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ORIGINAL ARTICLE

# DUSP6 Inhibitor (E/Z)-BCI Hydrochloride Attenuates Lipopolysaccharide-Induced Inflammatory Responses in Murine Macrophage Cells *via* Activating the Nrf2 Signaling Axis and Inhibiting the NF-κB Pathway

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*Abstract*— Macrophages play a fundamental role in human chronic diseases such as rheumatoid arthritis, atherosclerosis, and cancer. In the present study, we demonstrated that dual-specificity phosphatase 6 (DUSP6) was upregulated by lipopolysaccharide (LPS) treatment of macrophages. (E/Z)-BCI hydrochloride (BCI) functions as a small molecule inhibitor of DUSP6, and BCI treatment inhibited DUSP6 expression in LPS-activated macrophages. BCI treatment inhibited LPS-triggered inflammatory cytokine production, including IL-1β and IL-6, but not TNF-α, and also affected macrophage polarization to an M1 phenotype. In addition, BCI treatment decreased reactive oxygen species (ROS) production and significantly elevated the levels of Nrf2. Interestingly, pharmacological inhibition of DUSP6 attenuated LPS-induced inflammatory responses was independent of extracellular signal-regulated kinase (ERK) signaling. Furthermore, BCI treatment inhibited phosphorylation of P65 and nuclear P65 expression in LPS-activated macrophages. These results demonstrated that pharmacological inhibition of DUSP6 attenuated LPS-induced inflammatory mediators and ROS production in macrophage cells *via* activating the Nrf2 signaling axis and inhibiting the NF-κB pathway. These anti-inflammatory effects indicated that BCI may be considered as a therapeutic agent for blocking inflammatory disorders.

**KEY WORDS:** Dusp6; inflammation; LPS; macrophage; BCI.

## INTRODUCTION

Inflammation, a fundamental innate immune response caused by noxious stimuli such as infection and necrotic tissues, is commonly characterized by production of proinflammatory cytokines [1]. Evidence has demonstrated that inflammation is closely associated with the pathogenesis of human chronic diseases such as rheumatoid arthritis [2], atherosclerosis [3], diabetes and obesity [4], and cancer [5]. Hence, it is very important to control inflammatory processes in order to defend against these inflammation-associated diseases.

Macrophages are phagocytic cells derived from circulating monocytes and lymphocytes and are present in all mammalian tissue types [6, 7]. Macrophages are considered vital regulators and effectors of innate immune responses and are activated by external irritants and stress, such as cytokines, lipopolysaccharide (LPS), and the tumor

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microenvironment. Furthermore, in response to stimulation by pathogens and the local environment, macrophages polarize to the M1 (proinflammatory) phenotype or M2 (anti-inflammatory) phenotype [8]. LPS is found in the outer membrane of Gram-negative bacteria, and induces macrophage inflammatory responses and causes generation of proinflammatory mediators, including interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and reactive oxygen species (ROS) [9, 10].

Dual-specificity phosphatase 6 (DUSP6, also known as MKP3 or PYST1), is a member of the family of mitogen-activated protein kinase (MAPK) phosphatases that work as feedback regulators of the MAPK cascade [11, 12]. DUSP6 can also function as an oncogene and has tumor-suppressive effects depending on the type of cancer [13], and it also plays a vital role in embryogenesis [14], heart development [15], metabolism [16], and colitis [17]. DUSP6 has also been reported to work as an important mediator of inflammatory processes in T cell immunity and T ($T_{FH}$) cell differentiation [18, 19]. (E/Z)-BCI hydrochloride (BCI) is a small molecule inhibitor of DUSP6. As shown using a transgenic zebrafish chemical screening assays, BCI treatment blocked DUSP6 activity and enhanced FGF target gene expression in zebrafish embryos [20]. BCI treatment also suppressed gastric cancer growth and metastasis [21] and improved zebrafish cardiac regeneration [22]. However, little is known regarding the mechanism of BCI in LPS-induced proinflammatory responses in macrophages.

Oxygen metabolism also has a fundamental role in the pathogenesis of inflammatory responses. Increasing evidence suggests that ROS are key signaling molecules that play an important role in the progression of inflammatory disorders [23]. The transcription factor nuclear factor E2-related factor or nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is an essential regulator of oxidative stress and inflammation. In response to oxidative stress, Nrf2 is released from Kelch-like ECH-associated protein 1, after which it translocates to the nucleus, binds to the antioxidant response element (ARE), and triggers the expression of ARE-regulated genes [24].

The transcription factor NF-κB regulates immunity by controlling the expression of genes involved in inflammation. Activation of NF-κB is through two different pathways including the canonical and alternative pathways. The canonical pathway is triggered by TLRs and proinflammatory cytokines, resulting in the activation of RelA, whereas the alternative NF-κB pathway is activated by LTβ, CD40L, BAFF, and RANKL, leading to activation of RelB/p52 complexes [25]. LPS treatment activates several intracellular signaling pathways that include the IkappaB kinase-NF-kappaB pathway in macrophage cells.

