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Daphnetin reduces endotoxin lethality in mice and decreases LPS-induced inflammation in Raw264.7 cells via suppressing JAK/STATs activation and ROS production

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Abstract

Objective Here, we used various approaches to investigate the suppressive role of daphnetin in LPS-induced inflammatory response, with the goal to understand the underlining molecular mechanism by which daphnetin regulated these processes.

Methods We examined the survival rate and the lung injury in the mice model of LPS-induced endotoxemia. The production of pro-inflammatory factors including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, nitric oxide (NO), and prostaglandin E2 (PGE2) was measured by ELISA and nitrite analysis, respectively. The expression of inducible NO synthase (iNOS), cyclooxygenase 2 (COX-2), and the activation of signaling molecules was determined by immunoblotting. The production of reactive oxygen species (ROS) was measured by the ROS assay.

Results In vivo study showed that daphnetin enhanced the survival rate and reduced the lung injury in mice with LPS-induced endotoxemia. Both in vivo and in vitro study showed that daphnetin prevented the production of pro-inflammatory factors including TNF-α, IL-1β, IL-6, NO, and PGE2 after LPS challenge. In Raw264.7 cells, we found that daphnetin reduced LPS-induced expression of iNOS and COX-2, and suppressed LPS-induced ROS production. In addition, we found that daphnetin suppressed the activation of JAK/STATs pathway and inhibited the nucleus import of STAT1 and STAT3.

Conclusions Here, our results indicate that daphnetin shows anti-inflammatory properties, at least in part, through suppressing LPS-induced activation of JAK/STATs cascades and ROS production.

Keywords Daphnetin · COX-2 · iNOS · JAK/STATs · ROS

Abbreviations

DFN	Daphnetin
LPS	Lipopolysaccharide
IL	Interleukin
TNF	Tumor necrosis factor
NO	Nitric oxide
PGE2	Prostaglandin E2
iNOS	Inducible NO synthase
COX-2	Cyclooxygenase 2
JAK	Janus protein tyrosine kinase
STAT	Signal transducers and activators of transcription
MAPKs	Mitogen-activated protein kinases
ROS	Reactive oxygen species
I.P. injection	Intraperitoneal injection

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Introduction

Lipopolysaccharide (LPS), also known as endotoxin, is a major component of outer membrane of gram-negative bacteria and activates strong immune responses in cells and animals. Upon LPS stimulation, monocytes and macrophages are activated and generate pro-inflammatory mediators including TNF-α, IL-1β, IL-6, NO, and PGE2 [1, 2]. Among these pro-inflammatory mediators, NO and PGE2 are produced by iNOS and COX-2, respectively [3, 4]. These cytokines, in moderate dose, are required for host-defense and cell growth. However, the uncontrolled production of pro-inflammatory factors results in serve immunopathology, including rheumatoid arthritis [5], atherosclerosis [6, 7], and systemic inflammatory response syndrome (SIRS) [8, 9].

Mechanistically, upon LPS challenge, the activation of intracellular signaling pathways contributes to the immune responses. Nucleus factor κB (NF-κB) plays an important role in regulating gene expression and inflammatory process. In unstimulated conditions, Iκ-B binds to NF-κB and represses its transcription activity. Upon LPS stimulation, Iκ-B is phosphorylated by IKK and ubiquitinated. As a result, the NF-κB-IκB complex is disrupted and the nucleus import of NF-κB initiates transcription of target genes including iNOS, COX-2, TNF-α, IL-1β, and IL-6 [10–12]. The mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase1/2 (ERK1/2), p38MAPKs, and c-Jun NH_2-terminal kinase (JNK), are intracellular serine/threonine kinases and are activated by LPS to trigger the expression of iNOS and COX-2 [13, 14]. The janus kinase-signal transducers and activators of transcription (JAK/STATs) are important signaling pathways in regulating cytokines expression [15, 16]. In response to LPS stimulation, STATs can be phosphorylated on specific tyrosine residues by receptor-associated JAK, form homo or heterodimers, and then translocate into nucleus to drive the transcription of target genes which encode pro-inflammatory cytokines, chemokines, and inducible enzyme, such as iNOS and COX-2 [17–19].

Daphnetin (7,8-dithydroxycoumarin, DFN; Fig. 1a), a coumarin derivative from *Daphne koreana* Nakai (Ruixiang), is the primary component of Chinese herb medicine Zushima. Daphnetin has been widely used to treat clinic inflammatory disease including rheumatism and hypertension [20, 21]. However, the potential molecular mechanism of daphnetin is still unclear and requires further investigation.

In this study, we investigate the anti-inflammatory properties of daphnetin after LPS challenge and also its role in regulating related signaling pathways.

Materials and methods

Antibodies and reagents

Monoclonal antibodies recognizing iNOS, COX-2, p38-MAPKs, JNK, phospho-JNK (Thr183/Tyr185), IKK-α, IKK-β, phospho-IKKα/β (Ser176/180), JAK1, phospho-JAK1 (Tyr1022/Tyr1023), JAK2, and phospho-JAK2 (Tyr1007/Tyr1008) were purchased from Cell Signaling Technology. Antibodies against phospho-p38 MAPKs (Thr180/Tyr182), ERK, phospho-ERK (Thr202/Thr204), GAPDH, β-Actin, LaminB, phospho-STAT1 (Tyr701), phospho-STAT3 (Ser727), and phospho-STAT3 (Tyr705) were purchased from Bioworld Technology. Antibodies recognizing STAT1 and STAT3 were obtained from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies against mouse or rabbit IgG were purchased from Vazyme Biotech.

