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•Research article•

Esculetin protects against early sepsis *via* attenuating inflammation by inhibiting NF-κB and STAT1/STAT3 signaling

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[ABSTRACT] Esculetin, a natural derivative from the traditional and widely-used Chinese medicinal herb *Cortex Fraxini*, has a variety of pharmacological effects, especially in anti-inflammation. However, it is not clear whether esculetin has a therapeutic effect on sepsis. This study aimed to investigate the anti-inflammatory and protective effects of esculetin on early sepsis. The results showed that the lung injury was significantly relieved with the treatment of esculetin, accompanied with the restrained production of inflammatory factors including IL-1*β*, IL-6, TNF-*α*, CCL2 and iNOS during the early phase of *E.coli*-induced sepsis. Of note, activation of NF-*κ*B and STAT1/STAT3 signals, the main upstream signals of many inflammatory factors, were attenuated by esculetin in both lung tissues from septic mice and LPS-stimulated macrophage. These findings suggested that the protection of esculetin against early sepsis should be related to its anti-inflammatory effect, which was at least partly due to its inhibition on NF-*κ*B and STAT1/STAT3 signaling pathway in macrophage. Thus, esculetin could serve as a potential therapeutic agent by rebalancing innate immune response in macrophage for the treatment of early sepsis.

[KEY WORDS] Esculetin; NF-*κ*B; STAT1; STAT3; Early sepsis
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Introduction

Sepsis, charaterized as life-threatening organ injury, is caused by a dysregulated host response to invasive pathogens [1]. The uncontrolled inflammatory cytokines, produced by excessive activation of the host innate immune system during pathogenic infections, are generally considered to be the major driving force in the development of sepsis [2]. In the early stage of sepsis, innate immune cells including macrophages/monocytes and neutrophils are activated by pathogen-associated molecular patterns (PAMPs) and release hyperinflammatory mediators, which leads to exacerbated multi-organ dysfunction and high mortality [1]. Thus, rebalance of the dysregulated macrophage activation and restraint of the critical regulators in the inflammatory response could be a promising therapeutic approach for early sepsis treatment.

Transcription factors of the nuclear factor kappa B (NF-*κ*B)/Rel family play a key role in inflammatory and immune responses. It was widely reported that NF-*κ*B signaling is markedly activated in peripheral mononuclear cells and alveolar macrophages of septic patients, which is highly related to the severity of sepsis verity of sepsis [3-6]. In animal models of sepsis, the activation of NF-*κ*B signaling is solidly induced by bacteria infection, LPS or cecal ligation and puncture (CLP), leading to the production of inflammatory cytokines [7-9]. Therefore, NF-*κ*B signaling is critical in the development of sepsis and promising therapeutic targets.

JAK/STATs signal, the critical pathway of many cytokine receptor systems, is involved in many physiological functions including the response to pathogens. In experimental sepsis model, STAT1 and STAT3 are markedly activated in macrophages and monocytes [10, 11]. However, STAT1 knock-out mice are resistant to LPS-induced endotoxemia and CLP-induced septic shock [12, 13]. Although the conditional deletion of STAT3 in macrophages aggravates LPS-induced septic shock by increasing production of cytokines and

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decreasing survival [14], the pharmacological inhibition of STAT3 can protect against LPS-induced acute lung injury, and inhibit hyperactivation of inflammatory response in CLP-induced septic mice [15, 16]. Therefore, the balance of JAK/STATs signaling activation is important for the therapy of sepsis.

Cortex Fraxini, a well-known and widely used herb in China, is also named Qinpi in traditional Chinese medicine [17]. Qinpi is officially listed in the Pharmacopoeia of the People's Republic of China [18], and frequently utilized to cure colitis and bacillary dysentery [19, 20]. Esculetin is one of the main effective ingredients of Qinpi with strong therapeutic effect on anti-inflammation, such as inhibition of cartilage destruction in rheumatoid arthritis and osteoarthritis [21], attenuation of the disease progression of psoriatic mouse skin [22], amelioration of LPS-induced acute lung injury [23]. However, the therapeutic effect and the underlying mechanism of esculetin on sepsis remain unclear.

In this study, the models of *E. coli*-induced mice sepsis and LPS-stimulated macrophage were introduced, and the protective effect and the molecular mechanism of esculetin against sepsis were investigated. The results indicated that esculetin ameliorated acute lung injury in *E. coli*-induced sepsis by decreasing the levels of inflammatory cytokines and chemokines, which at least partly may be due to its inhibition on NF-κB, STAT1 and STAT3 signals in macrophages. All together, esculetin could serve as a potential therapeutic agent for treatment of sepsis.

Materials and Methods

Reagent

Esculetin (HPLC ≥ 98%) was purchased from National Institutes for Food and Drug Control (Peking, China) and prepared in DMSO. *Escherichia coli* (ATCC25922) was obtained from American Type Culture Collection. LPS (*Escherichia coli* O111: B4) was purchased from Sigma (Shanghai, China) and prepared in ddH_2O. Cell Counting Kit-8 was obtained from Dalian Meilun Biology Technology Co. Ltd. (Dalian, China). Enzyme-linked immunosorbent assay (ELISA) kits of cytokines CCL2, IL-6, IL-1β and TNF-α were ordered from R&D Systems (Minneapolis, MN, USA). Trizol reagent, HiScript 1st Strand cDNA Synthesis Kit and SYBR Green Master Mix were purchased from Vazyme (Nanjing, China). Antibodies used for western blot analysis were as follow: p-p65 (Ser536), p65, p-IκBα (Ser32), IκBα, p-IKKα/β (Ser176/180), IKKα, p-p38 (Thr180/Tyr182), p38, p-JNK (Thr183/Tyr185), JNK, p-STAT1 (Tyr701), STAT1, p-STAT3 (Tyr705), STAT3, p-ERK1/2 (Thr202/Tyr204), ERK1/2, H3, iNOS, SOCS3, and β-Actin were purchased from Cell Signaling Technology (Beverly, MA, USA). And JMJD3 was purchased from Abgent (San Diego, CA). Nuclear and Cytoplasmic Protein Extraction Kit, BCA Protein Assay Kit and NO Assay Kit were ordered from Beyotime Institute of Biotechnology (Jiangsu, China). MPO Kit was ordered from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Mice

Male ICR mice aged 6–7 weeks (weight: 20–25 g) were purchased from Yangzhou University (Yangzhou, China) and acclimatized for 1 week before molding. Use and experiment of mice were performed according to the Laboratory Animal Care & Use Committee at China Pharmaceutical University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The mice were randomly divided into five groups (n = 6−10 per group): (i) control group; (ii) sepsis group; (iii) sepsis with esculetin (20 mg·kg^{-1})-treated; (iv) sepsis with esculetin (40 mg·kg^{-1})-treated; (v) sepsis with esculetin (60 mg·kg^{-1})-treated. To induce spesis, *E. coli* was grown to mid-exponential phase, then centrifuged, washed and diluted in PBS (6×10^8 CFU·mL^{-1}). ICR mice were intraperitoneally injected with 0.3 mL *E. coli* suspension (total 3×10^8 CFU) or LPS (10 mg·kg^{-1}) to induce sepsis. Esculetin was injected intraperitoneally 2 h before sepsis induction. Finally, all animals were euthanized after sepsis induction for 12 h. Moreover, mice were injected with *E. coli* (9×10^8 CFU, i.p.) to investigate the effect of esculetin on septic mortality for four days. For histopathological analysis, the right lung tissues were fixed in 10% formalin, and paraffin-embedded sections were prepared for hematoxylin and eosin (H&E) staining.

Cell culture

RAW264.7 cells were purchased from China Center for Type Culture Collection (CCTCC, Shanghai, China) and cultured in DMEM medium (GIBCO, Beijing, China) with 10% fetal bovine serum (GIBCO, NY, USA), supplemented with 100 U·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin, at 37 °C in a humidified environment with 5% CO_2. For different experiments, RAW264.7 cells were pretreated with various concentrations of esculetin (10, 20 and 40 μmol·L^{-1}) for 1 h and then stimulated with LPS (100 ng·mL^{-1}). Cells and medium supernatants were harvested at 0.5, or 6, or 24 h after LPS stimulation.

RNA isolation and q-PCR

Total RNA was isolated using the Trizol reagent and reverse-transcribed to produce cDNA. q-PCR was performed on an Applied Biosystems Stepone plus Sequence Detection System (Applied Biosystems, CA, USA) using SYBR green dye (Vazyme Biotech, Jiangsu, China). For quantification, the relative mRNA level of specific gene expression was obtained using the $2^{-\Delta Ct}$ method.

Western Blot

RAW264.7 cells were lysed in RIPA buffer with PMSF. Tissue proteins from mice lungs were prepared in the lysis buffer (125 mmol·L^{-1} Tris, 2% SDS, pH 6.8) for 30 min followed by centrifugation. Nuclear proteins from cells were extracted with the Nuclear and Cytoplasmic Protein Extraction Kit. Quantified proteins were separated by SDS–PAGE and transferred onto polyvinylidene difluoride membrane. Bands were analyzed by ImageJ software.

ELISA assay

The lung tissues were weighed and homogenized with RIPA buffer (1 : 9 (*W*/*V*)) on ice and then centrifuged at

12 000 *g* for 15 min at 4 °C. The supernatants were harvested and used immediately or stored at −80 °C. The IL-1*β*, IL-6, TNF-*α* and CCL_2 were detected by ELISA kits according to the manufacturer's protocol.

MPO assay

Myeloperoxidase (MPO) activity in lung tissues was assessed by MPO Kit following the manufacturer's instructions.

Cell viability assay and quantification of nitrite

The cell viability was carried out by Cell Counting Kit-8 (CCK-8) in strict accordance with the manufacturer's instructions. The quantitative detection of NO was assayed with a Nitric Oxide Test Kit according to the manufacturer's instructions. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 550 nm.

Statistical analysis

The data are reported as mean ± SD. Statistically significant values were compared using the one-way ANOVA test and unpaired two tailed student's t test. Significance of differences were indicated when $P < 0.05$, $P < 0.01$, and $P < 0.001$. GraphPad Prism 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis.

Results

Esculetin protects against E. coli or LPS-induced sepsis and attenuates inflammatory response

Gram-positive bacteria are the most common cause of sepsis, which can ordinarily induce severe systemic inflammatory response and contribute to acute tissue injury. Lung dysfunction, referred to acute lung injury (ALI), is the primary cause of death in sepsis. To investigate whether esculetin could improve survival rates of sepsis, mice were pre-treated with esculetin (20, 40 or 60 $mg \cdot kg^{-1}$, i.p.) for 2 h, and then injected with *E. coli* (9×10^8 CFU, i.p.) (Fig. 1A and 1B). The result showed that esculetin visibly improved the survival rates of septic mice in the following four days (Fig. 1C). To investigate the anti-inflammatory effect of esculetin on sepsis, *E. coli* or LPS-induced septic models were introduced, which were ordinarily accompanied with severe lung inflammation and injury. Before injection with *E. coli* (3×10^8 CFU, i.p.) or LPS (10 $mg \cdot kg^{-1}$, i.p.) to induce experimental sepsis, the mice were pre-treated with esculetin for 2 h (Fig. 1A and 1B). Then the experimental mice were euthanized after sepsis induction for 12 h and lung injury was assessed using histological analysis. Lung tissues from *E. coli* or LPS-induced septic mice showed significant pathological changes including inflammatory cell infiltration and alveolar septal thickening, which were attenuated by esculetin pre-treatment (Fig. 1D and 1E). Neutrophil infiltration in lungs is a major pathophysiological hallmark of lung inflammation and injury, and the myeloperoxidase (MPO) is found to be an effective measure of neutrophil infiltration into tissues [24]. Consistent with the histological assessment, the MPO activities were significantly decreased by the treatment of esculetin comparing to the septic mice (Fig. 1F and 1G). These data demonstrated that esculetin has protective effects against *E. coli* or LPS-induced sepsis *in vivo*..

Cytokine storm refers to the rapid production of many cytokines in body fluids caused by severe stimulation, which can lead to systemic inflammatory response, multiple organ failure and even death. Cytokine storm has been reported in sepsis and ALI [25, 26]. Therefore, the inflammatory factors were detected in lung tissues and peritoneal macrophages of the septic mice. The ELISA assay showed that the protein levels of IL-1*β*, IL-6, TNF-*α* and CCL_2 in lung tissues from septic mice were significantly decreased by the pre-administration of esculetin (Fig. 2A and 2B). Moreover, the mRNA levels of IL-1*β*, TNF-*α* and iNOS in the peritoneal macrophages from *E. coli* induced septic mice were also signaficantly down-regulated by esculetin pre-treatment (Fig. 2C). These findings suggested that the protective effects of esculetin against sepsis should be related to the down-regulation of proinflammatory cytokines.