Here, we demonstrated the role of DUSP6 inhibitor BCI in LPS-triggered inflammation. We found that DUSP6 was upregulated in both mouse macrophage cells and human macrophage cells by LPS. BCI inhibited IL-1β and IL-6 expression, and ROS production after LPS treatment. In addition, BCI regulated LPS-induced proinflammatory processes in macrophages *via* inhibiting the NF-κB pathway and activating the Nrf2 pathway. Our findings demonstrate that DUSP6 is a potential therapeutic target to control the immune response.

## RESULTS

### DUSP6 Was Upregulated in LPS-Induced Murine Macrophages

It is well-known that macrophages produce inflammatory cytokines after LPS treatment, and are primary responders of inflammatory responses. In the previous study, RAW264.7 macrophage cells and peritoneal macrophages were used to measure the effect of LPS treatment [26, 27]. To determine the role of DUSP6 in LPS-activated murine macrophages, RAW264.7 macrophage cells and peritoneal macrophages were treated with different concentrations of LPS. DUSP6 mRNA was slightly upregulated with 50 ng/mL LPS treatment for 24 h. However, with increasing LPS concentrations, the mRNA of DUSP6 was significantly upregulated (Fig. 1a–b), suggesting that DUSP6 played an important role in regulation of LPS-induced inflammatory processes in murine macrophages.

### Pharmacological Inhibition of DUSP6 Inhibited Inflammatory Cytokines in LPS-Induced Murine Macrophages

The structure of BCI, a DUSP6 inhibitor, is shown in Fig. 2a. To investigate the functional effects of BCI in LPS-triggered inflammatory responses, RAW264.7 macrophage cells were treated with 100 ng/mL BCI for 24 h. DUSP6 protein was significantly downregulated with BCI treatment (Fig. 2b). To further examine the anti-inflammatory effect of BCI in LPS-induced macrophages, RAW264.7 macrophages were then treated with 100 ng/mL LPS plus 0–1 nM BCI for 24 h. RT-qPCR was used to determine the expression of inflammatory cytokine mRNA levels for IL-1β, IL-6, and TNF-α. With BCI treatment, the expression of IL-1β and IL-6 mRNA was significantly inhibited in LPS-activated macrophages (Fig. 2c, d). However, the TNF-α mRNA expression was suppressed in LPS-activated RAW264.7 macrophage cells (Fig. 2e).

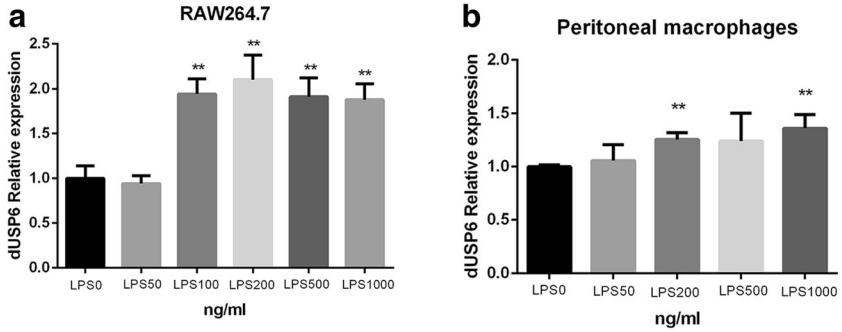


**Fig. 1.** DUSP6 was upregulated in LPS-activated macrophages. RAW264.7 macrophage cells and peritoneal macrophages were treated with 0–1000 ng/mL lipopolysaccharide for 24 h. (**a**, **b**) RT-qPCR was performed to determine DUSP6 mRNA expression in RAW264.7 macrophage cells (**a**) and peritoneal macrophages (**b**). β-actin was used as the internal control. Data are shown as the mean ± SD of at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

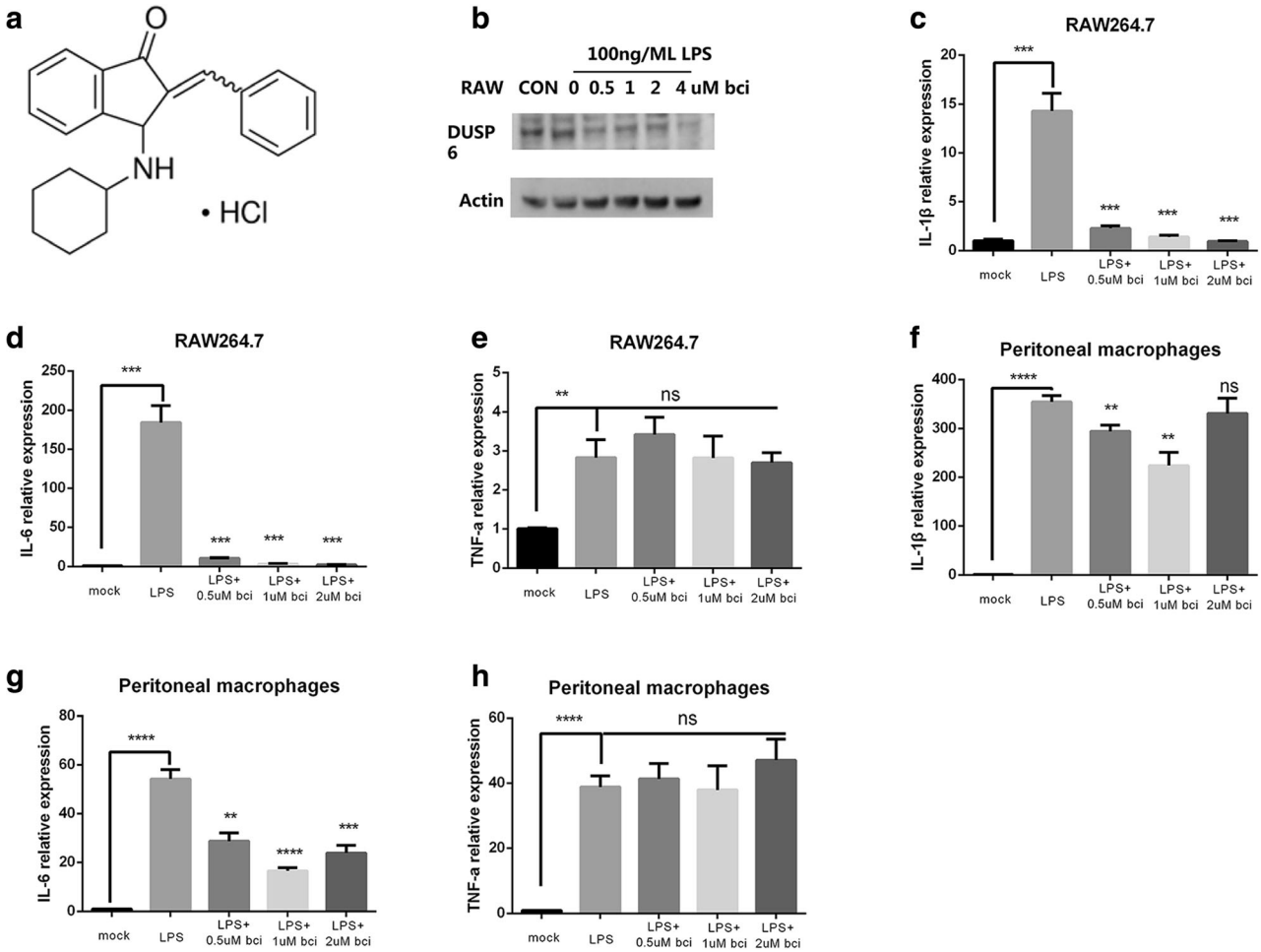


**Fig. 2.** Pharmacological inhibition of DUSP6 suppressed inflammatory cytokine production in lipopolysaccharide (LPS)-induced murine macrophages. The chemical structure of BCI is shown in (**a**) RAW264.7 macrophage cells and peritoneal macrophages were treated with increasing BCI concentrations plus 100 ng/mL LPS treatment. (**b**) Western blots were used to measure DUSP6 protein expression in RAW264.7 macrophage cells. (**c**–**e**) RT-qPCR was used to measure the IL-1β, IL-6, and TNF-α mRNA expressions in RAW264.7 macrophage cells. (**f**–**g**) RT-qPCR was used to measure the IL-1β, IL-6, and TNF-α mRNA levels in peritoneal macrophages. β-actin served as an internal control. Data are shown as the mean ± SD of at least three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

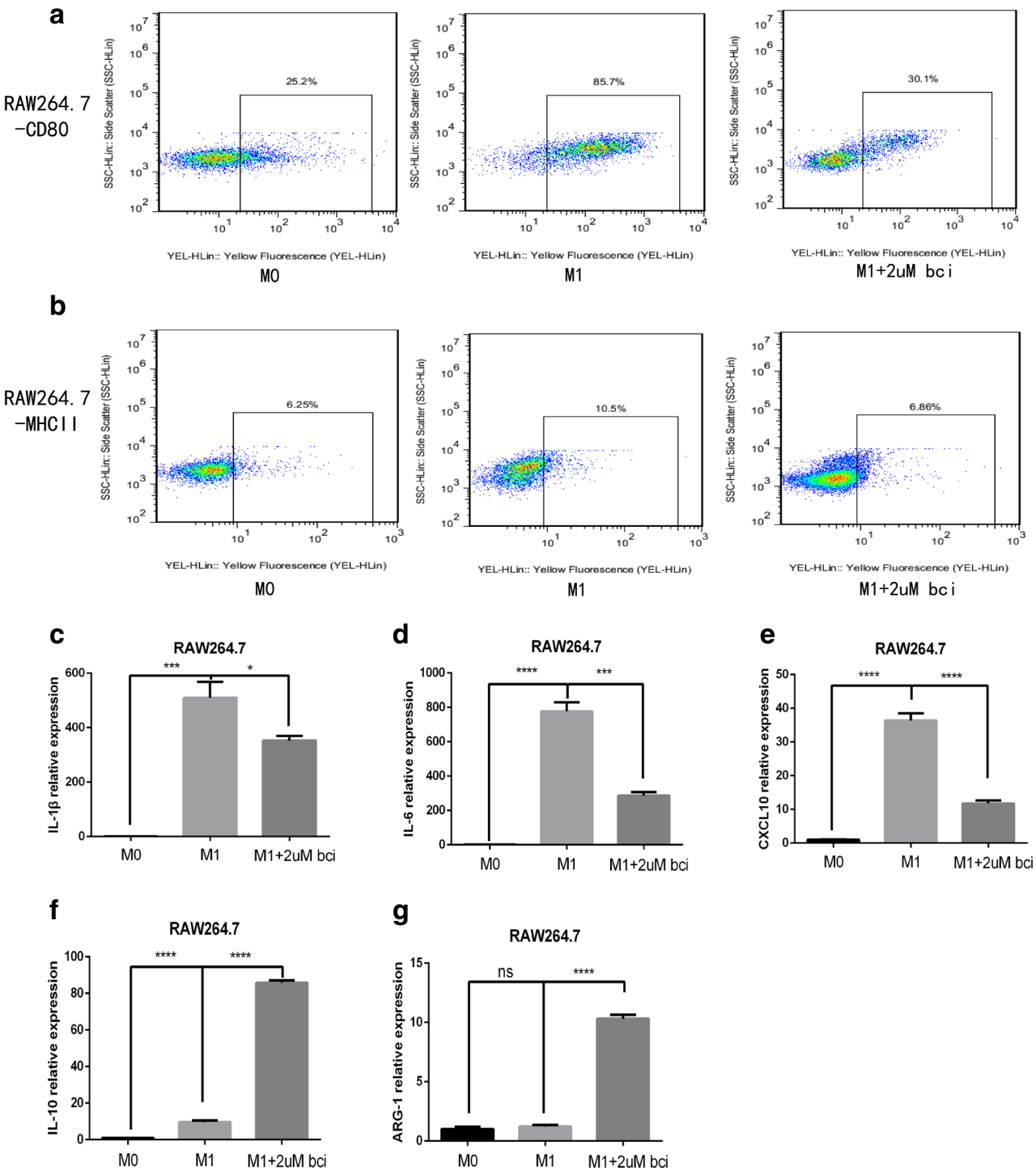


**Fig. 3.** BCI treatment inhibited M1-mediated maker expression and promoted M2-mediated anti-proinflammatory cytokine expression. RAW264.7 macrophage cells were stimulated with lipopolysaccharide + IFN-γ and incubated with increasing BCI concentrations for 24 h. (**a**–**b**) Flow cytometry was used to measure the expression of CD80 and MHC-II, the specific surface makers. (**c**–**g**) RT-qPCR was used to detect the levels of M1-mediated cytokines including IL-1β (**c**), IL-6 (**d**), and CXCL10 (**e**), and M2-mediated cytokines including Arg-1 (**f**) and IL-10 (**g**). β-actin served as an internal control. Data are shown as the mean ± SD of at least three independent experiments. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

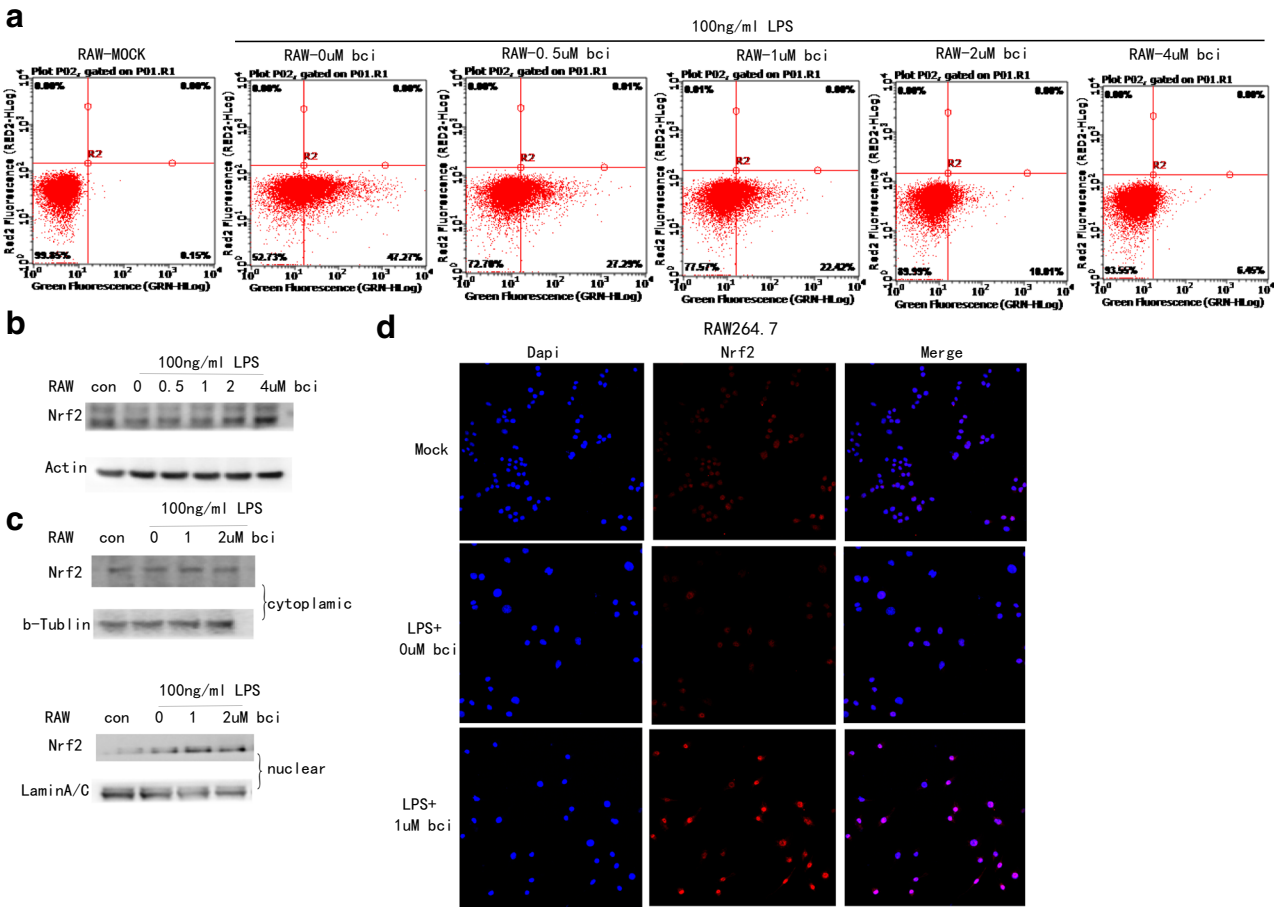


**Fig. 4.** BCI treatment decreased reactive oxygen species (ROS) production and activated the Nrf2 signaling pathway. RAW264.7 macrophages and peritoneal macrophages were treated with 100 ng/mL lipopolysaccharide with different concentrations of BCI for 24 h. (**a**) Flow cytometry was used to determine ROS production in RAW264.7 macrophages. (**b**, **c**) Western blots were used to measure the protein levels of total Nrf2, nuclear Nrf2, and cytoplasmic Nrf2 in RAW264.7 macrophages. (**d**) Immunofluorescence assays were used to measure the Nrf2 expression. β-actin served as an internal control. Data are shown as the mean ± SD of at least three independent experiments.

Peritoneal macrophages were also treated with 100 ng/mL LPS plus 0–1 nM BCI for 24 h. Similarly, with LPS treatment, the expression of IL-1β and IL-6 mRNA was suppressed in LPS-induced macrophages (Fig. 2f, g). However, there was no significant difference in the expression of TNF-α mRNA in LPS-stimulated macrophages treated with 0–2 nM BCI (Fig. 2h). These data suggested that BCI treatment inhibited LPS-induced inflammatory responses.

## BCI Treatment Inhibited Macrophage Polarization to the M1 Phenotype

In order to confirm the role of BCI in macrophage polarization, RAW264.7 macrophages were treated with 500 ng/mL LPS and 100 ng/mL IFN-r with different concentrations of BCI for 24 h. The M1 phenotypic changes in RAW264.7 macrophage cells were analyzed by flow cytometry and RT-qPCR. CD80 and MHC-II were served as a specific surface marker of the M1 phenotypes. Proinflammatory cytokines including IL-1β, IL-6, and CXCL10 were considered as M1-mediated cytokines. We found that the expression of CD80 and MHC-II was gradually downregulated with increasing BCI concentrations in LPS- and IFN-r-induced macrophages (Fig. 3a, b). Furthermore, after BCI treatment, M1-mediated cytokines, including IL-1β, IL-6, and CXCL10, were significantly inhibited in LPS and IFN-r triggered macrophages (Fig. 3c–e). To further investigate the anti-inflammatory effect of BCI in LPS and IFN-r triggered macrophages, we also measured the expression of IL-10 and ARG-1 mRNA, which could act as anti-inflammatory factors. Increasingly, the mRNA expressions of IL-10 and ARG-1 were significantly upregulated

in LPS and IFN-r activated macrophages (Fig. 3f, g). These data suggest that BCI treatment inhibited macrophage polarization to the proinflammatory macrophage class and enhanced anti-inflammatory cytokine production.

### BCI Treatment Decreased ROS Production and Activated the Nrf2 Pathway in LPS-Activated Macrophages

LPS has been reported to induce ROS production, which plays a fundamental role in inflammatory processes in macrophages. In the present study, RAW264.7 macrophages were treated with 100 ng/mL LPS plus 0–4 nM BCI for 24 h. Flow cytometry was used to determine ROS production. We found that ROS production was gradually inhibited with increasing BCI treatment in LPS-activated macrophages (Fig. 4a). In addition, BCI treatment significantly elevated Nrf2 expression. (Fig. 4b). In order to further elucidate the mechanisms by which BCI treatment suppressed the levels of ROS in LPS-activated macrophages, nuclear and cytoplasmic extractions and immunofluorescence assays were used to measure the Nrf2 expression. We found that BCI treatment enhanced nuclear Nrf2 expression but only slightly changed cytoplasmic Nrf2 levels in LPS-activated macrophages (Fig. 4c, d). These results indicated that BCI treatment inhibited ROS production and reduced oxidative stress through activating the Nrf2 signaling pathway components in LPS-stimulated macrophages.

### BCI Treatment Regulated LPS-Induced Inflammatory Responses Independently of ERK Signaling Cascades in Macrophages

To further investigate the mechanism of BCI on LPS-induced inflammatory processes in macrophages, RAW264.7 macrophages were treated with 100 ng/mL LPS with different concentrations of BCI treatment for 24 h. Western blots were used to measure changes in protein expression levels. DUSP6 has been characterized as a member of the ERK phosphatase family. We first determined the expression of P38 signaling pathway components. Surprisingly, the protein expression of P-P38 was slightly activated after BCI treatment in LPS-activated macrophages (Fig. 5a). We then measured the protein levels of JNK signaling pathway and ERK signaling pathway proteins, which functioned as critical components of MAPK signaling cascades. However, the levels of P-JNK and P-ERK proteins were also not changed after BCI treatment in LPS-triggered macrophages (Fig. 5b, c). These findings demonstrate that BCI treatment inhibited LPS-induced inflammatory processes, but did not rely on ERK signaling pathway components in macrophages.

### BCI Treatment Inhibited the NF-κB Signaling Pathway in LPS-Activated Macrophages

According to a previous study, NF-κB is an essential proinflammatory responder, which can be activated in LPS-induced macrophages. RAW264.7 cells were treated with 100 ng/mL LPS with different concentrations of BCI treatment for 24 h. BCI treatment significantly inhibited P65 phosphorylation expression with increasing BCI concentrations. The total P65 protein level was also suppressed (Fig. 6a). In addition, BCI treatment inhibited nuclear P65 expression, but barely affected the cytoplasmic P65 expression as determined using nuclear and cytoplasmic extraction and immunofluorescence assays (Fig. 6b, c). These results demonstrate that BCI treatment inhibited the NF-κB signaling pathway in LPS-triggered macrophages.

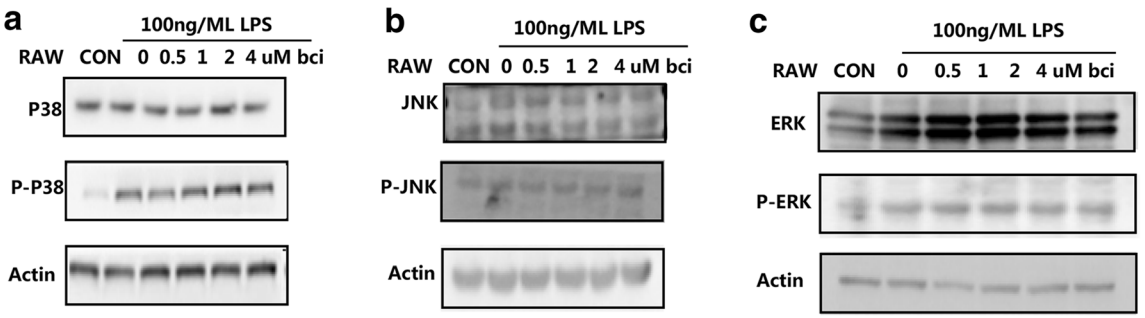


**Fig. 5.** BCI treatment regulated lipopolysaccharide (LPS)-activated inflammatory process independent of the ERK signaling pathway. RAW264.7 macrophages were treated with 100 ng/mL LPS with increasing BCI concentrations for 24 h. (**a**–**c**) Western blots were used to detect the protein levels of P38, P-P38, JNK, P-JNK, ERK, and P-ERK in RAW264.7 macrophages. β-actin was used as the internal control. Data are shown as the mean ± SD of at least three independent experiments.

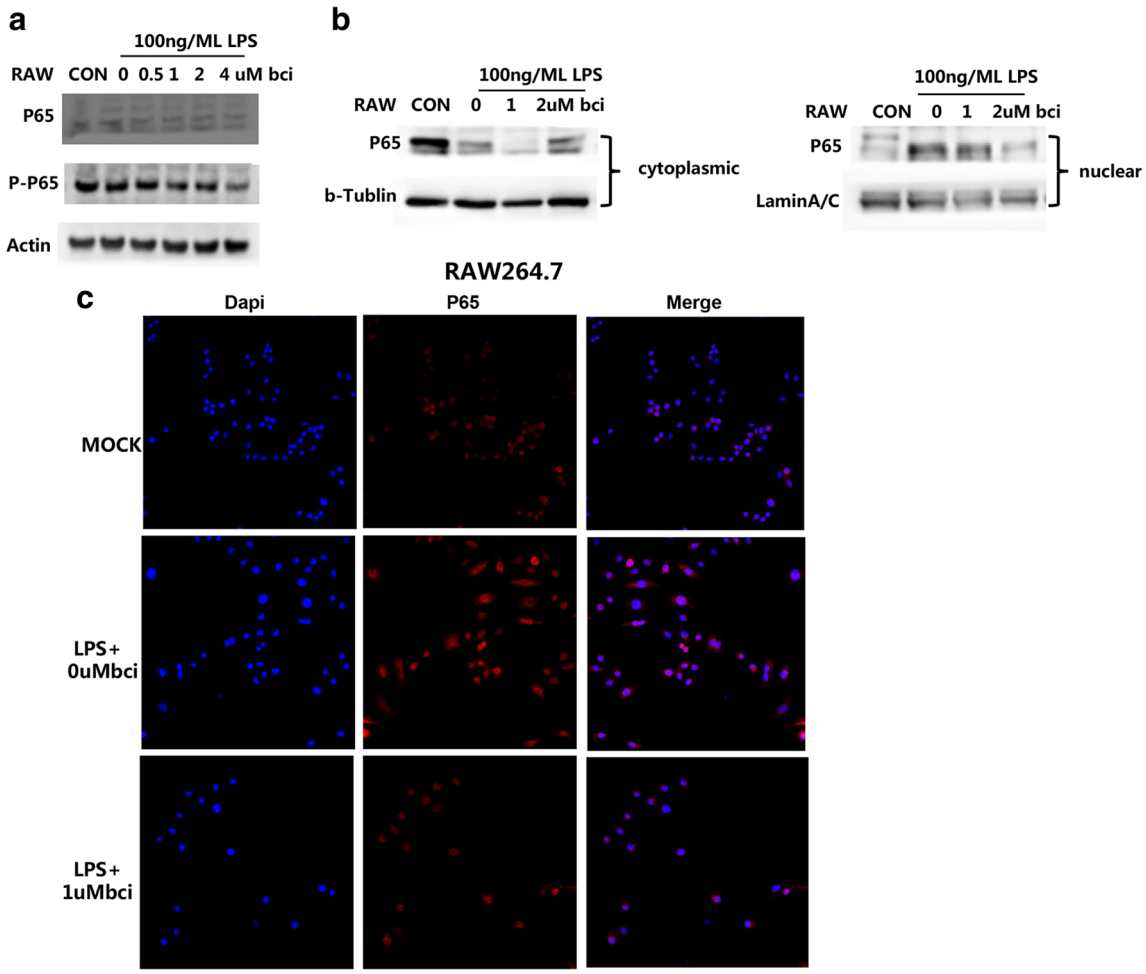


**Fig. 6.** BCI treatment suppressed the NF-κB signaling pathway. RAW264.7 macrophages were treated with 100 ng/mL lipopolysaccharide and with increasing BCI concentrations for 24 h. (**a**–**b**) Western blots were used to measure the levels of P65, P-P65, nuclear P65, and cytoplasmic P65 in RAW264.7 macrophages. (**c**) Immunofluorescence assays were used to measure nuclear P65 and cytoplasmic P65 expressions. Data are shown as the mean ± SD of at least three independent experiments.

## DISCUSSION

It has long been established that inflammation is a complex pathophysiological reaction necessary to defend against endogenous and exogenous noxious stimuli, and that it is closely associated with a wide variety of inflammatory disorders [28]. In immunological defense mechanisms, macrophages are considered as the primary defense against inflammation and act as a pivotal part of the host defense and immune responses [29]. LPS-triggered macrophages release various kinds of proinflammatory cytokines and chemokines. Furthermore, in LPS-stimulated macrophages, an imbalance of the intracellular reduction–oxidation state results in the production of ROS, which aggravate inflammation. Hence, in terms of controlling inflammatory processes in the macrophage, reducing pro-inflammatory factors and ROS will play central roles.

DUSP6 promotes endothelial inflammation-mediated pathological processes through inducible expression of ICAM-1 [30]. However, it remains unclear whether DUSP6 participates in LPS-induced inflammatory responses in macrophages. BCI is a small molecule inhibitor of DUSP6 and its potential mechanism of regulating LPS-triggered inflammation needs further elucidation. In our study, we found that DUSP6 was upregulated in LPS-induced macrophages. It is worth mentioning that the expression of DUSP6 was upregulated with increasing concentrations of LPS. Therefore, we concluded that

DUSP6 played an important role in LPS-triggered inflammation in macrophages. Further experiments showed that pharmacological inhibition of DUSP6 decreased IL-1β and IL-6 expression and attenuated LPS-induced inflammatory responses.

Next, we explored the mechanism of BCI during inhibition of LPS-triggered inflammatory responses in macrophages. It is well-documented that DUSP6 is a member of the MAPK phosphatase family, and functions as a negative regulator of ERK, playing an important role in the MAPK signaling pathway [31]. In our study, BCI treatment inhibited DUSP6 expression in LPS-triggered macrophages. However, its potential mechanism of controlling LPS-stimulated immune responses was independent of extracellular signaling-regulated kinase (ERK) signaling. We found that BCI treatment suppressed NF-κB phosphorylation in LPS-triggered macrophages. NF-κB, a nuclear transcription factor, which is an essential regulator of various genes involved in the production of a number of proinflammatory cytokines and enzymes, was associated with the inflammatory response [32]. We also found that BCI treatment inhibited NF-κB entry into the nucleus in LPS-activated macrophages. It is well-established that activated NF-κB can directly translocate to the nucleus and promote the production of proinflammatory factors, including IL-1β and IL-6. This suggested that BCI treatment exerted anti-inflammatory effects through inhibiting NF-κB phosphorylation and nuclear NF-κB expression in LPS-induced macrophages.

ROS are released from LPS-activated macrophages and are closely related to the inflammatory response [33]. In the present study, we also clarified the inhibitory effect of BCI on ROS production in LPS-triggered macrophages. Further studies showed that BCI treatment upregulated nuclear Nrf2 expression. Nrf2 is a member of the basic-leucine zipper transcription factor family that plays a fundamental role in maintaining ROS balance and alleviating the inflammatory response. Therefore, targeting the Nrf2 cascade could be a potential strategy to treat inflammation-associated diseases. BCI may therefore be considered as a therapeutic agent for blocking inflammatory disorders.

In summary, our study explored the anti-oxidative and anti-inflammatory effects of BCI on LPS-triggered macrophages, including human and mouse macrophage cells. BCI treatment inhibited inflammatory cytokines and ROS production in LPS-activated macrophages and also prevented macrophage polarization to the M1 phenotype after either LPS or IFN-r treatment. Furthermore, BCI treatment inhibited DUSP6 expression, suppressed the NF-κB signaling pathway, and activated the Nrf2 cascade in LPS-induced macrophages. In conclusion, DUSP6 inhibitor (E/Z)-BCI hydrochloride attenuates alleviated LPS-triggered inflammatory processes in macrophages through inhibiting the NF-κB signaling pathway and activating the Nrf2 signaling axis.

## MATERIALS AND METHODS

### Animals and Macrophages

Male C57BL/6 wild-type mice, which were 6–8 weeks old, were purchased from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). Peritoneal macrophages were extracted from C57BL/6 mice by intraperitoneal injection of 2–3 mL of sterile 4% Brewer's thioglycollate. Cells were harvested 2–3 days later by peritoneal lavage and cultured on plates. After 24 h at 37 °C in a constant-temperature incubator, non-adherent cells were removed by washing, and adherent macrophages were used in the experiments.

### Cell Cultures and LPS and BCI Treatments

The murine macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 and peritoneal macrophages were cultured in RPMI1640 media supplemented with 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, and 10% fetal bovine serum in 5% $CO_2$ at 37 °C in a constant-temperature incubator. The cells were treated with 100 ng/mL LPS with or without 0–4 nM BCI treatment for 24 h.

### Real-time PCR (RT-PCR)

Total RNA was isolated from RAW264.7 and peritoneal macrophages using TRIzol® reagent, and the reverse transcription of first-strand cDNA was performed using the TransScript Top Green qPCR supermix (TransGen, Guangzhou, China) according to the manufacturer's protocol.

### Western Blotting

Total protein was isolated from the RAW264.7 and peritoneal macrophages, which were lysed in radioimmunoprecipitation assay buffer (Beyotime Biotechnology, Shanghai, China), and the lysates were incubated at 4 °C in a constant-temperature incubator before the supernatants were collected. A wet western blotting system

was used with β-actin antibody as the internal control (Cell Signaling Technology, Beverly, MA, USA).

### Measurement of ROS Production

RAW264.7 macrophage cells were treated with 100 ng/mL LPS with or without 0–4 nM BCI for 24 h, then RAW264.7 macrophage cells were incubated in basic RPMI1640 media with probe DCFH-DA (Yeasen, Shanghai, China) for 20–30 min incubated at 4 °C in a constant-temperature incubator. Then, the cells were washed three times by cold PBS in order to wash the redundant probe. The wavelengths of the probe are 488 nM and 525 nM. In addition, the spectrum of DCF is similar to that of FITC. Flow cytometry was used to measure ROS generation.

### Flow Cytometry

Flow cytometry was performed to measure the phenotypic changes in M1-mediated markers in RAW264.7 macrophages. In the study, CD80 and MHC-II (Becton Dickinson, San Jose, CA, USA) were used as the specific surface marker of M1 phenotypic macrophage changes. All samples were analyzed using FLOWJO installed software.

### Immunofluorescence Assay

In order to measure the nuclear Nrf2, P65 levels, and cytoplasmic Nrf2 and P65 expressions, we used an immunofluorescence assay. DAPI was used to stain nuclei. All samples were analyzed using the FV10-ASW confocal microscope with the installed software (Olympus, Hamburg, Germany).

### Nuclear and Cytosolic Extractions

RAW264.7 macrophage cells were cultured at a density of $1 \times 10^6$ cells/mL, and treated with 100 ng/mL LPS with or without 0–4 nM BCI for 24 h. RAW264.7 macrophage cells were harvested using trypsin-EDTA, collected by centrifugation, and washed with cold phosphate-buffered saline three times. Extraction used Nuclear Extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

### Statistical Analysis

Statistical analyses were performed using SPSS, version 16.0 (SPSS, Chicago, IL, USA). