and phosphorylation among the six groups. Taken together, our data indicated that pretreatment with esculetin could attenuate the AKT, ERK, and NF-κB phosphorylation and activation in the lung in LPS-induced ALI.

Effects of Esculetin on RORγt and IL-17 Expression in the Lung

To investigate the possible anti-inflammatory mechanism of esculetin in LPS-induced ALI, lung sections were immunohistochemically stained with RORγt and IL-17 antibody. The LPS group showed significant RORγt and IL-17 expressions in the lung tissues compared with the control group. Moreover, the Dex (5 mg/kg) and esculetin (20 and 40 mg/kg) pretreated groups showed significantly decreased RORγt and IL-17 expressions in the lung tissues compared to the LPS group (Figs. 6A, 7). We further examined the RORγt expression in the lung tissues using the western blotting assay. The expression of ROR significantly increased in the LPS group compared with the control group. Furthermore, pretreatment with Dex (5 mg/kg) or esculetin (20 and 40 mg/kg) 30 min before the administration of LPS decreased the RORγt expression compared to the LPS group (Fig. 6B) Taken together, our result indicated that pretreatment with esculetin could reduce the expression of RORγt and IL-17 in the lung in LPS-induced ALI.

DISCUSSION

In the current study, we investigated the protective effect of esculetin on LPS-induced ALI in mice. Our data

