

Broussonin E suppresses LPS-induced inflammatory response in macrophages *via* inhibiting MAPK pathway and enhancing JAK2-STAT3 pathway

HUANG Shao-Peng^{1Δ}, GUAN Xin^{2, 3Δ}, KAI Guo-Yin², XU Ya-Zhou³, XU Yuan⁴,
WANG Hao-Jie³, PANG Tao³, ZHANG Lu-Yong^{1, 3*}, LIU Ying^{1*}

¹ School of Basic Medicine, Center for Drug Screening and Pharmacodynamics Evaluation, School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, China;

² College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou 311402, China;

³ Jiangsu Key Laboratory of Drug Screening, State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China;

⁴ School of Medicine and Life Sciences, Nanjing University of Chinese Medicine, Nanjing 210023, China

Available online 20 May, 2019

[ABSTRACT] Macrophages play an important role in inflammation, and excessive and chronic activation of macrophages leads to systemic inflammatory diseases, such as atherosclerosis and rheumatoid arthritis. In this paper, we explored the anti-inflammatory effect of broussonin E, a novel phenolic compound isolated from the barks of *Broussonetia kanzinoki*, and its underlying molecular mechanisms. We discovered that Broussonin E could suppress the LPS-induced pro-inflammatory production in RAW264.7 cells, involving TNF- α , IL-1 β , IL-6, COX-2 and iNOS. And broussonin E enhanced the expressions of anti-inflammatory mediators such as IL-10, CD206 and arginase-1 (Arg-1) in LPS-stimulated RAW264.7 cells. Further, we demonstrated that broussonin E inhibited the LPS-stimulated phosphorylation of ERK and p38 MAPK. Moreover, we found that broussonin E could activate janus kinase (JAK) 2, signal transducer and activator of transcription (STAT) 3. Downregulated pro-inflammatory cytokines and upregulated anti-inflammatory factors by broussonin E were abolished by using the inhibitor of JAK2-STAT3 pathway, WP1066. Taken together, our results showed that broussonin E could suppress inflammation by modulating macrophages activation state *via* inhibiting the ERK and p38 MAPK and enhancing JAK2-STAT3 signaling pathway, and can be further developed as a promising drug for the treatment of inflammation-related diseases such as atherosclerosis.

[KEY WORDS] Broussonin E; Macrophage polarization; Inflammation; Janus kinase 2; Signal transducer and activator of transcription 3

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2019)05-0372-09

Introduction

Inflammation is a kind of defensive reaction in response

[Received on] 24-Mar.-2019

[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81703530 and 81773995), the Natural Science Foundation of Jiangsu Province (Nos. BK20160032 and BK20170859), the Opening Project of Zhejiang Provincial Preponderant and Characteristic Subject of Key University (Traditional Chinese Pharmacology), Zhejiang Chinese Medical University (No. ZYAOX2018001), the Six Talent Peaks Project of Jiangsu Province (PANG Tao).

[*Corresponding authors] E-mail: lyonzhang@163.com (ZHANG Lu-Yong); 2404600230@qq.com (LIU Ying)

^ΔThese authors contributed equally to this work.

These authors have no conflict of interest to declare.

Published by Elsevier B.V. All rights reserved

to pathogens and the damaged cells. Therefore, inflammation is an important part of the innate immune system in physical condition [1]. However, disordered and excessive inflammatory reactions will attack the body's healthy tissues and have been recognized as harmful factors in various diseases such as atherosclerosis and arthritis [2]. Lipopolysaccharide (LPS) is a major component of the cell walls of Gram-negative bacteria, which plays a crucial role in the pathophysiology of inflammatory responses [3]. LPS invasion activates immune cells to produce large amounts of cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-8 (IL-8), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 [4-5]. Therefore, interfering the inflammatory response caused by LPS have been well recognized in the development of anti-inflammatory drugs.

Macrophages are key cellular components of innate immunity and play as the first line in defense against pathogens and cellular debris [6]. In the face of exogenous stimuli, such as LPS, macrophages exhibit remarkable phenotypic plasticity and heterogeneity [7]. In response to different environments signals, macrophages can polarize into two phenotypes, including classically activated (M1) macrophages and alternatively activated (M2) macrophages, which play contrasting roles during pathogenesis or tissue damage [8-9]. Polarization of macrophage to classically phenotype (M1) is usually stimulated by T helper 1 (Th1) cytokines, such as TNF- α and IFN γ [10]. M1 macrophages produce high levels of pro-inflammatory cytokines including TNF- α , IL-6, IL-1 β , IL-12 and IL-23 [11-12]. Macrophage polarization into M2 phenotype is activated by Th2 cytokines like IL-13 and IL-14 and macrophage colony-stimulating factor (M-CSF). M2 macrophages produce anti-inflammatory factors including IL-10, arginase-1 (Arg-1) and CD206, contributing to the tissue healing and repairing [13]. Studies have shown that M1 macrophages and M2 macrophages are interchangeable. This transformation allows macrophages to play a dual role in the progression of diseases [14].

Mitogen-activating protein kinases (MAPKs) are a family of serine/threonine protein kinases that can be activated by different extracellular stimuli such as cytokines, neurotransmitters, hormones, cellular stress and cell adhesion [15-16]. In LPS-stimulated macrophages, three major members of MAPK family, the extracellular signal-regulated kinases (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) are induced phosphorylation to activate the transcription factors such as nuclear factor κ B (NF- κ B) and activator protein-1 (AP-1) and play an important role in inflammatory responses [17-18]. Therefore, the MAPK signaling pathway has become an inspiring target for the development of anti-inflammatory drugs.

Janus kinase 2 (JAK2)/signal transducer and suppressor of cytokine signaling 3 (STAT3) cascade is an essential inflammatory signaling pathway in mediating immune responses [19-20]. STAT3 signaling pathway has been reported to govern macrophage polarization to M2 phenotype [21]. Macrophages from STAT3 knockout mice release higher level of pro-inflammatory cytokines such as TNF- α [22]. Researches demonstrated that, JAK2/STAT3 signaling pathway is highly phosphorylated and activated in M2 macrophages [23]. The activation of STAT3 pathway contributes to the production of anti-inflammatory factors in macrophages [24].

In the present study, we discovered that broussonin E, a novel phenolic compound isolated from the barks of *Broussonetia kanzinoki*, exhibited anti-inflammatory effects in LPS-stimulated macrophages. Furthermore, we implied that the anti-inflammatory activity of broussonin E was dependent on inhibiting MAPKs (ERK, p38, but not JNK) pathway and stimulating JAK2/STAT3 pathways.

Materials and Methods

Materials

Broussonin E was purchased from BioBioPha (Kunming, China) and the purity of Broussonin E was about 96%. Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). The ELISA kit was purchased from Dakewe (Shen Zheng, China). Trizol reagent, cDNA synthesis kit and SYBR Green were purchased from Vazyme (Nanjing, China). Rabbit anti-p-ERK1/2, p-JNK and p-p38 MAPK and rabbit anti-ERK1/2, JNK and p38 MAPK and JAK2, STAT3 were obtained from Cell Signal Technology (Beverly, MA, USA). Rabbit anti-p-JAK2 (Tyr1008), p-STAT3 (Tyr705) and mouse anti- β -actin antibody were purchased from Abclonal (Wuhan, China). WP1066 was purchased from Beyotime Biotechnology (Shanghai, China).

Cell culture

RAW264.7 cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 100 U·mL⁻¹ penicillin and 100 μ g·mL⁻¹ streptomycin at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air. When reached 80% confluence, RAW264.7 cells were seeded onto 96-well plate at a density of 5 × 10⁴/mL cells or 24-well at a density of 1 × 10⁵/mL cells for further experiments.

MTT assay

Cell viability was tested by MTT assay. RAW 264.7 cells seeded in 96-well plate were pretreated with various concentrations of broussonin E for 24 h. Then the culture medium in each well were replaced with DMEM containing MTT (0.5 mg·mL⁻¹) and the cells were cultured for further 4 h. After that, 100 μ L of DMSO was added to each well, and the mixture was gently shook to fully dissolve the crystals and absorbance was measured at 570 nm in a Thermo plate-reader.

Measurement of TNF- α by ELISA

TNF- α protein released from macrophages was measured as previously described [25]. Briefly, the RAW264.7 cells were pretreated with different concentrations of broussonin E for 3 h and then incubated with LPS (100 ng·mL⁻¹) for another 3 h. The culture supernatant was collected for ELISA experiments. The content of TNF- α protein in culture medium was detected by using ELISA assay according to the manufacturer's instructions.

RNA isolation and quantitative PCR

The total RNA was extracted from RAW264.7 cells by using Trizol reagents. Isolated RNA was reverse-transcribed into cDNA using cDNA synthesis kit according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed as reported previously [26]. GAPDH was utilized as a control gene. Primers sequences for qPCR were listed in Table 1.

Table 1 Primers for RT-PCR

Gene	Sense (5'-3')	Anti-sense (5'-3')
GAPDH	AAC TTTGGCATTGTG GAAGG	GGATGCAGGGATGATGTTCT
TNF- α	GCTGAGCTCAAACCTGGTA	CGGACTCCGCAAAGTCTAAG
IL-1 β	TGTGAAATGCCATTGTA	GGTCAAAGGTTTGGAAAGCAG
IL-6	CCAGTTGCCTTCTTGGGACTG	CAGGTCTGTTGGGAGTGGTATCC
iNOS	CCCAGAGTTCAGCTTCTGG	CCAAGCCCCTCACCATTATCT
COX-2	TGGGGTGATGAGCAACTATT	AAGGAGCTCTGGGTCAAAC
IL-10	TACCTGGTAGAAGTGATGCC	CATCATGTATGCTTCTATGC
CD206	CTTCGGGCCTTTGGAATAAT	TAGAAGAGCCCTTGGGTTGA
Arginase-1	CTGGTCGGTTTGATGCTA	TGCTTAG CTCTGTCTGCTTTGC

Western blot analysis

RAW264.7 cells were collected and homogenized with RIPA buffer (Beyotime Biotechnology) containing protease inhibitors and phosphatase inhibitors (Roche, Basel, Switzerland). The extracted protein was quantified by bicinchoninic acid (BCA) protein assay kit (Thermo, Waltham, MA, USA). Western blot was performed as previously described [27]. Briefly, the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 3% bovine serum albumin (BSA, Sunshine Biotechnology, Nanjing, China) for 2 h at room temperature. Then the membranes were incubated with primary antibodies against p-ERK1/2 (1 : 1000), p-JNK (1 : 1000), p-p38 MAPK (1 : 1000), p-JAK2 (Tyr1008) (1 : 1000), p-STAT3 (Tyr705) (1 : 1000) and β -actin (1 : 2000) overnight at 4 °C. After washing adequately with TBST for five times, the membranes were incubated with the secondary antibodies at room temperature for 1 h. Then the membranes were washed for five times and the bands were visualized with a Bio-Rad Chemi-Doc XRS. The density of the western blot bands were quantified using Quantity one software (Bio-rad, CA, USA).

Statistical analysis

Statistical analysis were performed using GraphPad Prism Software 5 (La Jolla, CA, USA). All data were expressed as mean \pm standard deviation (SD) from at least three independent experiments. The differences were considered significant if the $P < 0.05$.

Results

Broussonin E dose-dependently inhibited LPS-induced TNF- α release

The chemical structure of broussonin E is shown in Fig. 1A. To determine the safe concentrations of broussonin E in RAW264.7 cells, various concentrations of broussonin E (2.5, 5, 10 and 20 $\mu\text{mol}\cdot\text{L}^{-1}$) were added to RAW 264.7 cells. MTT assay results indicated that Broussonin E (2.5, 5, 10 and 20 $\mu\text{mol}\cdot\text{L}^{-1}$) did not induce toxicity in RAW 264.7 cells (Fig. 1B). In order to measure the anti-inflammatory effect of broussonin E, RAW 264.7 cells were incubated with verifying concentrations of broussonin E for 3 h, followed by exposure

to LPS for 3 h. ELISA assay result showed that broussonin E dose-dependently inhibited LPS-induced TNF- α release in RAW264.7 cells (Fig. 1C). As the 20 $\mu\text{mol}\cdot\text{L}^{-1}$ concentration of broussonin E exhibited the maximum anti-inflammatory effect (Fig. 1C), we selected 20 $\mu\text{mol}\cdot\text{L}^{-1}$ broussonin E to be used in the further study on RAW264.7 cells. In addition, broussonin E significantly prevented LPS-induced TNF- α release in RAW264.7 cells when treated at 3 h or 1 h before LPS incubation, but had no effect on TNF- α release when added simultaneously with LPS or 1 h after LPS treatment (Fig. 1D).