Esculetin reduces LPS-induced inflammatory responses in macrophage

Macrophage, one of the main cells of innate immunity system, is heavily involved in the inflammatory response during the early phase of sepsis. Since esculetin suppressed inflammatory responses in septic mice, the underlying mechanism was investigated using the RAW264.7 macrophage cells *in vitro*. To determine the non-cytotoxic concentration of esculetin, RAW264.7 cells were treated with various concentrations of esculetin for 24 h, and the cell viability was tested using CCK-8. Esculetin had few effect on the cell viability at concentrations below 40 $\mu mol \cdot L^{-1}$ (Fig. 3A). Therefore, the concentrations of esculetin below 40 $\mu mol \cdot L^{-1}$ were used for the subsequent experiments.

LPS is considered to be responsible for the clinical manifestations of septic inflammation, thus the LPS-stimulated macrophage was introduced. To investigate the anti-inflammatory activity of esculetin, RAW264.7 cells were pre-treated with esculetin in the indicated concentrations for 1 h and then stimulated with LPS for another 6 h or 24 h. The cells and cultural supernatants were collected to detect the mRNA and protein levels of proinflammatory factors by q-PCR and ELISA. Consistent with the results of the sepsis models (Fig. 2), both mRNA and protein levels of IL-6, IL-1*β*, CCL_2 and iNOS induced by LPS were significantly decreased with the treatment of esculetin (Fig. 3B, 3C and 3E). Moreover, the LPS-induced NO generation was also significantly down-regulated (Fig. 3D). Taken together, these data suggested that the anti-inflammatory activity of esculetin should be mediated by the inhibition on the transcription level of these proinflammatory cytokines.

Esculetin suppresses NF-κB activation induced by LPS in macrophages

NF-*κ*B is a central participant in modulating the expression of various proinflammatory genes in response to LPS, and the suppression of which is a potent strategy for the treatment of endotoxic sepsis [27]. To investigate whether NF-*κ*B signaling could be regulated by esculetin, the active forms of the typical NF-*κ*B family members were evaluated in LPS-

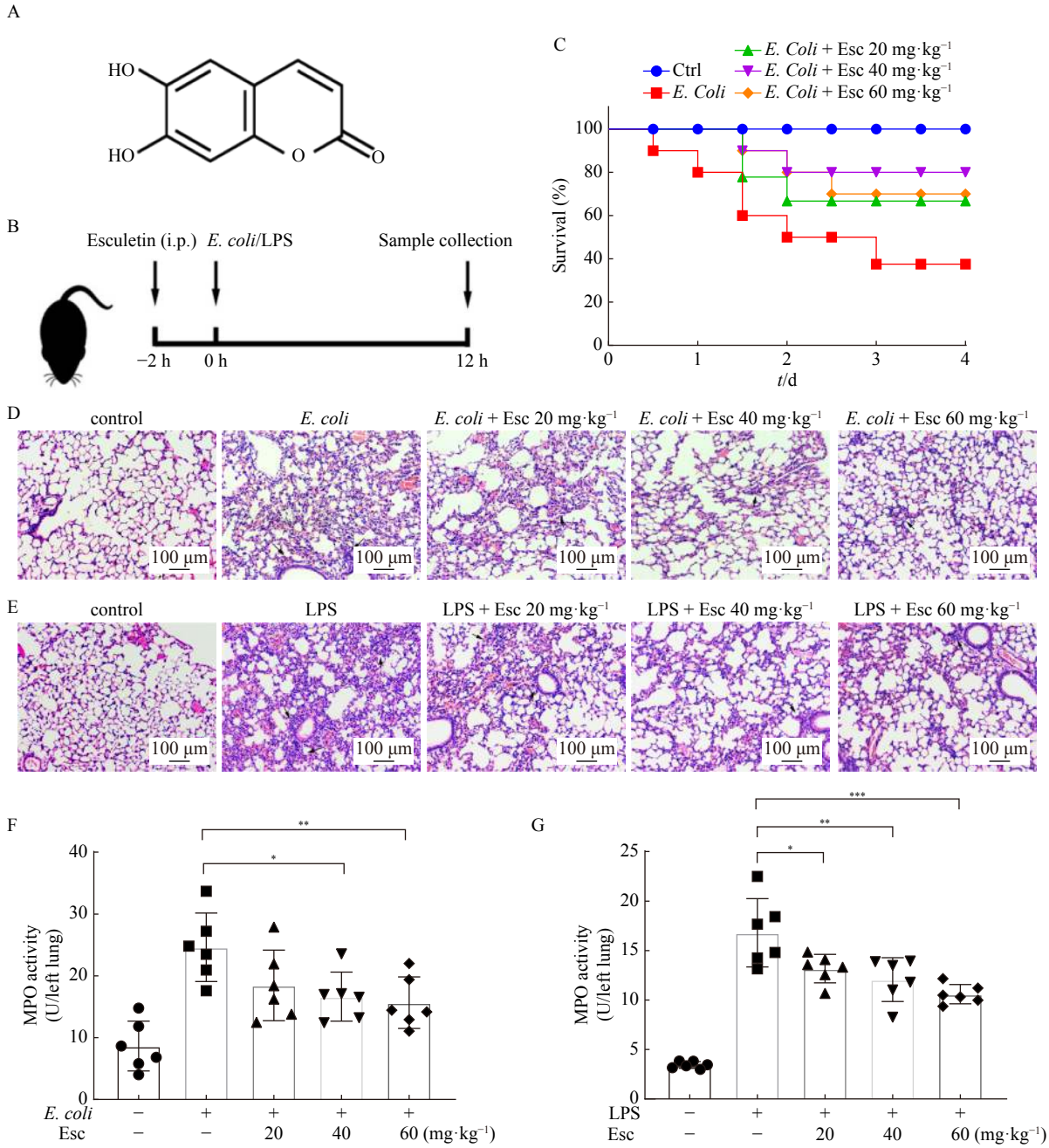

Fig. 1 Esculetin ameliorated *E. coli*- or LPS-induced sepsis. (A) The structural formula of esculetin. (B) Mice were intraperitoneally injected with esculetin or vehicle (PBS) before 2 h of sepsis induction by *E. coli* or LPS. (C) Mice were injected with *E. coli* (9×10^8 CFU, i.p.), and septic survival rates were evaluated in the following four days (n = 9−10 mice per group). Lung sections from *E. coli* (D) and LPS-induced septic mice (E) were examined by H&E staining to monitor lung injury. The arrows indicate the inflammatory cell infiltration and alveolar septal thickening in lung tissues. (F−G) The MPO activity from the left lung was determined to assess neutrophil infiltration. Esc, esculetin. (n = 6 mice per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

stimulated RAW264.7 cells. The phosphorylated levels of IKKα/β, IκBα (the upstream regulators of p65) and p65 were extremely upregulated in LPS-stimulated RAW264.7 cells as reported, which were significantly attenuated by esculetin (Fig. 4A). Furthermore, JMJD3, a downstream target of NF-κB signal, was also down-regulated in LPS-stimulated RAW264.7 cells (Fig. 4A). In addition, the translocation of p-p65 from cytoplasm to nucleus was enhanced in LPS-stimulated cells, which was inhibited by esculetin (Fig. 4B). Taken together, these results indicated that esculetin suppressed LPS-induced NF-κB activation in macrophages, suggesting that the anti-inflammatory activity of esculetin may rely on its inhibition on NF-κB activation.