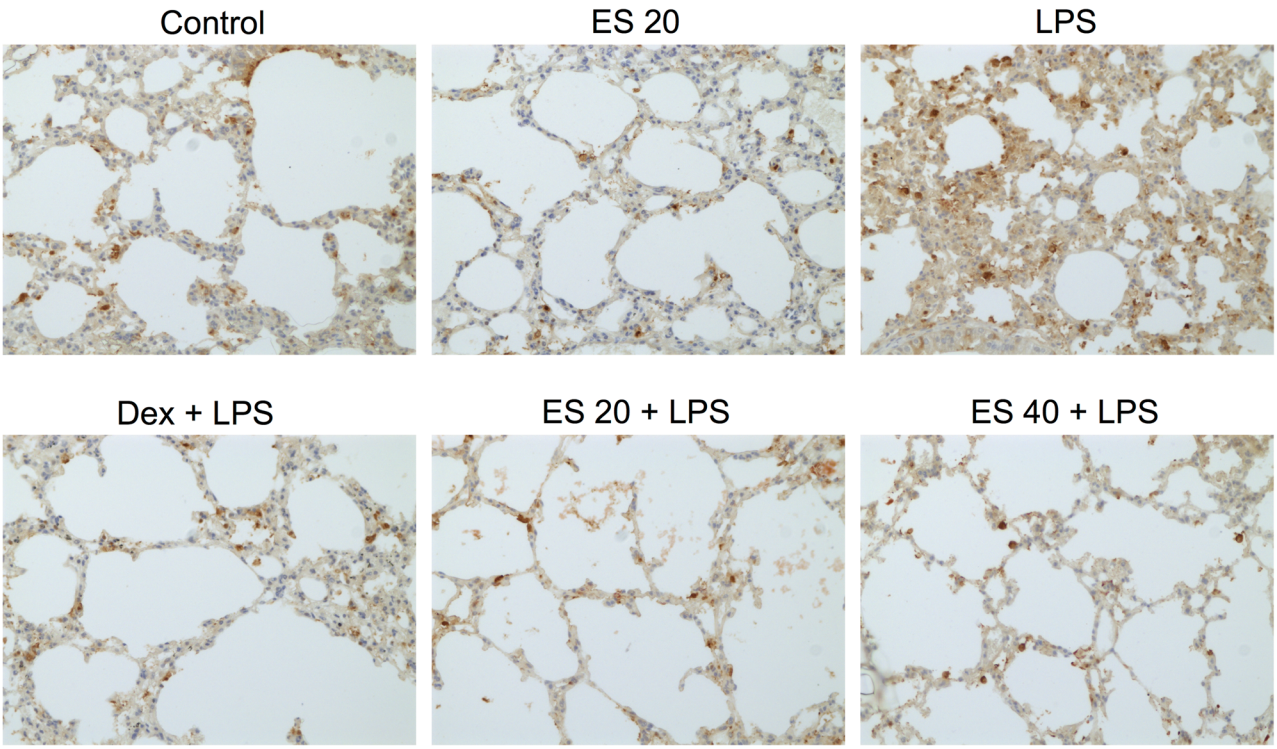

Fig. 3. Effects of esculetin on macrophage infiltration in the lung induced by LPS. Dex (5 mg/kg), esculetin (ES, 20 and 40 mg/kg), or equal volume of saline was administered intraperitoneally 30 min before intratracheal LPS challenge. Mice were sacrificed after 6 h of LPS challenge and lungs were harvested. The lung sections were immunostained with Mac-2 antibody (brown). Representative immunohistochemical staining of the lung tissues obtained from six groups. (200 × magnifications are shown).

showed that pretreatment with esculetin significantly attenuated LPS-induced histopathological changes, decreased the infiltration of inflammatory cells, and reduced the production of pro-inflammatory cytokines (*i.e.*, TNF-α, IL-1β, and IL-6) in the lung tissue. Moreover, pretreatment with esculetin suppressed the phosphorylation of AKT, ERK, and NF-κB proteins and inhibited the expression of RORγt and IL-17 in the LPS-induced lung tissue. Taken together, our results indicated that esculetin has protective effects against LPS-induced ALI in mice, and the anti-inflammatory mechanism might involve the inhibition of AKT/ERK/NF-κB and RORγt/IL-17 pathways.

ALI is characterized by acute inflammatory reaction, infiltration of inflammatory cells, release of exudative secretions, pulmonary cell death, and subsequent parenchyma injury [5, 6, 38]. Growing evidence has shown that neutrophils are essential in initiating and aggravating inflammatory responses and pathological processes [39]. The inhibition of neutrophil recruitment could attenuate LPS-induced ALI by reducing the production of ROS and the secretion of pro-inflammatory cytokines [40]. Moreover, macrophages are also crucial in immune reactions and inflammatory processes in the acute phase of ALI mediated by attenuating neutrophil recruitment and reducing pro-inflammatory mediators in the lungs [41]. Based on the histopathological findings of the current study, pretreatment with esculetin significantly attenuated pulmonary parenchyma injury and inhibited neutrophil and macrophage infiltration and accumulation in the lungs. In addition, pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) are secreted from active pulmonary and immune cells and are important in eliciting the inflammatory cascade in ALI, including inflammatory cell infiltration, histopathological changes, and severity of injury [17, 42]. Consistent with the previous literature, our results showed that esculetin has anti-inflammatory properties and pretreatment with esculetin significantly reduces the release and production of pro-inflammatory cytokines in LPS-induced ALI.

Accumulating evidence has shown that NF-κB, a crucial transcription factor of inflammation, plays a key role in the development and pathogenesis of ALI [15, 43]. The phosphorylation and activation of NF-κB contributes to the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [16, 22]. In this study, we found

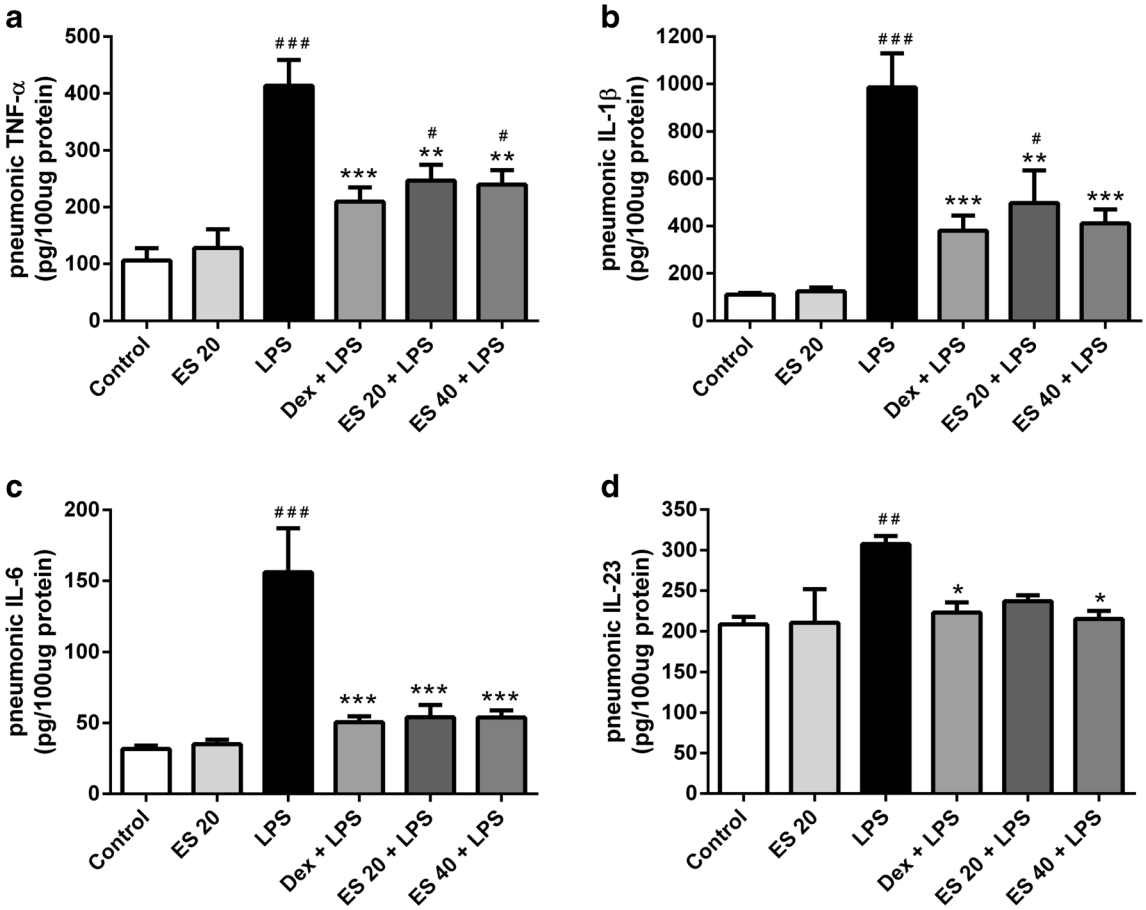

Fig. 4. Effects of esculetin on TNF-α (**a**), IL-1β (**b**), IL-6 (**c**), and IL-23 (**d**) levels in the lung induced by LPS. Dex (5 mg/kg), esculetin (ES, 20 and 40 mg/kg), or equal volume of saline was administered intraperitoneally 30 min before intratracheal LPS challenge. Mice were sacrificed after 6 h of LPS challenge and lungs were harvested for ELISA assay. All values are represented as mean ± SEM ($n = 6$ per group). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$ *vs.* the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ *vs.* the LPS group.

that pretreatment with esculetin obviously reduced the phosphorylation of NF-κB induced by LPS. Moreover, the MAPK protein family plays a key role in inducing the activation of NF-κB and the secretion of pro-inflammatory mediators, leading to inflammation and lung damage in ALI [18, 44]. The ERK, one of the MAPKs, is involved in various cellular processes, including oxidative stress, survival, apoptosis, and inflammatory process [45]. A previous study has shown that ERK signaling contributes to TNF-α production and plays an important role in ALI, whereas the inhibition of ERK provides protective effects against pulmonary inflammatory diseases [46]. Furthermore, a recent study has shown that the activation of the NF-κB pathway that is dependent on ERK can contribute in upregulating the gene of the pro-inflammatory cytokine [20]. In our study, pretreatment with esculetin 30 min prior to the administration of LPS can effectively attenuate the phosphorylation of ERK and NF-κB in the lung tissues, which indicates that esculetin suppresses the ERK and NF-κB activity, leading to the downregulation of pro-inflammatory cytokines. Taken together, our results indicated that pretreatment with esculetin reduces the secretion and production of pro-inflammatory cytokines *via* the inhibition of the ERK/NF-κB pathway.

AKT, an important signaling pathway involved in various cellular processes, is crucial in cell survival, proliferation, and inflammatory responses against extracellular stimuli [21, 47]. Moreover, recent studies have revealed that the inhibition of the AKT signaling ameliorated LPS-induced ALI by downregulating the NF-κB activity and reducing the production of ROS and pro-inflammatory cytokines [22, 48]. In this study, pretreatment with esculetin significantly reduced the phosphorylation of AKT in the lung tissues. Our results indicated that the protective

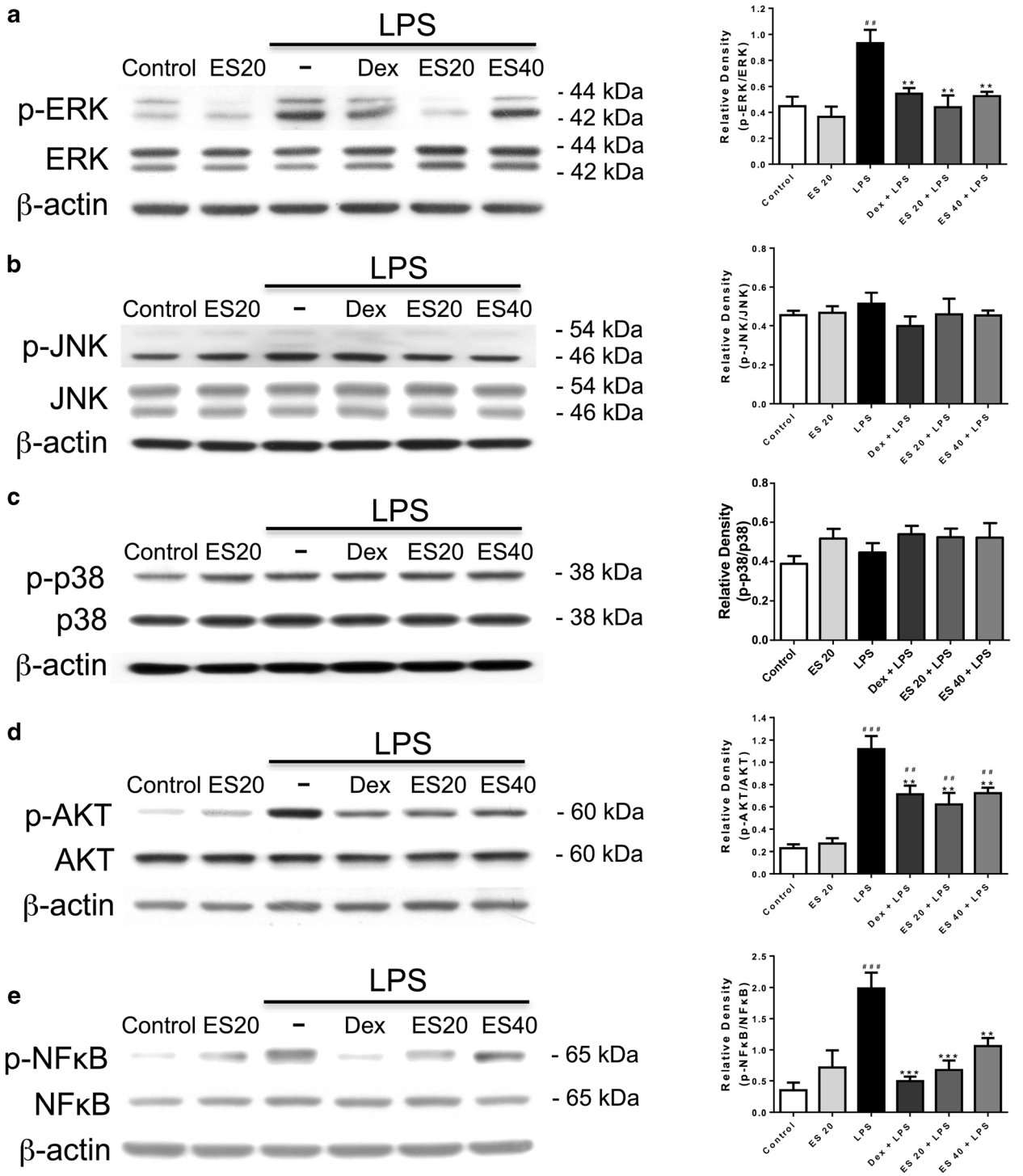

Fig. 5. Effects of esculetin on ERK (**a**), JNK (**b**), p38 (**c**), AKT (**d**), and NF-κB (**e**) expression and phosphorylation in the lung induced by LPS. Dex (5 mg/kg), esculetin (ES, 20 and 40 mg/kg), or equal volume of saline was administered intraperitoneally 30 min before intratracheal LPS challenge. Mice were sacrificed after 6 h of LPS challenge, and lungs were harvested for western blotting analysis. The bands were analyzed using densitometry. All values are represented as mean ± SEM ($n = 5$–6 per group). $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$ *vs.* the control group; $^{**}p < 0.01$, $^{***}p < 0.005$ *vs.* the LPS group.

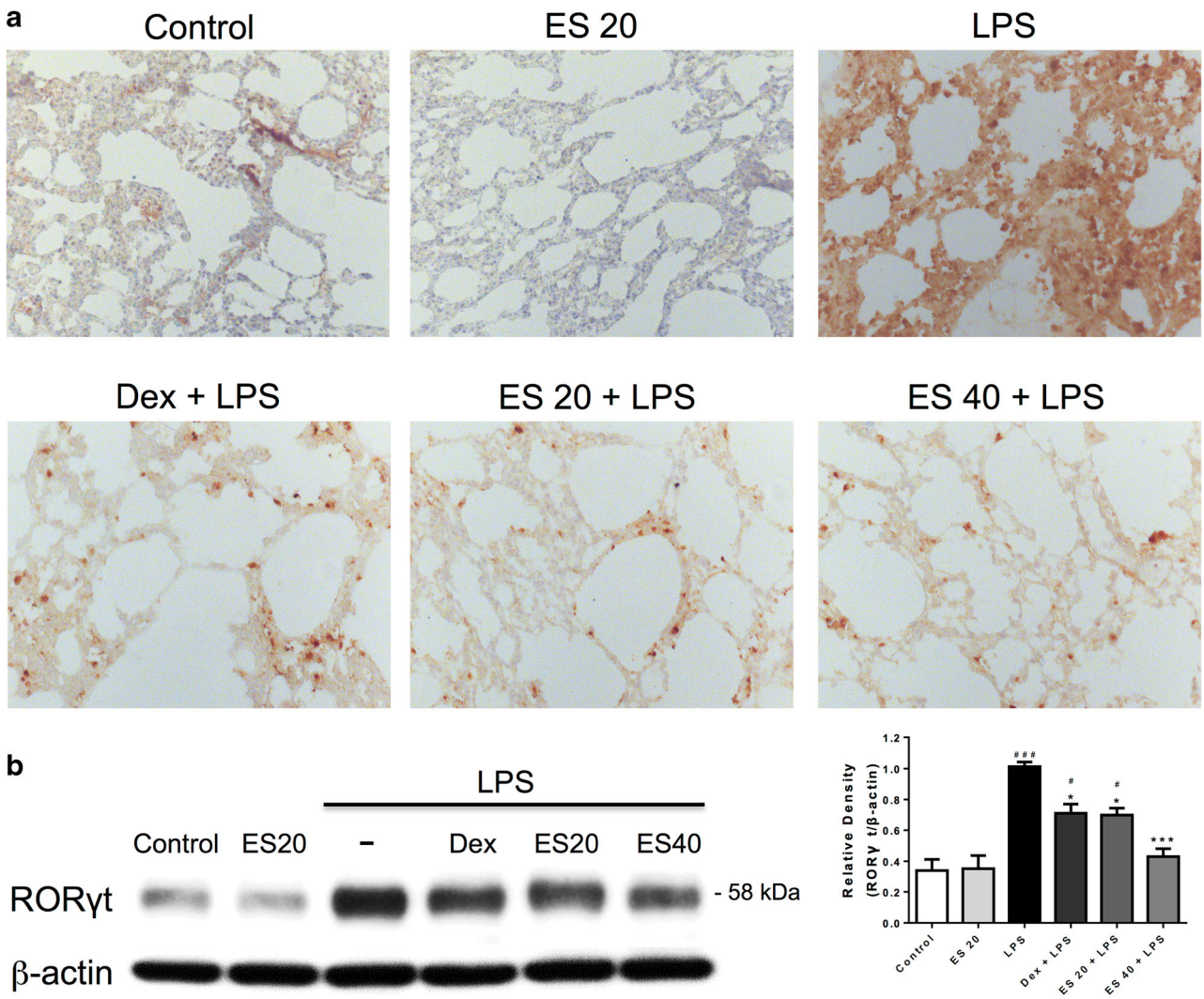

Fig. 6. Effects of esculetin on RORγt expression in the lung induced by LPS. Dex (5 mg/kg), esculetin (ES, 20 and 40 mg/kg), or equal volume of saline was administered intraperitoneally 30 min before intratracheal LPS challenge. Mice were sacrificed after 6 h of LPS challenge and lungs were harvested. (A) The lung sections were immunostained with RORγt antibody (brown). Representative immunohistochemical staining of the lung tissues obtained from six groups. (200 × magnifications are shown). (B) Protein extracted from the lung tissues analyzed by western blotting. The bands were analyzed using densitometry. All values are represented as mean ± SEM (n = 5–6 per group). $^{\#}p < 0.05$, $^{\#\#\#}p < 0.005$ *vs.* the control group; $^{*}p < 0.05$, $^{***}p < 0.005$ *vs.* the LPS group.

activity of esculetin might involve the suppression of the phosphorylation of AKT and the subsequent NF-κB pathway.

IL-17, a pro-inflammatory cytokine produced mainly by Th17, plays an essential role in the recruitment of neutrophils and immune responses against extracellular infection [23, 49]. Previous studies have also shown that IL-17 facilitates neutrophil recruitment and accumulation by upregulating the expression of chemokines in LPS-induced ALI [23, 50]. In the current study, we found that IL-17 was significantly expressed in the lungs of the LPS group *via* immunohistological staining; meanwhile, pretreatment with esculetin substantially reduced the expression of IL-17 in the lung tissues. Recent studies have also shown that neutralizing IL-17 with antibody could attenuate the severity of LPS-induced ALI by the downregulation of pro-inflammatory cytokines [30, 31]. To understand the detailed mechanism of the protective effects of esculetin, we further examined the expression of RORγt, one of the most important nuclear transcription factors in the differentiation of Th17 cells [51]. Growing evidence has demonstrated that RORγt-dependent IL-17A pathway is

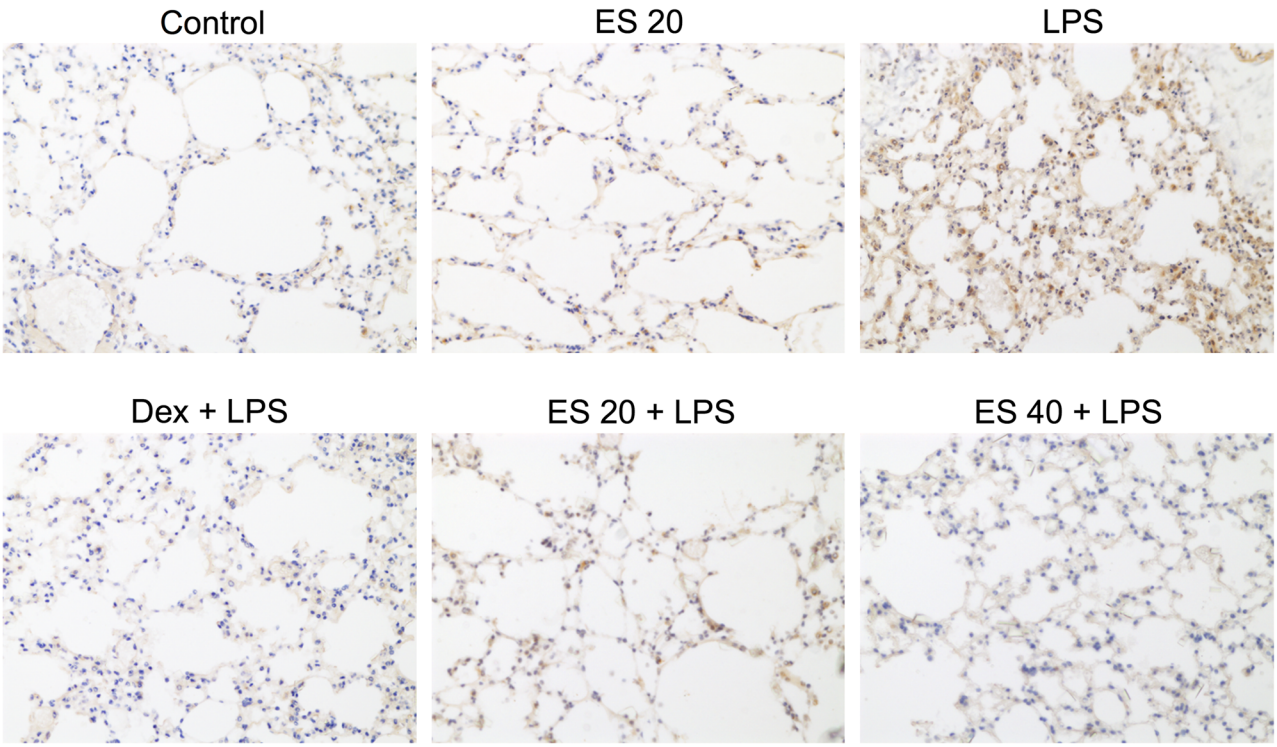

Fig. 7. Effects of esculetin on IL-17 expression in the lung induced by LPS. Dex (5 mg/kg), esculetin (ES, 20 and 40 mg/kg), or equal volume of saline was administered intraperitoneally 30 min before intratracheal LPS challenge. Mice were sacrificed after 6 h of LPS challenge and lungs were harvested. The lung sections were immunostained with IL-17 antibody (brown). Representative immunohistochemical staining of the lung tissues obtained from six groups. (200 × magnifications are shown).

crucial for pathogenesis of intestinal and pulmonary inflammation [52, 53]. A previous study had shown that the level of IL-17 was significantly elevated and the degree of neutrophilic pulmonary inflammation was also enhanced following mycobacteria infection in RORγt-overexpressing mice [54]. Moreover, a recent study found that RORγt inverse agonist could reduce the expression of IL-17 and diminish the neutrophil infiltration in the lung in asthmatic mice model [55]. In the current study, the expression of RORγt in the LPS group was increased; meanwhile, pretreatment with esculetin significantly reduced the expression of RORγt in the lung tissues. Taken together, our data indicated that pretreatment with esculetin protects against LPS-induced ALI partly *via* the RORγt/IL-17 pathway.

In addition, a previous study has indicated that the AKT signaling pathway could enhance the nuclear translocation of RORγt and facilitate the differentiation of Th17 cells [56]. Recent studies have also shown that IL-23 facilitates the differentiation of Th17 cells and the IL-23/IL-17 axis plays a critical role in the pathogenesis of acute lung injury [57, 58]. Furthermore, another study has demonstrated that treatment with anti-IL-23 antibody could markedly attenuate airway hyperresponsiveness and pulmonary inflammatory cell infiltration *via* IL-17 pathway [59]. In our study, pretreatment with esculetin significantly reduced the level of IL-23, which indicated that esculetin might inhibit IL-23/IL-17 axis in LPS-induced ALI model. However, there are potential limitations for our study. Administration of esculetin 30 min before LPS challenge was chosen for the present study. It is unknown whether esculetin posttreatment has beneficial effects in LPS-induced ALI. To make clear this concern, we would add posttreatment experimental design and evaluate the application of esculetin for the treatment of ALI in our future studies.

In conclusion, esculetin has protective effects against LPS-induced ALI in mice by attenuating inflammatory immune cell infiltration and reducing the production of pro-inflammatory cytokines. The anti-inflammatory mechanism of esculetin might involve the inhibition of the AKT/ERK/NF-κB and RORγt/IL-17 pathways (Fig. 8). Therefore, esculetin may be a therapeutic agent for ALI. In the future, comprehensive studies must be conducted to validate the clinical applications of esculetin.

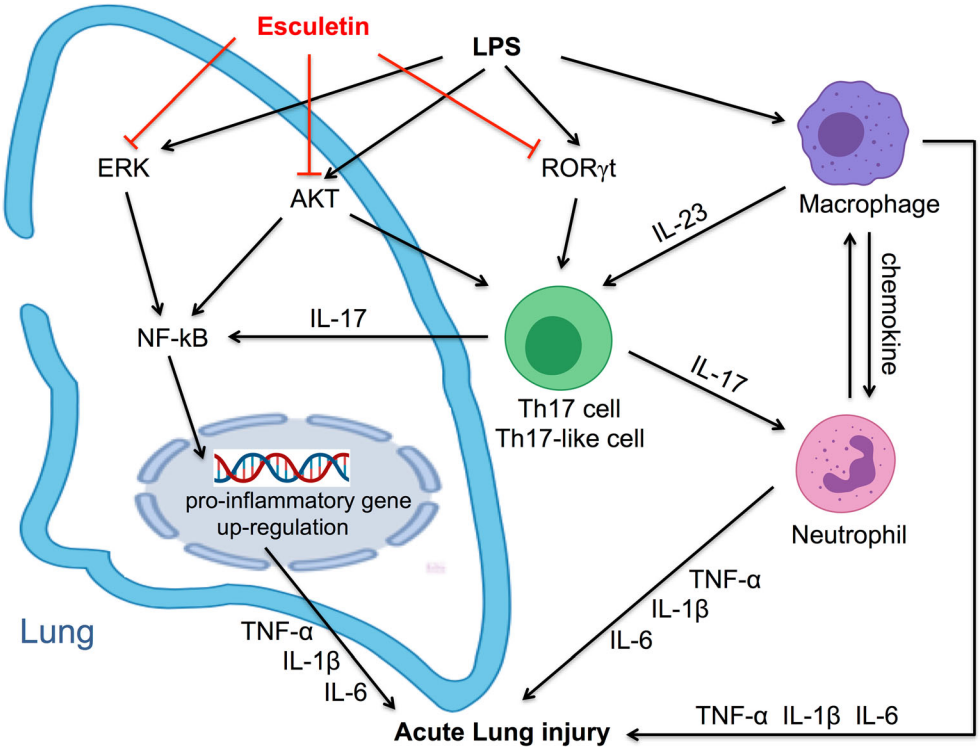

Fig. 8. Schematic summary for the protective and anti-inflammatory effects of esculetin on LPS-induced acute lung injury in mice. Esculetin pretreatment effectively protects against LPS-induced acute lung injury by reducing inflammatory responses *via* the inhibition of the AKT/ERK/NF-κB and RORγt/IL-17 pathways.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that there is no conflict of interests regarding the publication of this paper.

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