Broussonin E inhibited the LPS-stimulated mRNA expression of proinflammatory factors

In order to further investigate the anti-inflammatory activity of broussonin E, the effects of broussonin E on TNF- α , iNOS, IL-6, IL-1 β and COX-2 mRNA expression were measured by qPCR. As demonstrated in Figs. 2A–E, LPS significantly increased the expression of TNF- α , IL-1 β , IL-6, iNOS and COX-2 mRNA in RAW 264.7 cells compared with control group. However, treatment with broussonin E significantly inhibited LPS-induced upregulation of TNF- α , iNOS, IL-6, IL-1 β and COX-2 mRNA. These results suggested that broussonin E effectively inhibited proinflammatory cytokine production in LPS-induced RAW264.7 cells.

Broussonin E increased the mRNA expression of anti-inflammatory mediators

We further performed qPCR assay to determine the effect of broussonin E on anti-inflammatory factors, including IL-10, CD206 and Arg-1 in LPS-induced RAW264.7. As illustrated in Figs. 3A–C, Broussonin E (20 $\mu\text{mol}\cdot\text{L}^{-1}$) treatment significantly upregulated the mRNA levels of IL-10, CD206 and Arg-1 compared with the LPS group. These results indicated that broussonin E promotes anti-inflammatory mediator expression in LPS-stimulated RAW264.7 cells.

Broussonin E decreased LPS-induced phosphorylation of ERK and p38 MAPK in LPS-stimulated RAW264.7 cells

To explore the target pathway underlying the effect of broussonin E on LPS-induced proinflammatory factors production, we further detected the changes in MAPK signaling pathway. RAW264.7 cells were pretreated with broussonin E for 3 h, and the further incubated with LPS for 15, 30, 60, 120 or 180 min. As shown in Fig. 4, LPS stimulation markedly

upregulated the phosphorylation of ERK, p38 and JNK MAPK in RAW 264.7 cells. Noticeably, LPS-induced phosphorylation of ERK and p38 MAPK was time-dependently attenuated by pretreated with broussonin E. However, brous-

sonin E treatment had no effect on LPS-stimulated phosphorylation of JNK MAPK. The results suggested that the MAPKs (ERK, p38, but not JNK) pathways were involved in the broussonin E-mediated inflammation inhibition.

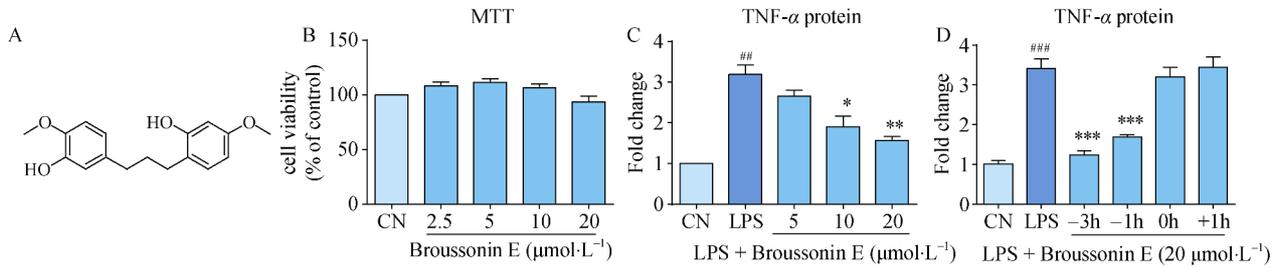


Fig. 1 Broussonin E dose-dependently inhibited LPS-induced TNF- α release. (A) Structure of broussonin E. (B) RAW264.7 cells were incubated with various concentrations of compound broussonin E for 24 h and MTT assay was utilized to measure the viability of RAW264.7 cells. (C) Broussonin E dose-dependently inhibited LPS-induced TNF- α release as determined by ELISA assay. RAW264.7 cells were incubated with different concentrations of compound broussonin E for 3 h, followed by incubated with 100 ng·mL⁻¹ LPS for another 3 h. (D) RAW264.7 cells were administrated with broussonin E at 3 h or 1 h before LPS addition, the same time with LPS addition or 1 h after LPS addition, followed by stimulation with 100 ng·mL⁻¹ LPS for another 3 h, and the protein level of TNF- α release in culture media was measured by ELISA assay. The experiments were performed in triplicate and repeated at least three times. Results are expressed as means \pm SD ($n = 3$). ^{##} $P < 0.01$ vs the control group (CN); * $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs the LPS group

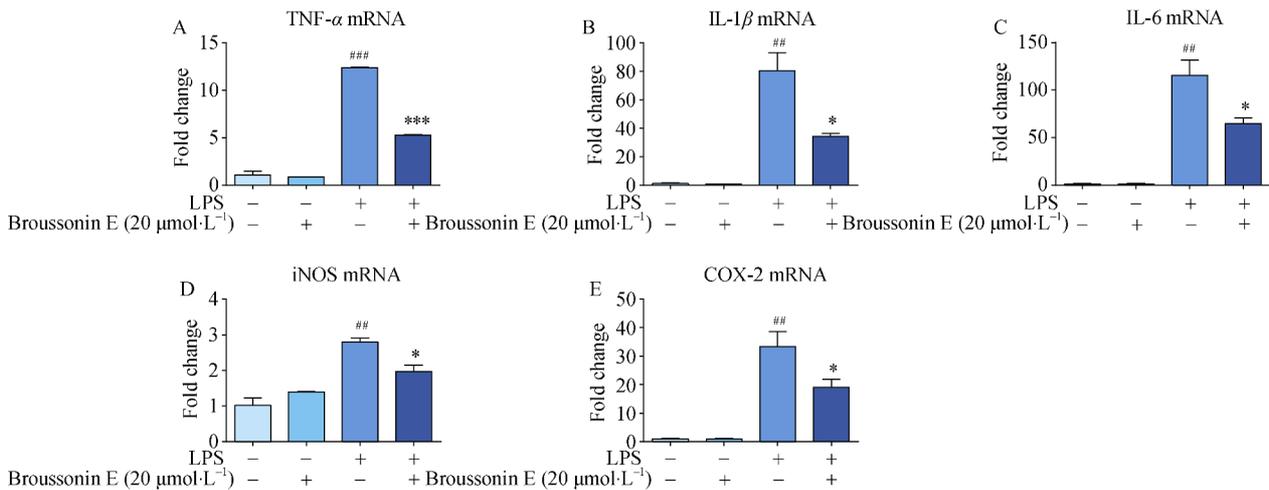


Fig. 2 Broussonin E inhibited the LPS-stimulated mRNA expression of proinflammatory factors. RAW264.7 cells were pretreated with compound broussonin E (20 $\mu\text{mol}\cdot\text{L}^{-1}$) for 3 h, followed by exposure to 100 ng·mL⁻¹ LPS for 3 h. Quantitative PCR results showed that broussonin E significantly inhibited LPS-induced mRNA level of TNF- α (A), IL-1 β (B), IL-6 (C), iNOS (D) and COX-2 (E). The experiments were performed in triplicate and repeated at least three times. Results are expressed as means \pm SD ($n = 3$). ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs the untreated group; * $P < 0.05$, ^{***} $P < 0.001$ vs the LPS group

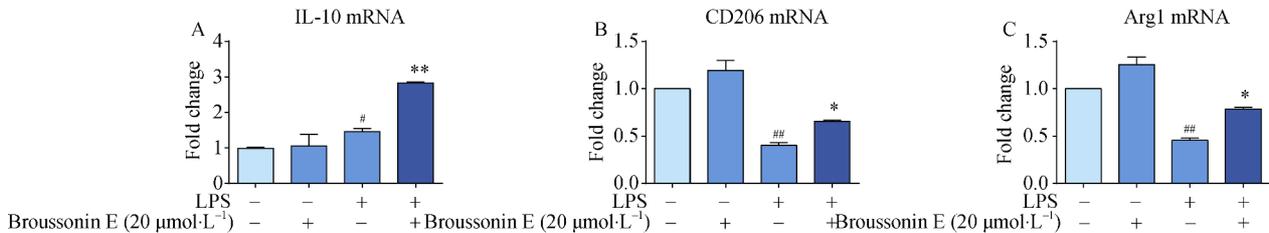


Fig. 3 Broussonin E promoted the mRNA expression of anti-inflammatory mediators. RAW264.7 cells were pretreated with broussonin E (20 $\mu\text{mol}\cdot\text{L}^{-1}$) for 3 h, followed by incubated with 100 ng·mL⁻¹ LPS for 9 h. Quantitative PCR results indicated that broussonin E significantly upregulated the mRNA level of anti-inflammatory mediators, including IL-10 (A), CD206 (B) and Arginase-1 (C). The experiments were performed in triplicate and repeated at least three times. Results are expressed as means \pm SD ($n = 3$). ^{##} $P < 0.01$ vs the untreated group; * $P < 0.05$ vs the LPS group

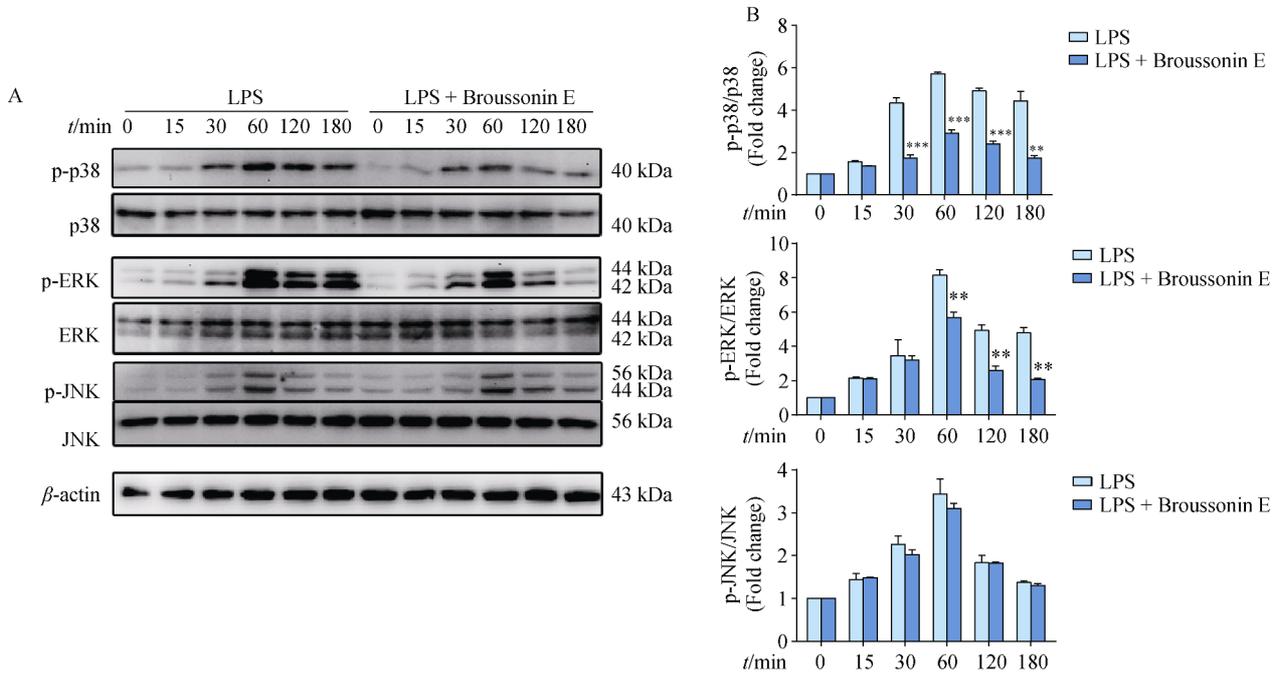


Fig. 4 Broussonin E inhibits p-ERK and p-p38 MAPK, but not p-JNK MAPK expression in LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells were pretreated with broussonin E ($20 \mu\text{mol}\cdot\text{L}^{-1}$) for 3 h and then further incubated with LPS ($100 \text{ ng}\cdot\text{mL}^{-1}$) for indicated times. The expressions of p-ERK, p-p38 and p-JNK MAPK were determined by Western blot. (B) Statistical analysis of Western blot results. Results are expressed as means \pm SD ($n = 3$). ** $P < 0.01$, *** $P < 0.001$ vs the corresponding time point in LPS groups

Broussonin E activated JAK2/STAT3 signaling pathway in RAW264.7 cells.

The JAK2-STAT3 signaling pathway plays a crucial role in M2 macrophage polarization. Thus we tested the effect of broussonin E on the phosphorylation levels of JAK2 and

STAT3 in RAW264.7 cells by using Western blot. As described in Fig. 5, treated with broussonin E significantly upregulated the protein levels of p-JAK2 and p-STAT3 in RAW264.7 cells. The results revealed that broussonin E could activate JAK2-STAT3 pathway.

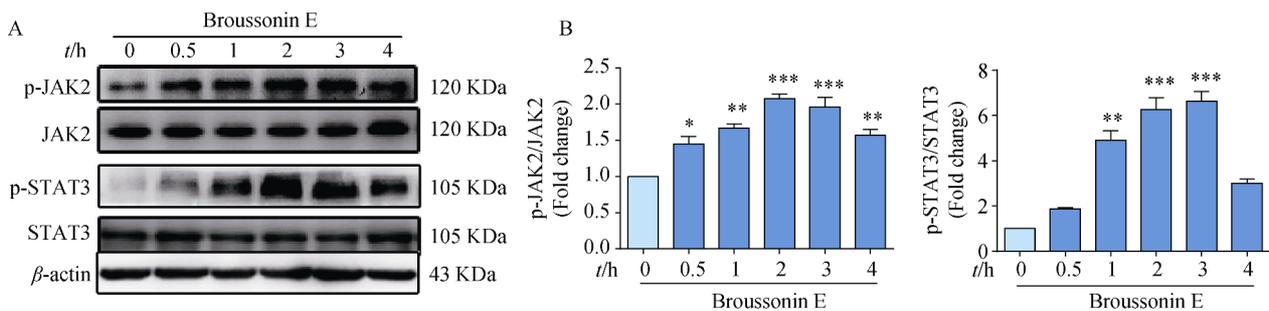


Fig. 5 Broussonin E activated JAK2-STAT3 signaling pathway in RAW264.7 cells. RAW264.7 cells were treated with broussonin E ($20 \mu\text{mol}\cdot\text{L}^{-1}$) for indicated times, and the phosphorylation of JAK2 and STAT3 was determined by Western blot. (B) Statistical analysis of Western blot results. Results are expressed as means \pm SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control group (0 h)

Broussonin E inhibited M1 macrophages polarization and enhanced M2 macrophages polarization via JAK2/STAT3 signaling pathway.

To further investigate whether JAK2/STAT3 signaling pathway was involved in Broussonin E effects on modulating macrophages polarization, the specific inhibitor of JAK2-STAT3 pathway, WP1066 was utilized in the following

study. As demonstrated in Figs. 6A–B, enhanced phosphorylation of JAK2 and STAT3 by Broussonin E was abolished by WP1066. Figs. 6C–E showed that Broussonin E significantly inhibited production of $\text{TNF}\cdot\alpha$, $\text{IL}\cdot 1\beta$, COX-2 in LPS-induced RAW264.7 cells, which was reversed by the administration of WP1066. Furthermore, the upregulated M2 macrophage associated mediators (IL-10, CD206, and

Arg-1) by broussonin E treatment were inhibited by WP1066 (Figs. 6F–H). These results suggested that brou-

ssonin E promoted macrophage M2 polarization by activating the JAK-STAT3 signaling pathway.

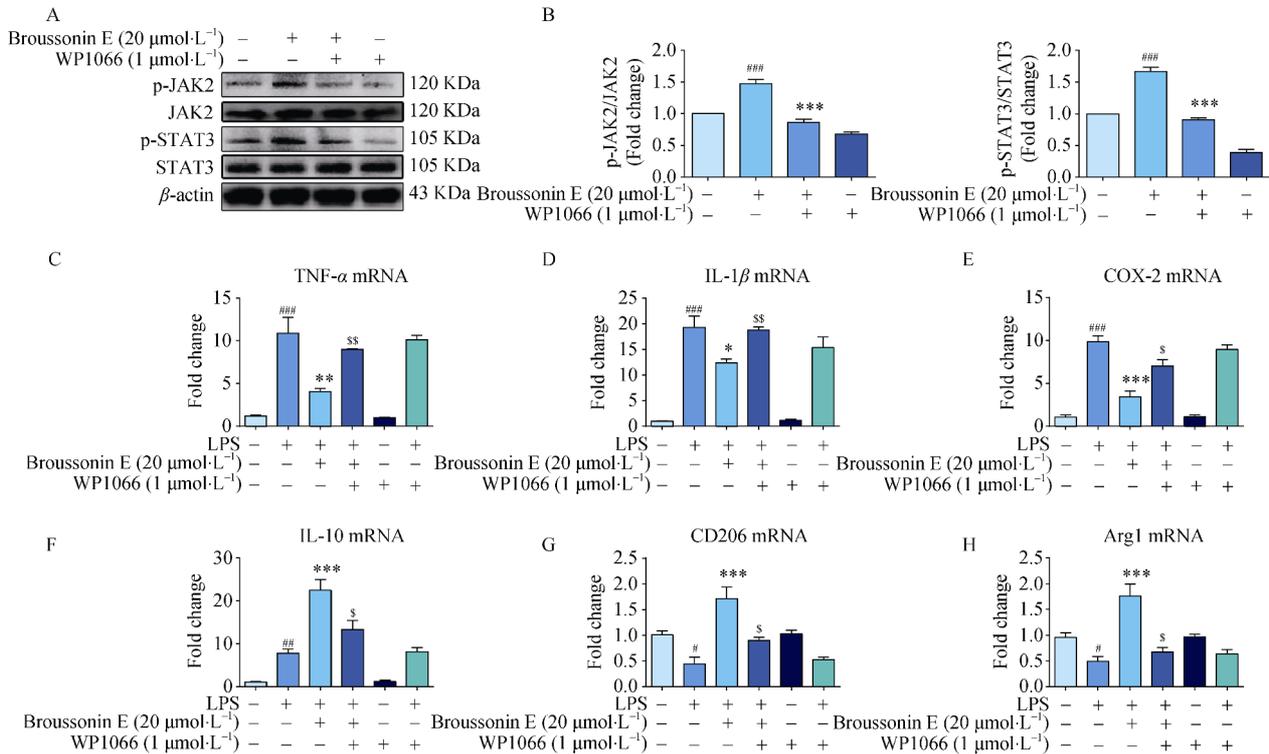


Fig. 6 Broussonin E suppressed M1 macrophage polarization and promoted M2 macrophage polarization via JAK2/STAT3 signaling pathway. (A–B) RAW264.7 cells were pretreated with WP1066 (1 μmol·L⁻¹) for 12h and then co-treated with broussonin E for 3 h. The phosphorylation of JAK2 and STAT3 was determined by Western blot. Results are expressed as means ± SD (n = 3). ###P < 0.001 vs the untreated group; ***P < 0.001 vs broussonin E group. (C–E) RAW264.7 cells were pretreated with WP1066 (1 μmol·L⁻¹) for 12 h, and then incubated with broussonin E (20 μmol·L⁻¹) for 3 h, followed by exposure to 100 ng·mL⁻¹ LPS for another 3 h. The mRNA expression of proinflammatory factors, including TNF-α, IL-1β and COX-2 was measured by using qPCR. (F–H) RAW264.7 cells were pretreated with WP1066 (1 μmol·L⁻¹) for 12 h, and then incubated with broussonin E (20 μmol·L⁻¹) for 3 h, followed by exposure to 100 ng·mL⁻¹ LPS for another 9 h. The mRNA expression of anti-inflammatory factors, including IL-10, CD206 and Arginase-1 was determined by using qPCR. Results are expressed as means ± SD (n = 3). #P < 0.05, ##P < 0.01, ###P < 0.001 vs the untreated group (CN); *P < 0.05, **P < 0.01, ***P < 0.001 vs the LPS group; §P < 0.05, §§P < 0.01 vs the LPS + broussonin E group

Discussion

Inflammation, a self-protected immune process in physical condition, is largely adjusted by macrophages during the innate immune response [28]. Macrophages show a great phenotypic plasticity and heterogeneity and polarize into classically activated (M1) macrophages and alternatively activated (M2) macrophages in response to different stimuli [10]. M1 macrophages polarization are induced when exposed to inflammatory stimuli such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ) and considered as proinflammatory phenotype, whereas M2 phenotype can be stimulated by inflammatory cytokines such as interleukin-4 (IL-4) or IL-13, which are considered as an anti-inflammatory phenotype [29–30]. Various studies have demonstrated that macrophages polarize to M1 phenotype and secrete large amounts of the inflammatory mediators such as TNF-α, IL-1β, IL-6, PGE2 and NO

after exposure to LPS stimuli [31]. LPS stimulation model is well-established for the research of inflammation in macrophages, and therefore applied as an *in vitro* model in the present study.

Broussonin E, a novel phenolic compound isolated from the barks of *Broussonetia kanzinoki*, has been reported to exert anti-tumor activity and induce NF-κB inhibition in hepatocellular carcinoma cells [32]. In this study, we described that broussonin E reduced TNF-α release in LPS-stimulated RAW264.7 cells with a dose-dependent manner and showed no obvious cytotoxicity. Furthermore, we discovered that treatment with broussonin E significantly inhibited the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, iNOS and COX-2 and enhanced the expression of anti-inflammatory mediators such as IL-10, CD206 and Arg-1 in LPS-stimulated RAW264.7 cells. These results indicated that broussonin E reversed LPS-induced M1 macrophages

polarization and promoted macrophages conversion to M2 phenotype.

MAPKs are a family of intracellular protein kinases, and the activity of MAPKs is adjusted by the phosphorylation of their activation loop on a conserved residues during inflammatory process [33]. MAPKs can be activated by various pro-inflammatory stimuli and stress. Therefore, MAPKs play a significant role in response to inflammatory condition [34]. Once activated by LPS, MAPKs can phosphorylate and activate other kinases or transcription factors in the cytoplasm and nucleus such as NF- κ B [35]. Accumulating evidences suggested that inhibiting the activity of MAPK signaling cascades can exhibit anti-inflammatory effects [15-16, 36]. Consistent with previous reports, our results described that MAPK (ERK, p38, JNK) signaling pathway could be phosphorylated and activated after exposure to LPS in RAW264.7 cells. Moreover, treatment with broussonin E inhibited the expression of phosphorylated ERK and p38 MAPK, but had no influence on JNK MAPK phosphorylation. These results implied that inhibition of MAPK (ERK and p38, but not JNK) signaling pathway may be responsible for the anti-inflammatory effect of broussonin E.

JAK2/STAT3 signaling pathway has been reported to regulate the expression of cytokines in response to inflammatory stimuli [37-38]. Numbers of papers describe that suppressing JAK/STAT3 pathway contributes to preventing LPS-induced inflammatory response [37-40]. Emerging evidences show that STAT3 serves as one of the primary transcription factors for macrophage polarization to M2 phenotype and JAK2/STAT3 activation results in production of anti-inflammatory factors such as IL-10 and Arg-1 [9, 41-42]. In this study, we detected the effect of broussonin E on the

JAK2/STAT3 signaling pathway in RAW264.7 cells. The results showed that broussonin E treatment promoted the phosphorylation of JAK2 (Tyr1008) and STAT3 (Tyr705). Furthermore, pre-treated with the inhibitor of JAK2/STAT3 pathway, WP1066, abolished the upregulation of anti-inflammatory mediators (IL-10, Arg-1 and CD206) by broussonin E. These findings indicated that the effect of broussonin E on promoting M2 macrophages polarization was dependent on the regulation of JAK2/STAT3 signaling pathway.

In summary, broussonin E was demonstrated to attenuate the expression of pro-inflammatory cytokines and enhance the expression of anti-inflammatory factors such as IL-10, CD206 and Arg-1 in LPS-stimulated RAW264.7 cells. And the effect of broussonin E on promoting macrophages to M2 phenotype were mediated by inhibiting ERK and p38 MAPK and activating JAK2/STAT3 pathway (Fig. 7). Thus, we anticipate that broussonin E may serve as a promising agent for the treatment of inflammation-related diseases such as atherosclerosis and arthritis. However, more experiments on rodent and non-human primate models should be conducted to further study the anti-inflammatory effect of broussonin E, which may provide more evidence for broussonin E translation to clinical application.

References

- [1] Park SB, Park GH, Kim HN, et al. Anti-inflammatory effect of the extracts from the branch of *Taxillus yadoriki* being parasitic in *Neolitsea sericea* in LPS-stimulated RAW264.7 cells [J]. *Biomed Pharmacother*, 2018, **104**: 1-7.
- [2] Du C, Bhatia M, Tang SC, et al. Mediators of inflammation: Inflammation in cancer, chronic diseases, and wound healing [J]. *Mediators Inflamm*, 2015, **2015**: 570653.
- [3] Breithaupt H. The new antibiotics [J]. *Nat Biotechnol*, 1999, **17**(12): 1165-1169.
- [4] Sun Y, Shang D. Inhibitory effects of antimicrobial peptides on lipopolysaccharide-induced inflammation [J]. *Mediators Inflamm*, 2015, **2015**: 167572.
- [5] Jin X, Han J, Yang S, et al. 11-O-Acetylcyathatriol inhibits MAPK/p38-mediated inflammation in LPS-activated RAW 264.7 macrophages and has a protective effect on ethanol-induced gastric injury [J]. *Mol Med Rep*, 2016, **14**(1): 874-880.
- [6] Parisi L, Gini E, Baci D, et al. Macrophage polarization in chronic inflammatory diseases: Killers or builders? [J]. *J Immunol Res*, 2018, **14**: 8917804.
- [7] Chistiakov DA, Myasoedova VA, Revin VV, et al. The impact of interferon-regulatory factors to macrophage differentiation and polarization into M1 and M2 [J]. *Immunobiology*, 2018, **223**(1): 101-111.
- [8] Koh YC, Yang G, Lai CS, et al. Chemopreventive effects of phytochemicals and medicines on M1/M2 polarized macrophage role in inflammation-related diseases [J]. *Int J Mol Sci*, 2018, **19**(8): 2208.
- [9] Zhang X, Xu F, Liu L, et al. (+)-Borneol improves the efficacy

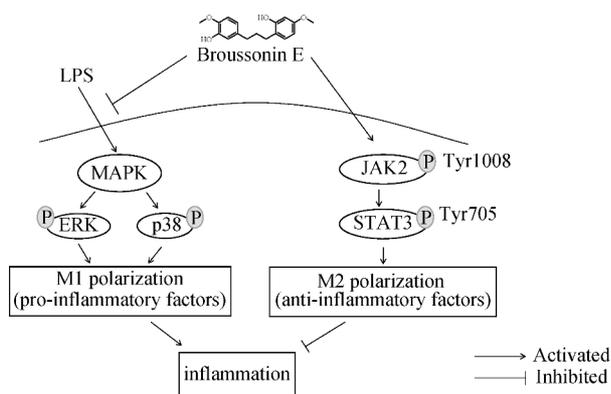


Fig. 7 Potential anti-inflammatory mechanism of broussonin E on activated RAW 264.7 cells. Broussonin E suppresses inflammation by inhibiting the expression of pro-inflammatory factors (TNF- α , IL-1 β , iNOS and COX-2) and enhancing the expression of anti-inflammatory factors (IL-10, CD206 and Arg-1) in LPS-stimulated RAW264.7 cells. And the effect of broussonin E on promoting macrophages to M2 phenotype is mediated by inhibiting ERK and p38 MAPK and activating JAK2/STAT3 pathway.

- of edaravone against DSS-induced colitis by promoting M2 macrophages polarization via JAK2-STAT3 signaling pathway [J]. *Int Immunopharmacol*, 2017, **53**: 1-10.
- [10] Abdolmaleki F, Gheibi Hayat SM, Bianconi V, et al. Atherosclerosis and immunity: A perspective [J]. *Trends Cardiovasc Med*, 2018. DOI: 10.1016/j.tcm.2018.09.017
- [11] Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development [J]. *J Clin Invest*, 2007, **117**(5): 1155-1166.
- [12] Verreck FA, de Boer T, Langenberg DM, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco) bacteria [J]. *Proc Natl Acad Sci*, 2004, **101**(13): 4560-4565.
- [13] Labonte AC, Tosello-Trampont AC, Hahn YS. The role of macrophage polarization in infectious and inflammatory diseases [J]. *Mol Cells*, 2014, **37**(4): 275-285.
- [14] Eichenfield DZ, Troutman TD, Link VM, et al. Tissue damage drives co-localization of NF- κ B, Smad3, and Nrf2 to direct Rev-erb sensitive wound repair in mouse macrophages [J]. *Elife*, 2016, **5**: e13024.
- [15] Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits [J]. *Biochim Biophys Acta*, 2005, **1754**(1-2): 253-262.
- [16] Yong HY, Koh MS, Moon A. The p38 MAPK inhibitors for the treatment of inflammatory diseases and cancer [J]. *Expert Opin Investig Drugs*, 2009, **18**(12): 1893-1905.
- [17] Cheng Y, Li X, Tse HF, et al. Gallic acid-L-leucine conjugate protects mice against LPS-induced inflammation and sepsis via correcting proinflammatory lipid mediator profiles and oxidative stress [J]. *Oxid Med Cell Longev*, 2018, **2018**: 1081287.
- [18] Yang Y, Kim SC, Yu T, et al. Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses [J]. *Mediators Inflamm*, 2014, **2014**: 352371.
- [19] Park EJ, Park SY, Joe EH, et al. 15d-PGJ2 and rosiglitazone suppress Janus kinase-STAT inflammatory signaling through induction of suppressor of cytokine signaling 1 (SOCS1) and SOCS3 in glia [J]. *J Biol Chem*, 2003, **278**(17): 14747-14752.
- [20] Okugawa S, Ota Y, Kitazawa T, et al. Janus kinase 2 is involved in lipopolysaccharide-induced activation of macrophages [J]. *Am J Physiol Cell Physiol*, 2003, **285**(2): C399-408.
- [21] Choi JW, Kwon MJ, Kim IH, et al. *Pyropia yezoensis* glycoprotein promotes the M1 to M2 macrophage phenotypic switch via the STAT3 and STAT6 transcription factors [J]. *Int J Mol Med*, 2016, **38**(2): 666-674.
- [22] Matsukawa A, Kudo S, Maeda T, et al. Stat3 in resident macrophages as a repressor protein of inflammatory response [J]. *J Immunol*, 2005, **175**(5): 3354-3359.
- [23] Hollander L, Guo X, Velazquez H, et al. Renalase expression by melanoma and tumor-associated macrophages promotes tumor growth through a STAT3-mediated mechanism [J]. *Cancer Res*, 2016, **76**(13): 3884-3894.
- [24] Lin N, Yao XG, Li NF. Research advances in suppressor of cytokine signaling 3 [J]. *Acta Acad Med Sci*, 2012, **34**(2): 178-182.
- [25] Wang Y, Ruan W, Mi J, et al. Balasubramide derivative 3C modulates microglia activation via CaMKK β -dependent AMPK/PGC-1 α pathway in neuroinflammatory conditions [J]. *Brain Behav Immun*, 2018, **67**: 101-117.
- [26] Ruan W, Li J, Xu Y, et al. MALAT1 up-regulator polydatin protects brain microvascular integrity and ameliorates stroke through C/EBP β /MALAT1/CREB/PGC-1 α /PPAR γ pathway [J]. *Cell Mol Neurobiol*, 2019, **39**(2): 265-286.
- [27] Xu Y, Kabba JA, Ruan W, et al. The PGC-1 α activator ZLN005 ameliorates ischemia-induced neuronal injury *in vitro* and *in vivo* [J]. *Cell Mol Neurobiol*, 2018, **38**(4): 929-939.
- [28] Woo JH, Lee JH, Kim H, et al. Control of inflammatory responses: a new paradigm for the treatment of chronic neuronal diseases [J]. *Exp Neurobiol*, 2015, **24**(2): 95-102.
- [29] Atri C, Guerfali FZ, Laouini D. Role of human macrophage polarization in inflammation during infectious diseases [J]. *Int J Mol Sci*, 2018, **19**(6): 1801.
- [30] Gordon S. Phagocytosis: An immunobiologic process [J]. *Immunity*, 2016, **44**(3): 463-475.
- [31] Philip S, Tom G, Vasumathi AV. Evaluation of the anti-inflammatory activity of *Tinospora cordifolia* (Willd.) Miers chloroform extract-a preclinical study [J]. *J Pharm Pharmacol*, 2018, **70**(8): 1113-1125.
- [32] Yao GD, Sun Q, Song XY, et al. 1, 3-Diphenylpropanes from *Daphne giraldii* induced apoptosis in hepatocellular carcinoma cells through nuclear factor kappa-B inhibition [J]. *Bioorg Chem*, 2018, **77**: 619-624.
- [33] Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation [J]. *Physiol Rev*, 2001, **81**(2): 807-869.
- [34] Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions [J]. *Endocr Rev*, 2001, **22**(2): 153-183.
- [35] Herlaar E, Brown Z. p38 MAPK signalling cascades in inflammatory disease [J]. *Mol Med Today*, 1999, **5**(10): 439-447.
- [36] Wang Y, Huang Y, Xu Y, et al. A dual AMPK/Nrf2 activator reduces brain inflammation after stroke by enhancing microglia M2 polarization [J]. *Antioxid Redox Signal*, 2018, **28**(2): 141-163.
- [37] Qi Z, Yin F, Lu L, et al. Baicalein reduces lipopolysaccharide-induced inflammation via suppressing JAK/STATs activation and ROS production [J]. *Inflamm Res*, 2013, **62**(9): 845-855.
- [38] Zhou Y, Wang J, Yang W, et al. Bergapten prevents lipopolysaccharide-induced inflammation in RAW264.7 cells through suppressing JAK/STAT activation and ROS production and increases the survival rate of mice after LPS challenge [J]. *Int Immunopharmacol*, 2017, **48**: 159-168.
- [39] Yu Q, Zeng KW, Ma XL, et al. Ginsenoside Rk1 suppresses pro-inflammatory responses in lipopolysaccharide-stimulated RAW264.7 cells by inhibiting the Jak2/Stat3 pathway [J]. *Chin J Nat Med*, 2017, **15**(10): 751-757.

- [40] Wang LC, Liao LX, Zhao MB, *et al.* Protosappanin A exerts anti-neuroinflammatory effect by inhibiting JAK2-STAT3 pathway in lipopolysaccharide-induced BV2 microglia [J]. *Chin J Nat Med*, 2017, **15**(9): 674-679.
- [41] Goswami KK, Sarkar M, Ghosh S, *et al.* Neem leaf glycoprotein regulates function of tumor associated M2 macrophages in hypoxic tumor core: Critical role of IL-10/STAT3 signaling [J]. *Mol Immunol*, 2016, **80**: 1-10.
- [42] Luo YP, Zhou H, Krueger J, *et al.* The role of proto-oncogene Fra-1 in remodeling the tumor microenvironment in support of breast tumor cell invasion and progression [J]. *Oncogene*, 2010, **29**(5): 662-673.

Cite this article as: HUANG Shao-Peng, GUAN Xin, KAI Guo-Yin, XU Ya-Zhou, XU Yuan, WANG Hao-Jie, PANG Tao, ZHANG Lu-Yong, LIU Ying. Broussonin E suppresses LPS-induced inflammatory response in macrophages *via* inhibiting MAPK pathway and enhancing JAK2-STAT3 pathway [J]. *Chin J Nat Med*, 2019, **17**(5): 372-380.