Effects of esculetin on MAPK signaling in LPS-stimulated RAW264.7 macrophages

MAPKs are well known as a specific class of

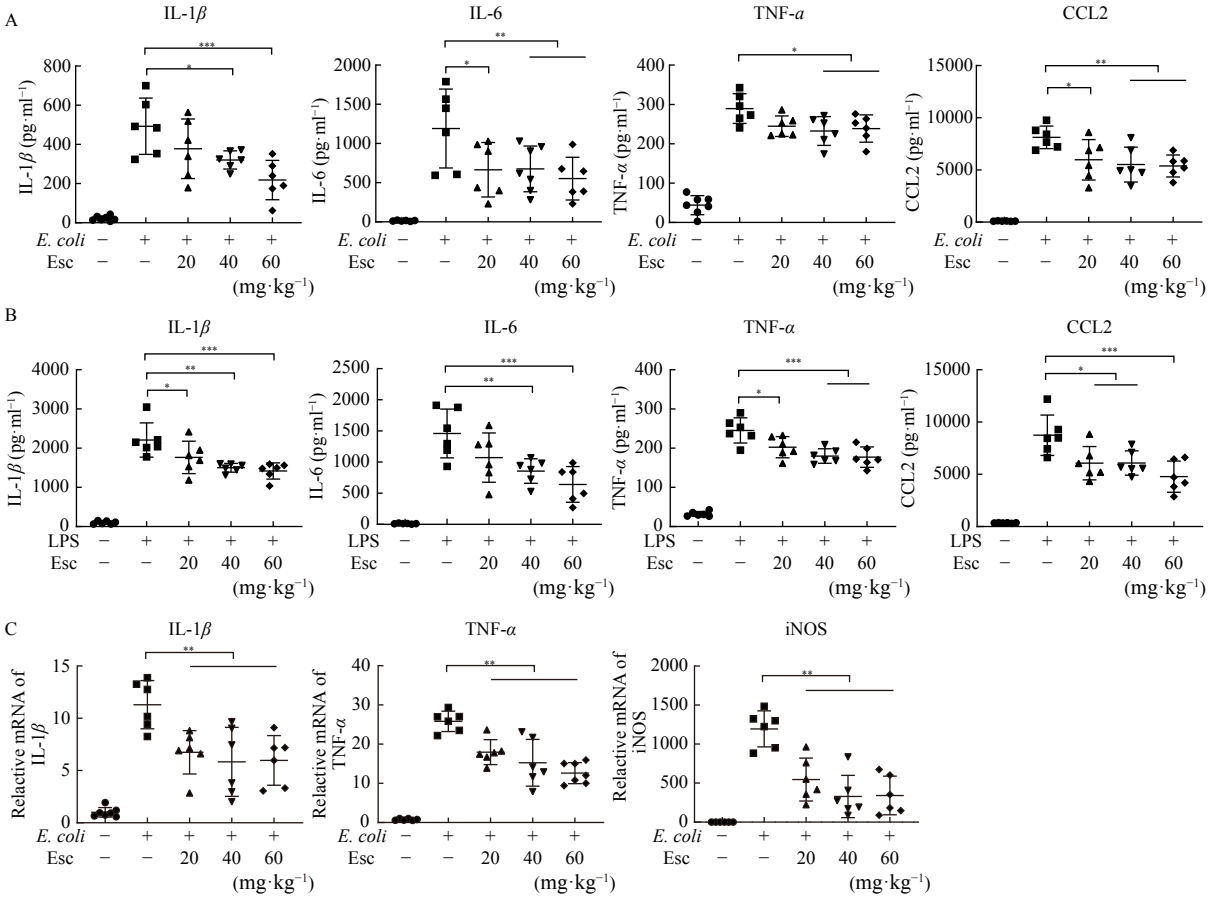

Fig. 2 The effects of esculetin on the proinflammatory cytokines in the lungs from septic mice. The expression of IL-1*β*, IL-6, TNF-*α* and CCL2 in the lungs of *E. coli*-induced sepsis (A) and LPS-induced sepsis (B) were measured by ELISA. (C) The mRNA expression level of IL-1*β*, TNF-*α* and iNOS in peritoneal macrophages from the septic mice were determined by q-PCR. iNOS, inducible nitric oxide synthase. The results were representative of three independent experiments and expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

serine/threonine kinases, playing an important role in the regulation of LPS-induced inflammatory responses in macrophages. The classical MAPKs cascades are comprised of ERK1/2, JNK and p38. Therefore, it was not clear whether the anti-inflammatory response of esculetin was mediated through the MAPK pathways. The phosphorylation levels of ERK1/2, JNK, and p38 were significantly increased by LPS stimulation for 30 min, which was barely affected by the pretreatment with esculetin (Fig. 5). These results suggested that esculetin has few regulatory effect on the classical MAPK pathways.

Esculetin suppresses STAT1 and STAT3 activation induced by LPS in macrophages

The signal transducers and activators of transcription (STATs) play important roles in LPS-induced production of inflammatory factors such as iNOS, IL-6 and other cytokines. Suppression of STAT1 and/or STAT3 activation is considered to be a therapeutic target for sepsis through regulating inflammation [15, 16]. To address whether esculetin has an inhibitory effect on STATs activation in LPS-stimualted macrophges, the phosphorylation levels of both STAT1 (Tyr701) and STAT3 (Tyr705) were detected. The results showed that the expression levels of both phosphorylated STAT1 and STAT3 in LPS-stimualted RAW264.7 cells were dose-dependently down-regulated by the treatment of esculetin in the whole cell lysate (Fig. 6A), cytoplasm and nucleus (Fig. 6B). Furthermore, SOCS3 (Fig. 6A) and iNOS (Fig. 3E), the STAT1 and STAT3 downstream targets, were also down-regulated by esculetin. These data suggested the anti-inflammatory activity of esculetin should also be related to the suppressed activation of STAT1 and STAT3.

Esculetin suppresses NF-κB, STAT1 and STAT3 activation in septic mice

To further confirm the effects of esculetin on NF-*κ*B, STAT1 and STAT3 signals, the expression levels of the key pathways components were detected in the *E. coli*-induced septic mice. The elevated phosphorylation levels of IKK-*α*/*β*, I*κ*B-*α*, p65, STAT1 and STAT3 in the lungs of the septic mice were significantly down-regulated by esculetin, and the elevated levels of iNOS and JMJD3 were also decreased (Fig. 7A and 7B). These results were consistent with our experiments *in vitro*, suggesting the protection of esculetin against sepsis is at least partly due to the down-regulated activation of NF-*κ*B, STAT1 and STAT3 signals.

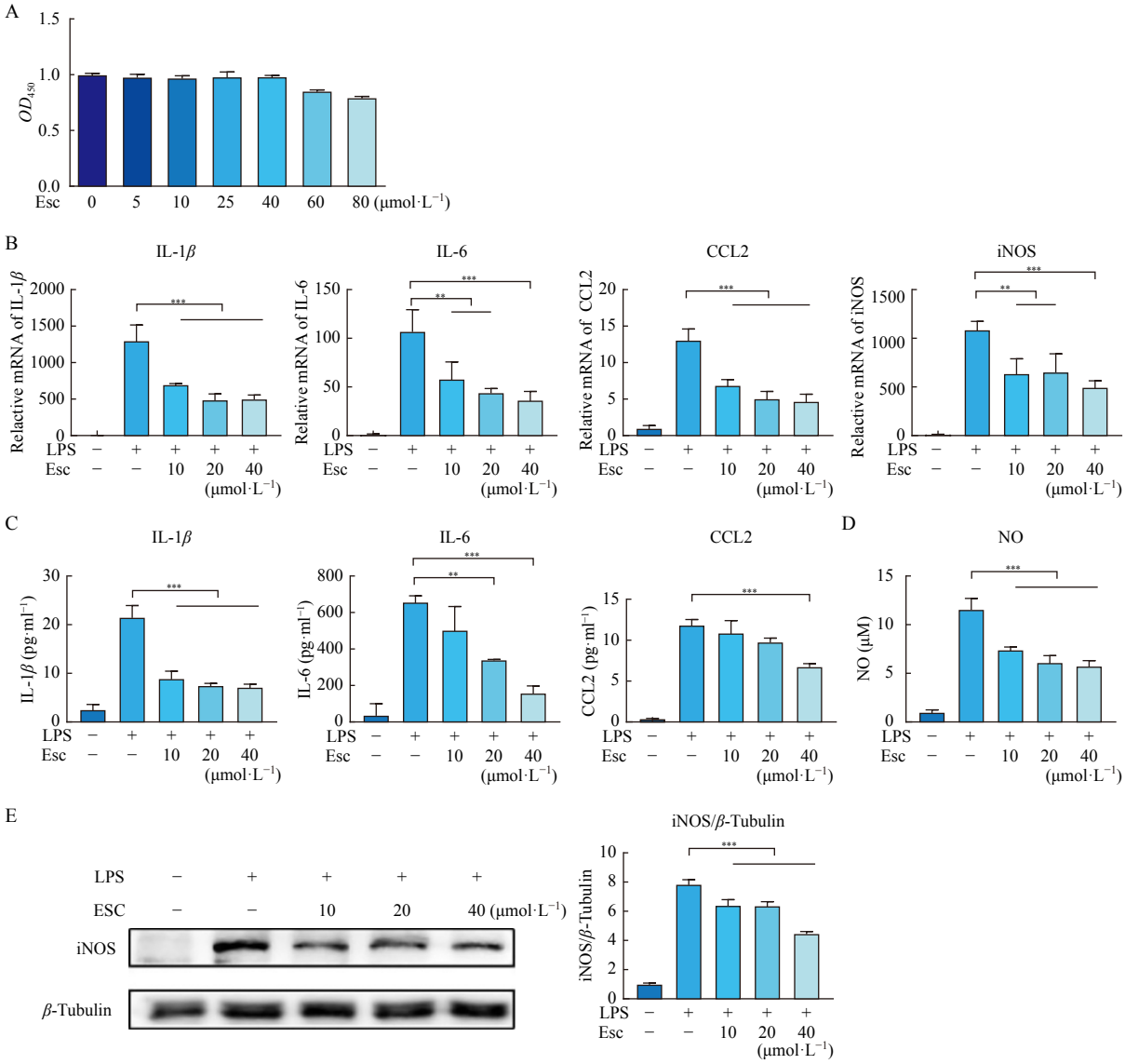

Fig. 3 Esculetin reduced LPS-induced inflammatory responses in macrophage. (A) RAW264.7 cells were pretreated with the indicated concentrations of esculetin for 24 h. The cytotoxicity of esculetin on macrophage was tested by the cell counting kit-8 (CCK-8) assay. (B) The relative mRNA levels of IL-1*β*, IL-6, CCL2 and iNOS were determined by q-PCR. (C) The proinflammatory cytokines levels of IL-1*β*, IL-6 and CCL2 were measured by ELISA. (D) The NO levels were measured by Griess reagent in triplicate. (E) The protein levels of iNOS were determined by western blotting. Densitometric analysis was performed to determine the relative ratios of each protein. NO, nitric oxide. RAW264.7 cells were pretreated with esculetin for 1 h and stimulated with LPS (100 ng·mL^{-1}) for another 6 h (B−C), or 24 h (D−E). Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Discussion

Esculetin is a coumarin compound, extracted from a traditional and widely-used Chinese medicine named *Cortex Fraxini*. In the past few years, it was reported that esculetin has many kinds of biological activities, such as antibacterial, anti-inflammatory, immunomodulatory and sedative activities [17]. In this study, esculetin exhibited a significant protection against *E. coli*-induced and LPS-induced sepsis *in vivo*, and relieved inflammation of the sepsis at least partly through inhibition of NF-*κ*B pathway, STAT1 and STAT3 signals *in vivo* and *in vitro*. Therefore, the elucidation of esculetin regulation on inflammation can shed light on therapeutic strategies for early sepsis.

Sepsis is a condition of life-threatening organ dysfunction caused by both invading pathogens and the dysregulated host response [28]. The systemic hyperinflammatory response syndrome (SIRS) is a cardinal feature and the major cause of multiple organ failure (MOF) during the early phase of sepsis [29]. Therefore, the inhibition of hyperinflammatory response has become the focus of therapeutic strategies for early sepsis. Moreover, SIRS and sepsis are closely related to the dysregulated inflammatory immune network. As a critical effector cells of innate immune system, macrophage plays

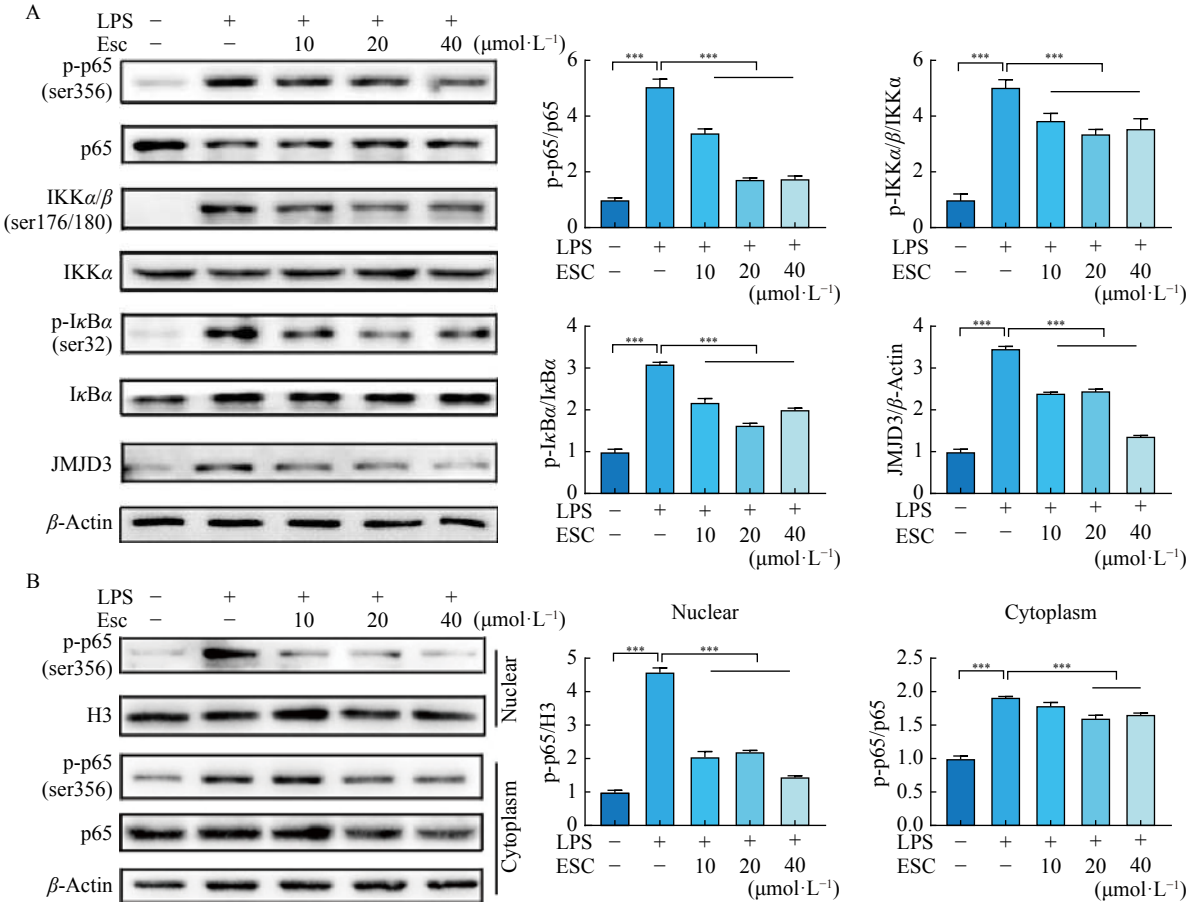

Fig. 4 Esculetin suppressed NF-κB activation induced by LPS in macrophages. RAW264.7 cells were pretreated with esculetin for 1 h and then stimulated with LPS (100 ng·mL^{-1}) for 0.5 h. (A) The protein levels of p-p65 (ser356), p-IKKα/β (ser176/180), p-IκBα (ser32) and JMJD3 were determined by western blotting. (B−C) The nuclear and cytoplasmic protein levels of p-p65. H3 was used as a nuclear internal control while β-actin as the cytoplasmic internal control. Densitometric analysis was performed to determine the relative ratios of each protein. The results are representative of three independent experiments and expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

a key role in host defense against invading microbe and its excessive activation contributes to hyperinflammatory response and induces organ dysfunction in early sepsis. Hence, the rebalance of the hyperactivated macrophages might be a rational therapeutic strategy for the early sepsis.

NF-κB plays an central role in the regulation of cytokine production, and the level of NF-κB activity is strongly correlated with the severity of sepsis [3, 30, 31]. In bacterial sepsis, NF-κB can be activated by verious Toll-like receptors, leading to the synthesis and release of various proinflammatory cytokines and factors, including TNF-α, IL-1β, IL-6, iNOS, JMJD3 etc. These cytokines and factors were markedly increased in patients with sepsis compared to those in healthy control subjects [32]. JMJD3, a key enzyme for demethylation of H3K27, is a critical regulator on the transcription of many inflammatory genes. Pharmacological inhibition of JMJD3 by GSKJ4 protected mice against early septic death and reduced proinflammatory cytokine production [33]. Interestingly, the expression of JMJD3 can be can quickly induced by the activation of NF-κB, and the interaction of JMJD3 and NF-κB co-regulates the expression of the inflammatory cytokines [33, 34]. In this study, the mRNA and protein levels of TNF-α, IL-1β, IL-6, iNOS and JMJD3 were downregulated by esculetin in lungs of *E. coli*-induced sepsis, as well as in LPS-induced RAW264.7 cells. Furthermore, the phosphorylated levels of IKKα/β, IκBα and p65 were repressed by the treatment of esculetin. All these results above suggested that the protection against *E. coli*-induced sepsis of esculetin is at least partly due to its inhibition on the NF-κB/JMJD3 signaling pathway.

The JAK/STATs pathway, mediating the singal transduction of many cytokines, is considered to be involved in the pathogenesis of sepsis [10]. Of note, STAT1 is a critical mediator for the transcription of proinflammatory factors in macrophage, such as IL-1β, TNF-α, iNOS and high mobility group box (HMGB)-1, and contributes to hyperinflammatory state [12, 35]. On the other hand, the plasma concentrations of IL-6, MIP-2, CXCL10 and IFN-α were significantly lower in STAT1-deficient mice, and the STAT1-knockout mice are resistant to LPS- or CLP- induced sepsis [12]. Moreover, it is suggested that the occurrence of SIRS is mainly mediated by STAT1 activation, and then leading to tissue damage and

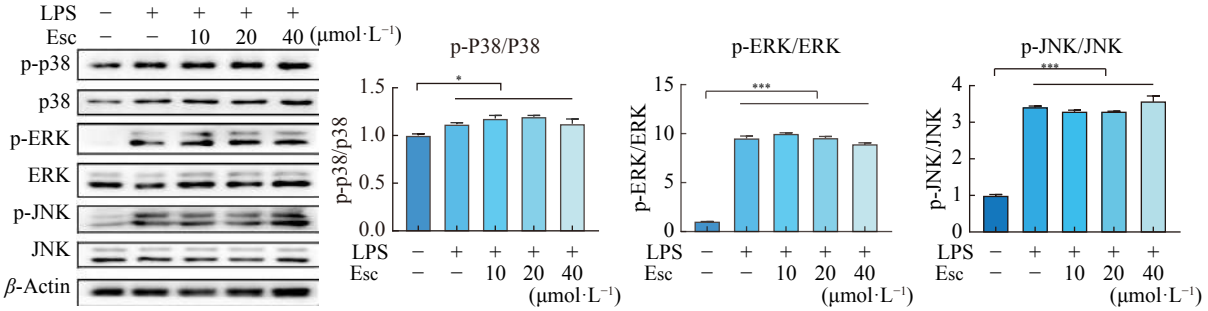

Fig. 5 Effects of esculetin on MAPK signals in LPS-stimulated macrophages. RAW264.7 cells were pretreated with esculetin for 1 h and then stimulated with LPS (100 ng·mL^{-1}) for 0.5 h. The levels of p-p38, p-JNK, and p-ERK were detected by western blotting. Densitometric analysis was performed to determine the relative ratios of each protein. The results are representative of three independent experiments and expressed as the mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$

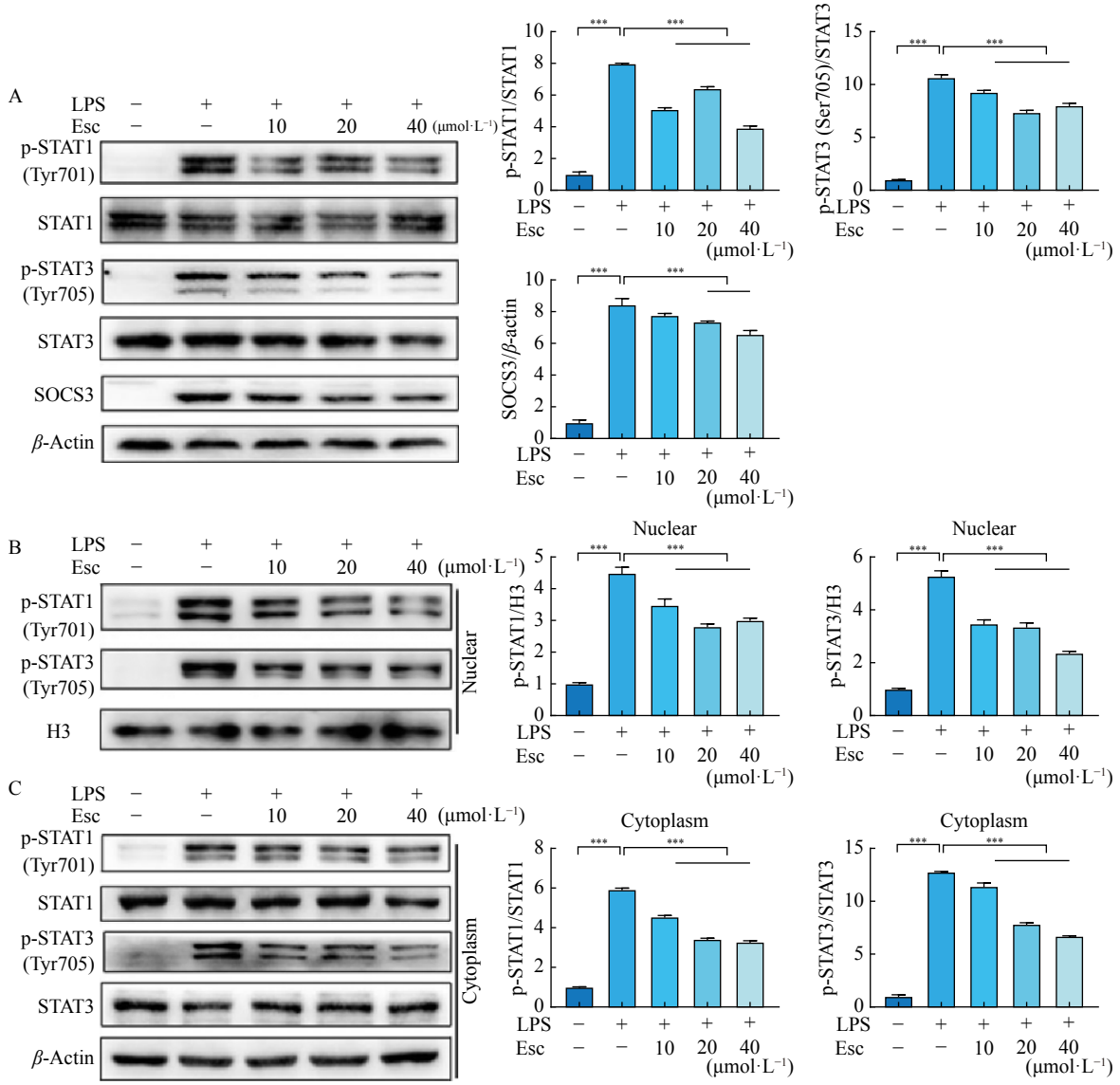

Fig. 6 Esculetin suppressed STAT1 and STAT3 activation induced by LPS in macrophages. RAW264.7 cells were pretreated with esculetin for 1 h and then stimulated with LPS for another 6 h. The protein levels of p-STAT1 (Tyr701), p-STAT3 (Tyr705), and SOCS3 were detected by western blotting in the whole cell lysate (A), or nuclear (B), or cytoplasm (C). Densitometric analysis was performed to determine the relative ratios of each protein. The results are representative of three independent experiments and expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

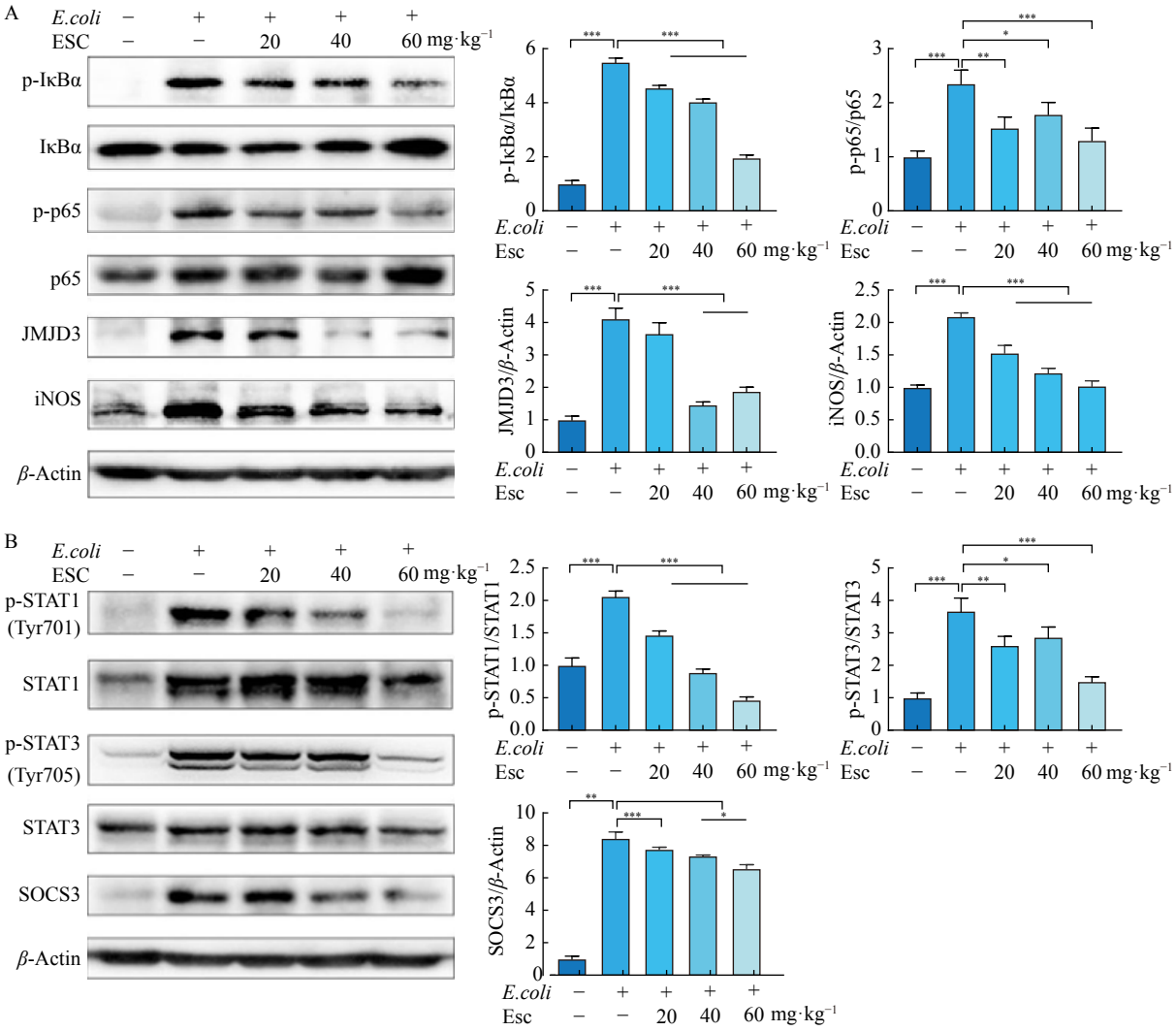

Fig. 7 Esculetin suppressed NF-κB, STAT1 and STAT3 activation in *E. coli*-induced septic mice. The total proteins were seperated from lung tissues of *E. coli*-induced septic mice, and the protein levels of (A) p-IκB*α*, p-p65, JMJD3 and iNOS, (B) p-STAT1 (Tyr701), p-STAT3 (Tyr705) and SOCS3 were determined by western blotting. Densitometric analysis was performed to determine the relative ratios of each protein. The results are representative of three independent experiments and expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

multiple organ failure syndrome, even to mortality [10, 12]. STAT3, another critical component of JAK/STATs pathway, was reported to be activated in an experimental mammalian sepsis model, and the IL-6/JAK2/STAT3 pathway was mainly involved in regulating the inflammatory response [10, 11, 36]. Interestingly, STAT3 is activated before the occurrence of severe lung injury, which is related to production of the proinflammatory molecules TNF-α, IL-1β and CCL_2. However, the suppression of STAT3 activity ameliorates lung inflammatory responses in LPS-induced acute lung injury (ALI) [15, 36], suggesting that STAT3 may play a critical role in initiating pulmonary inflammation in ALI [37]. Although mice with conditional deletion of STAT3 in macrophages or endothelial cells are susceptible to LPS-induced septic shock, and accompanied with increased production of cytokines and adverse survival [14, 38], the inhibition of STAT3 can markedly limit the hyperactivation of the inflammatory response, alleviate lung injury and improve the survival in septic mice [15, 16]. Therefore, STAT1 and STAT3 are considered to be potential therapeutic targets of sepsis. In this paper, esculetin treatment dose-dependently down-regulated the expression levels of both phosphorylated STAT1 and STAT3 in LPS-stimualted RAW264.7 cells and in *E. coli*-induced sepsis mice. Meanwhile, the levels of p-STAT1 and p-STAT3 were downregulated in the nucleus by esculetin. Taken together, these findings suggested the protective effect of esculetin against sepsis is multi-targets and the most potent esculetin activity also needs further research.

In summary, esculetin shows the protective effect on the histological indices and biochemical indexes of *E.coli* or LPS-induced sepsis at least partly through regulation of NF-κB pathway, STAT1 and STAT3 signals. These data suggested that, though rebalancing the innate immune response in macrophage, esculetin may serve as a potential therapeutic agent

for the clinical treatment of early sepsis.

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