Daphnetin (7,8-dihydroxy-2H-1-benzofuran-2-one) was purchased from Selleck. LPS (from *Escherichia coil* 0111:B4), *N*-acetyl-L-cysteine (NAC), was purchased from Sigma Aldrich. Dimethylsulfoxide (DMSO) was obtained from Amresco. CCK8 Cell Viability Assay Kit was purchased from Vazyme Biotech. RIPA lysis buffer and ROS Assay Kit are purchased from Beyotime Biotechnology.

Animals

Eight-week-old male mice (C57BL/6) were purchased from Nanjing Laboratory Animal Center, Nanjing University (Jiangsu, PR China) and maintained in the normal conditions. Laboratory animal handling and experimental procedures were conducted in accordance with the principles and procedures outlined in the Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Science and Technology Department of Jiangsu Province. To investigate the effect of daphnetin in mice survival, mice (10 mice/group) were received daphnetin (5 mg/kg, body weight) and LPS (37.5 mg/kg) by intraperitoneal injection. Survival was monitored every another hour up to 3 days. For ELISA assay and histology analysis, mice (5 mice/group) were received daphnetin (5 mg/kg, body weight) and LPS (37.5 mg/kg) by intraperitoneal injection, serum, and lung tissue which were collected 16 h after LPS injection.

H&E staining

For histopathological study, lung tissue was collected, fixed in 4% formaldehyde, and embedded in paraffin (Leica). Sections (10 μm thickness) were stained with hematoxylin and eosin (H&E). Photomicrographs were captured under

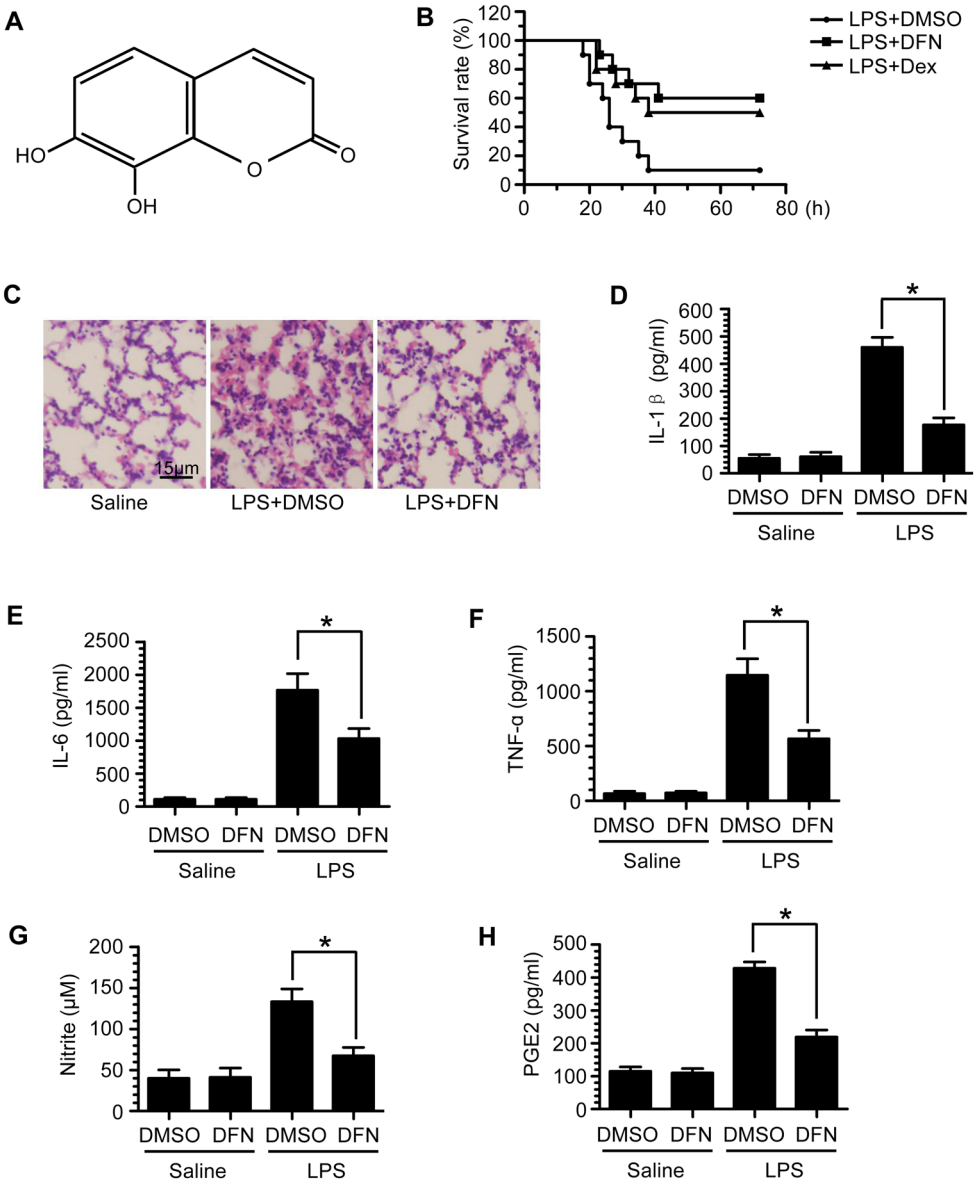

Fig. 1 Daphnetin reduces endotoxin lethality in mice. **a** Chemical structure of daphnetin (DFN). **b** C57/BL6 mice (10 mice/group) were intraperitoneally injected with daphnetin (DFN, 5 mg/kg) or DMSO, and then challenged with LPS (37.5 mg/kg) or saline. The time of death after LPS challenge was recorded. The diagram showed the survival rate in each group. **c**–**h** C57/BL6 mice (5 mice/group) were intraperitoneally injected with daphnetin (DFN, 5 mg/kg) or DMSO, and then challenged with LPS (37.5 mg/kg) or saline. 16 h after LPS challenge, mice were sacrificed, and then lung and serum were collected. **c** Representative photomicrographs showed H&E staining of lung tissue. **d**–**h** Results from ELISA assay showed the content of IL-1β, IL-6, TNF-α, NO, and PGE2 in the serum. The results were expressed as mean ± SD, $n=3$. *$p<0.05$, comparing with matched controls

the FSX100 intelligent biological image navigator (Olympus, Tokyo, Japan).

Cell culture

Murine macrophage cells Raw264.7 were purchased from Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, PR China), and were cultured in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Wisent Corporation) at 37 °C in an atmosphere of 5% CO_2.

Cell viability

Cell viability was determined using CCK8 Cell Viability Assay Kit. Raw264.7 cells were treated with indicated concentrations of daphnetin for 24 h, respectively, and then cell viability was measured using CCK8 Cell Viability Assay Kit according to the manufacturer's instructions. The absorbance was measured at 500 nm using the Synergy 2 Multi-Mode Microplate Reader (BIO-TEK, INC).

Determination of cytokines

Mice serum and the culture media were collected and centrifuged at 10,000 rpm, 4 °C for 10 min. The content of cytokines in the supernatants was determined using Sandwich ELISA according to the manufacturer's instructions (R&D System).

Nitrite analysis

Mice serum and the culture media were collected and centrifuged at 10,000 rpm, 4 °C for 10 min. The amount of nitrite was determined using NO Assay Kit (JianChen) according to the manufacturer's instructions. Sodium nitrite was used as standard. The absorbance was measured at 550 nm using the Synergy 2 Multi-Mode Microplate Reader (BIO-TEK, INC).

Western blotting

Whole cells lysates were prepared with RIPA lysis buffer supplement with protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor (Roche). Cytoplasmic and nucleus proteins were extracted using NE-PER Nucleus and Cytoplasmic Extraction Reagent (Thermo Scientific) according to the manufacturer's instructions. Protein concentrations were assayed using Modified BCA Protein Assay Kit (Bioworld Technology).

For immunoblotting, a total of 20 μg protein of each sample was electrophoresed in 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) western membrane (Bio-Rad). Following blocking with 5% non-fatty milk in TBST, membranes were incubated with indicated primary antibodies at 4 °C overnight. HRP-conjugated secondary antibodies (Vazyme Biotech co., ltd) were used and the antibody-antigen complexes were visualized by chemiluminescence method using the enhanced ECL immunoblotting system (Tanon, Shanghai, China).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from Raw264.7 cells using High Pure RNA Extraction Kit (Roche) according to the manufacturer's instructions. Reverse transcription was performed using a two-step RT-PCR System kit (Invitrogen). Quantitative polymerase chain reaction (qPCR) was carried out using the SYBR Premix Ex Taq (TaKaRa). GAPDH levels were taken for endogenous control, and the fold changes of iNOS and COX-2 expression were calculated by applying $2^{-\Delta\Delta C_t}$. The following primers were used: iNOS: sense primer 5′-cccttccgaagttctggcagc-3′, antisense primer 5′-ggctgtcagagcctgggctt-3′; COX-2: sense primer 5′-tctccaacctctcctactac-3′, antisense primer 5′-gcacgtagtcttcgatcact-3′; GAPDH: sense primer 5′-tgaaggtcggtgtgaacggatttggc-3′, antisense primer 5′-tggttcacacccatcacaaacatgg-3′.

Detection of ROS production

Raw264.7 cells were seeded on 96-well plate and treated with LPS for 30 min. Following washing with serum free medium, 10 μM DCFH-DA was added and the plate was incubated at 37 °C for 30 min. Then, fluorescence was measured using Synergy 2 Multi-Mode Microplate Reader (BIO-TEK, INC) after excitation at 485 nm and emission at 535 nm. The relative ROS level was expressed as percentage of control.

Statistical analysis

Unless otherwise mentioned, individual animal and cell culture experiments were performed in triplicate and repeated at least twice, and the data were pooled. The data were shown as mean ± SD. Statistical significance of differences was assayed by Student's *t* test and one-way ANOVA (version 17.0 SPSS, Chicago, IL). In all analyses, a value of $*p < 0.05$ was taken as significant, whereas n.s. means no significance.

Results

Daphnetin reduces endotoxin lethality in mice

To investigate the anti-inflammatory properties of daphnetin in vivo, the mice model of LPS-induced endotoxemia was established as described in the "Materials and methods". Before LPS challenge, half of mice were intraperitoneally injected with daphnetin (5 mg/kg), the other with vehicle DMSO. As shown in Fig. 1b, daphnetin treatment enhanced the survival rate compared with the vehicle. In LPS-induced inflammatory process, acute lung injury is caused by the overproduction of pro-inflammatory factors, and leads to pulmonary dysfunction, with a mortality rate of 40%. H&E staining of lung tissue showed that daphnetin treatment reduced LPS-induced pulmonary interstitial edema and inflammatory infiltrates (Fig. 1c). In addition, results of ELISA assay and nitrite analysis showed that daphnetin administration suppressed LPS-induced production of pro-inflammatory mediators including IL-1β, IL-6, TNF-α, nitrite, and PGE2 in mice serum (Fig. 1d–h). Collectively, these findings suggest that daphnetin protects mice from LPS-induced mortality, lung injury and reduces the production of pro-inflammatory factors in serum.

Daphnetin suppresses LPS-induced inflammatory response in Raw264.7 cells

To investigate the anti-inflammatory effect of daphnetin in macrophages, Raw264.7 cells were exposed to different concentrations of daphnetin. First, CCK8 assay was carried out to exclude the toxicity of daphnetin. As shown in Fig. 2a, daphnetin showed no notable effect on the cell viability at concentrations from 5 to 20 μM. Pro-inflammatory

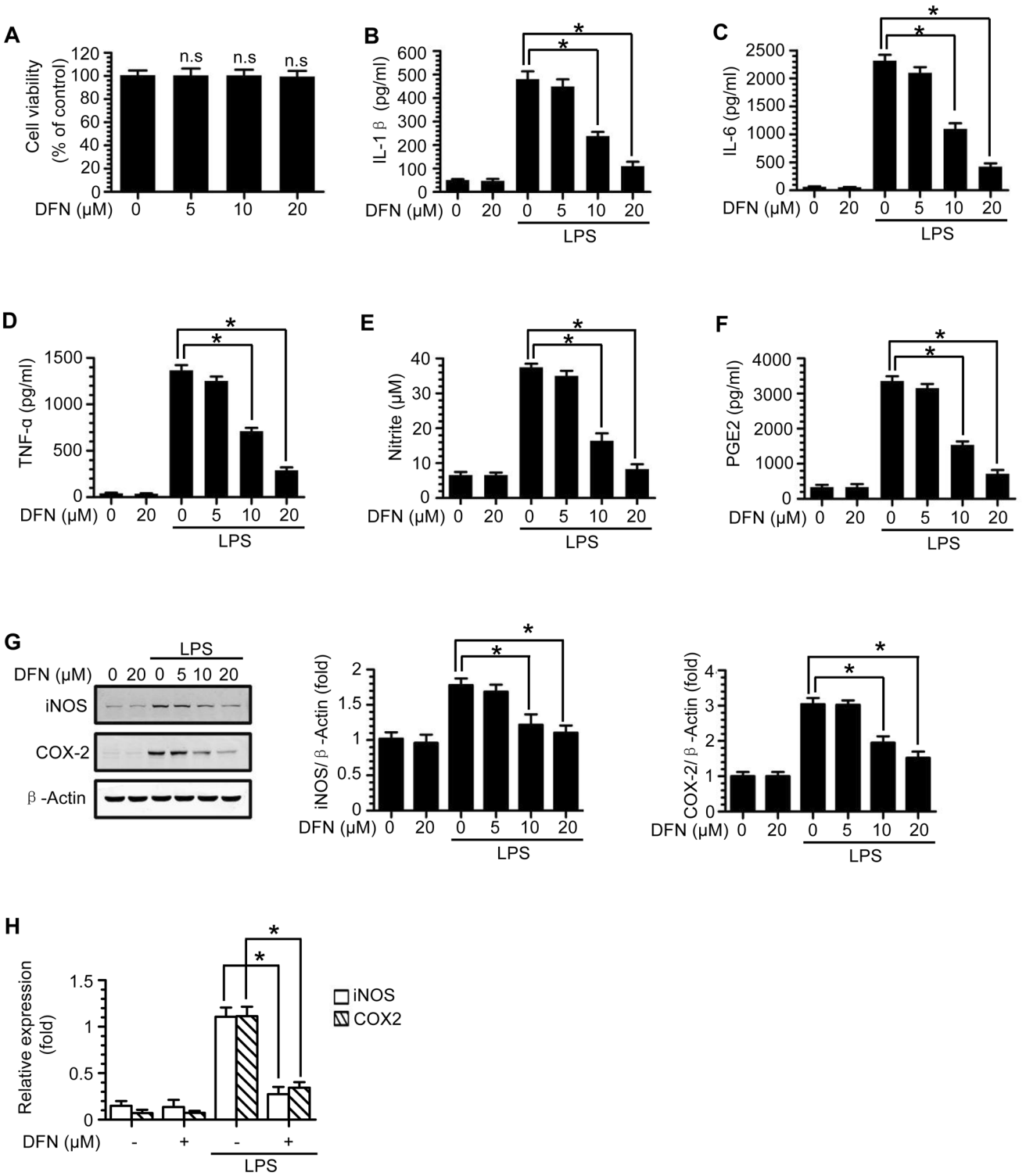

Fig. 2 Daphnetin suppresses LPS-induced inflammatory response in Raw264.7 cells. **a** Raw264.7 cells were treated with indicated concentrations of daphnetin (DFN) for 24 h, and then, cell viability was detected by CCK8 assay. **b–g** Raw264.7 cells were treated with indicated concentrations of daphnetin (DFN) for 2 h, followed by 16 h treatment with LPS (100 ng/ml). **b–f** Results from ELISA assay showed the content of IL-1β, IL-6, TNF-α, NO, and PGE2 in the culture medium. **g** Immunoblotting and densitometric analysis showed the protein levels of iNOS and COX-2 in Raw264.7 cells. **h** Raw264.7 cells were treated with daphnetin (DFN 20 μM) for 2 h, followed by 6 h treatment with LPS (100 ng/ml). The message RNA levels (expressed as the fold changes above the control group) were detected by the real-time PCR. The results were expressed as mean ± SD, $n = 3$. *$p < 0.05$, comparing with matched controls

factors play an important role in LPS-induced inflammatory response. Consistent with our previous findings (Fig. 1d–h), results from the ELISA assay and nitrite analysis showed that daphnetin reduced the production of LPS-induced pro-inflammatory mediators including IL-1β, IL-6, TNF-α, NO, and PGE2 in a dose-dependent manner in Raw264.7 cells (Fig. 2b–f).

Among these pro-inflammatory factors, NO and PGE2 are produced by iNOS and COX-2, respectively. To investigate the role of daphnetin in regulating iNOS and COX-2 expression, Raw264.7 cells were treated with indicated concentrations of daphnetin for 2 h, respectively, followed by administration of LPS for 16 h. We observed that daphnetin suppressed LPS-induced expression of iNOS and COX-2 in a dose-dependent manner (Fig. 2g). Furthermore, results from qPCR showed that daphnetin reduced the transcription of iNOS and COX-2 (Fig. 2h). Taken together, these results indicate that daphnetin exhibits anti-inflammatory properties in Raw264.7 cells.

Daphnetin inhibits LPS-induced activation of JAK/STATs, but not of MAPKs or NF-κB

Transcription factors are critical to regulating the pro-inflammatory genes expressing. NF-κB has been reported to drive the expression of iNOS, COX-2, TNF-α, and IL-6 [22, 23]. However, results from immunoblotting showed that daphnetin exhibited no notable effect on LPS-induced phosphorylation of IKKα/β, suggesting that daphnetin does not affect the LPS-induced activation of NF-κB (Fig. 3a). In addition, signal transducer and activator of transcription (STATs) play important role in LPS-induced production of pro-inflammatory components including iNOS and COX-2 and other cytokines [24–27]. As shown in Fig. 3b, daphnetin down-regulated the phosphorylation of both STAT1 (Tyr701) and STAT3 (Tyr705, Ser727). The phosphorylation of STATs results in dimerization, nucleus translocation, and then initiates the transcription of target genes [27–29]. As shown in Fig. 3c, daphnetin suppressed LPS-induced nucleus import of STAT1 and STAT3. These findings indicate that daphnetin suppresses the activation of STAT1 and STAT3.

Furthermore, in response to LPS stimulation, STAT1 and STAT3 can be phosphorylated by the Janus kinases (JAKs) [30]. Results from immunoblotting showed that daphnetin suppressed LPS-induced phosphorylation of JAK1 and JAK2 (Fig. 3d). MAPKs have been also reported to play important role in inflammatory response and to activate the STATs transcription factors [31–33]. However, as shown in Fig. 3e, daphnetin did not suppress the LPS-induced phosphorylation of p38MAPK, ERK or JNK. Taken together, these results suggest that daphnetin suppresses LPS-induced activation of JAK/STATs cascade, without altering the activation of NF-κB or MAPKs.

Daphnetin reduces LPS-induced production of ROS

Reactive oxygen species (ROS), the chemically reactive molecules containing oxygen, is important to regulate intracellular signaling and homeostasis. During LPS exposure, ROS is accumulated and results in cell injury and cell death [34]. We, therefore, investigated whether daphnetin showed anti-oxidant activity. *N*-acetylcysteine (NAC), a generally accepted anti-anti-oxidant, was used as positive control. As shown in Fig. 4a, consistent with the results from NAC, daphnetin reduced LPS-induced ROS accumulation. In addition, ROS has been regarded as the signaling molecules in LPS-induced inflammatory process [33, 35]. As shown in Fig. 4b, NAC suppressed LPS-induced expression of iNOS and COX-2, suggesting that ROS is critical factors involved in LPS-induced inflammatory process.

Furthermore, it has been reported that ROS contributes to LPS-induced activation of JAK/STATs cascade [25, 36]. We, therefore, investigated whether the inhibitory effect of daphnetin on LPS-induced JAK/STATs activation was correlated to its anti-oxidant properties on ROS. As shown in Fig. 4c and d, consistent with the role of daphnetin, NAC suppressed LPS-induced phosphorylation of JAK1, JAK2, STAT1, and STAT3, indicating the inhibition of JAK/STATs cascade by NAC. Taken together, these results suggest that the anti-oxidant activity of daphnetin is correlated to its anti-inflammatory properties.

Discussion

Daphnetin is the primarily component of Chinese herb medicine Zushima which has the beneficial effect on rheumatic arthritis, vasculitis, and coronary heart disease [37, 38]. Recent report has shown that daphnetin exerts anti-inflammatory property through inhibiting NF-κB activation [39]. However, the more detailed molecular mechanism of daphnetin is still unclear and requires further investigation. Thus, in this work, we explore the anti-inflammatory potential of daphnetin. We demonstrate that daphnetin reduces the endotoxin lethality in the mice model of LPS-induced endotoxemia and decreases LPS-induced inflammatory response in Raw264.7 cells. Our results indicate that daphnetin suppresses LPS-induced activation of JAK1 and JAK2, facilitates the inhibition of STAT1 and STAT3 phosphorylation, and then blocks the nucleus import of STAT1 and STAT3. In addition, daphnetin reduces LPS-induced ROS production.

Inflammation is the protective reaction in response to multiple harmful stimuli including microbial invasion

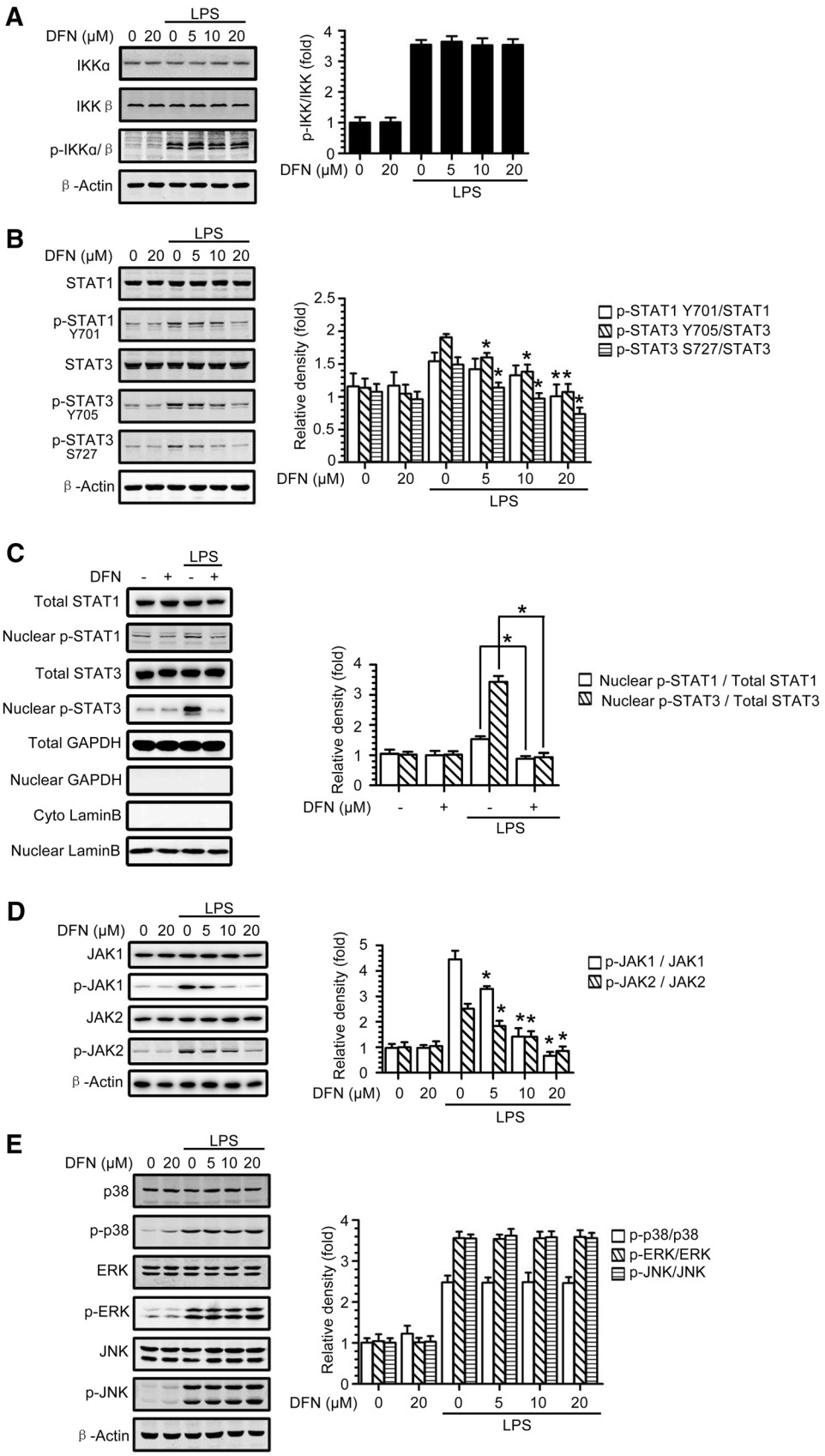

Fig. 3 Daphnetin inhibits LPS-induced activation of JAK/STATs, but not MAPK or NF-κB. **a** Raw264.7 cells were treated with daphnetin (DFN) for 2 h, followed by 30 min treatment with LPS (100 ng/ml). Immunoblotting and densitometric analysis showed the total IKKα, total IKKβ, and phosphorylated IKKα/β protein levels in Raw264.7 cells. **b** Raw264.7 cells were treated with daphnetin (DFN) for 2 h, followed by 4 h treatment with LPS (100 ng/ml). Immunoblotting and densitometric analysis showed the total STAT1, total STAT3, phosphorylated STAT1 (Y701), phosphorylated STAT3 (Y705), and phosphorylated STAT3 (S727) protein levels in Raw264.7 cells. **c** Raw264.7 cells were treated with daphnetin (DFN, 20 μM) for 2 h, followed by 4 h treatment with LPS (100 ng/ml). Immunoblotting and densitometric analysis showed the nucleus import of STAT1 and STAT3. The purity of nucleus extracts was determined by nucleus LaminB (nucleus envelop protein) and GAPDH. **d** Raw264.7 cells were treated with daphnetin (DFN) for 2 h, followed by 1 h treatment with LPS (100 ng/ml). Immunoblotting and densitometric analysis showed the total JAK1, total JAK2, phosphorylated JAK1, and phosphorylated JAK2 protein levels in Raw264.7 cells. **e** Raw264.7 cells were treated with daphnetin for 2 h, followed by 30 min treatment with LPS (100 ng/ml). Immunoblotting and densitometric analysis showed the total p38, total ERK, total JNK, phosphorylated p38, phosphorylated ERK, and phosphorylated JNK protein levels in Raw264.7 cells. The results were expressed as mean ± SD, $n = 3$. *$p < 0.05$, comparing with matched controls

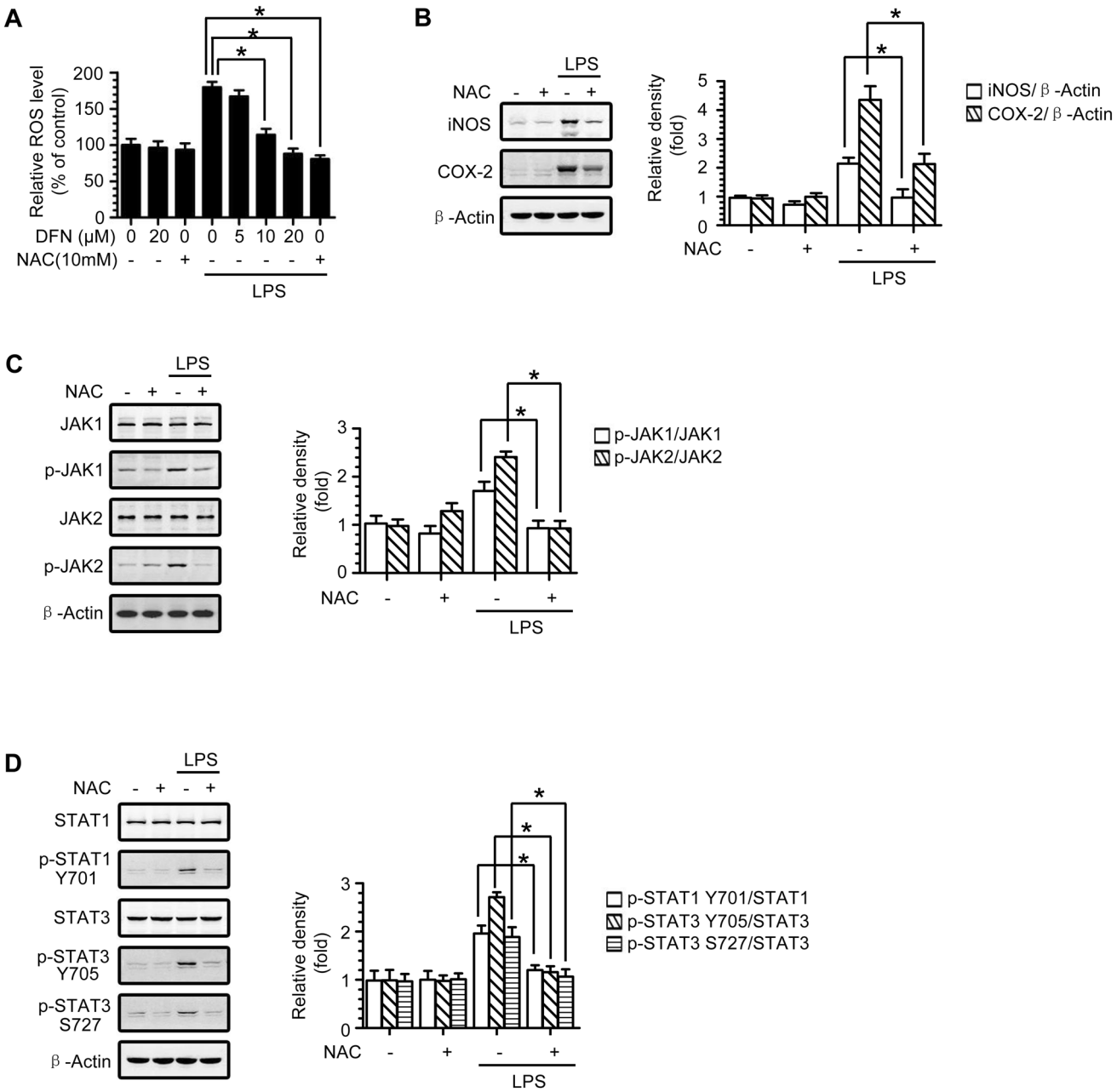

Fig. 4 Daphnetin reduces LPS-induced ROS production. **a** Raw264.7 cells were treated with daphnetin (DFN 20 μM) or NAC (10 mM) for 2 h, followed by 30 min treatment with LPS (100 ng/ml). The production of ROS was detected using DCFH-HA. NAC was used as a positive control. **b** Raw264.7 cells were treated with NAC (10 mM) for 2 h, followed by 16 h treatment with LPS. Immunoblotting and densitometric analysis showed the protein levels of iNOS and COX-2 in Raw264.7 cells. **c** Raw264.7 cells were treated with NAC (10 mM) for 2 h, followed by 1 h treatment with LPS (100 ng/ml). Immunoblotting and densitometric analysis showed the total JAK1, total JAK2, phosphorylated JAK1 and phosphorylated JAK2 protein levels in Raw264.7 cells. **d** Raw264.7 cells were treated with NAC (10 mM) for 2 h, followed by 4 h treatment with LPS (100 ng/ml). Immunoblotting and densitometric analysis showed the total STAT1, total STAT3, phosphorylated STAT1 (Y701), phosphorylated STAT3 (Y705) and phosphorylated STAT3 (S727) protein levels in Raw264.7 cells. The results were expressed as mean ± SD, $n = 3$. *$p < 0.05$, comparing with matched controls

and tissue injury. During inflammatory process, the macrophages are activated and produce a series of pro-inflammatory cytokines which are responsible for the pathphysiology of sepsis. Normally, the inflammatory responses can initiate tissue repair and eliminate cell injury. However, the uncontrolled inflammatory responses lead to tissue lesion, organ dysfunction, and even severe inflammatory responses syndrome with high fatality rate, such as

systemic inflammatory responses syndrome (SIRS) [6, 7, 40, 41]. Under LPS-induced endotoxemia, the acute lung injury is the excessive inflammatory response in lung and can process to the deficiency of lung [42]. In this study, we first investigated the anti-inflammatory activity of daphnetin in vivo and in vitro. We found that daphnetin obviously protected animals from mortality, reduced the LPS-induced alveolar edema and inflammation cells infiltration, and suppressed the production of pro-inflammatory factors including IL-1β, IL-6, and TNF-α (Figs. 1, 2). These observations are in agreement with the find of Yu et al., in which the authors found the anti-inflammatory and protect properties of daphnetin in endotoxin-induced lung injury [39]. Furthermore, we observed that daphnetin reduced of the production of NO and PGE2 (Figs. 1g, h, 2e, f). NO particularly works in the early stage of inflammation to regulate the transmission of inflammatory cells, and plays its role in almost all stages of inflammation. Though, PGE2 has been shown to have both anti- and pro-inflammatory actions. In some studies, PGE2 is considered as anti-inflammatory prostaglandin, whereas PGF2 is known as a inflammatory prostaglandin [43, 44]. It is also well accepted that PGE2 plays an important role in inflammatory response generating. In response to LPS stimulation, the biosynthesis of PGE2 is rapidly increased and it participates in the development of the cardinal signs (redness, heat, swelling, and pain) of acute inflammatory [45, 46]. In LPS-induced inflammation, NO and PGE2 are expressed by iNOS and COX-2, respectively [3, 4, 47]. We then observed that daphnetin suppressed the expression of iNOS and COX-2 in Raw264.7 cells (Fig. 2g, h). These results suggest that daphnetin exerts anti-inflammatory functions in LPS-induced inflammation both in vivo and in vitro.

LPS causes harmful disorders with complicated mechanism. During LPS-induced inflammatory response, the initiation of intracellular signaling cascades leads to the activation of macrophages, and contributes to the production of and the release of pro-inflammatory factors. STAT1 and STAT3 have been reported to regulate the expression of iNOS and COX-2 [18, 28, 48]. The inhibition of STATs activation is beneficial in inhibiting LPS-induced inflammatory process by reducing NO, IL-1β, IL-6, and IFN-γ production [24–27, 29, 36]. The phosphorylation of STATs is required for their nucleus import and transcriptional activity [49, 50]. Therefore, we investigate whether the anti-inflammatory effect of daphnetin is related to STATs activation in Raw264.7 cells. We found that daphnetin not only suppressed LPS-induced phosphorylation of STAT1 and STAT3, but also blocked the nucleus import of them (Fig. 3b, c). These results indicate that daphnetin suppresses LPS-induced inflammatory responses through, at least in part, inhibiting STAT1 and STAT3 activation. We further study the effect of daphnetin on the upstream signaling pathways of STATs. In response to cytokines stimulation, JAKs can be phosphorylated on tyrosine residues, and then activates downstream transcription factors including STATs [30, 32]. The JAK-STATs pathway shows important role in immune and inflammatory responses and is involved in LPS-induced iNOS expression [15, 16, 49]. As shown in Fig. 3d, we found that daphnetin suppressed the phosphorylation of JAK1 and JAK2. In addition, MAPKs can be activated by multiple intracellular disorders and environmental stimulation. In addition, the MAPKs can be involved in the phosphorylation of STATs, which is correlated to the production of IL-6 [13, 31, 51, 52]. However, in response to LPS stimulation, we did not observe the inhibitory effect of daphnetin on MAPKs (Fig. 3e). Furthermore, upon LPS stimulation, NF-κB signaling is also triggered and plays important role in regulating iNOS, COX-2, TNF-α, and IL-6 expression [23]. The LPS-induced phosphorylation of IKK disrupts the interaction between IκB and NF-κB, and facilitates the activation of NF-κB signaling [10, 53]. The interaction of STAT3 and NF-κB has also been found to suppress iNOS transcription [28]. The previous work showed an important role of NF-κB signaling and a potential cross-talk between NF-κB and STATs pathways. However, we did not observe the suppressive effect of daphnetin on the phosphorylation of IKK (Fig. 3a), indicating that the inhibitory role of daphnetin on inflammatory is not through NF-κB. These observations are inconsistent with the data of Yu et al., in which the authors reported that daphnetin inhibited NF-κB or MAPKs signaling. We reason that the discrepancy may result from the differences in the source, purity, and concentration of the daphnetin used. In this study, we only detected the phosphorylation of IKK to show the activation of NF-κB pathway. For more precise study, it is worth detecting the nucleus import of p65 and p50. Collectively, these foundings indicate that daphnetin suppresses LPS-induced inflammatory responses via inhibiting JAKs/STATs signaling. Moreover, daphnetin showed no notable effect on cell viability, suggesting the potential use of daphnetin in clinical activity.

ROS is a by-product of metabolism. In response to LPS stimulation, ROS is largely accumulated by the activated macrophages. Besides its role in differentiation and signaling transduction at normal concentrations, the excessive production of ROS disrupts the intracellular oxygen supply-demands balance and is lethal. The uncontrolled production of ROS can result in cell apoptosis, necrosis, and tissue injury [54]. As shown in Fig. 4a, daphnetin showed anti-oxidant activities by suppressing LPS-induced ROS production. As second messengers, ROS activates a series of signaling pathways including NF-κB, MAPKs, and JAK/STATs [52, 55–58]. Therefore, NAC was used as a positive control to study the effect of ROS in inflammatory response and the activation of JAK/STATs. We observed that NAC

suppressed the release of pro-inflammatory factors, inhibited the expression of iNOS and COX-2, and blocked the activation of JAK/STATs cascades (Fig. 4b–d). These results suggest that daphnetin exerts anti-oxidant activities which contribute to its suppressive effect on LPS-induced inflammatory response and the activation of JAK/STATs cascades. Given the potential link between ROS and STAT-related pro-inflammatory genes expression, further investigation can focus on the precise cross-talk in ROS and JAK-STATs cascades.

In summary, we demonstrate that daphnetin suppresses LPS-induced inflammatory responses, at least through inhibiting JAK/STATs activation and ROS production. This work not only highlights a potential clinic use of daphnetin in treating LPS-induced acute inflammation, but also provides a new insight into the underling molecular mechanism.

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Author contributions Conceived and designed the experiments: ZMY LS. Performed the experiments: LS. Analysis the data: ZMY L. Lan LS L. Luo TZ JW XMS. Contributed reagents/materials/analysis tools: ZMY L. Lan L. Luo LS. Wrote the paper: LS ZMY L. Luo.

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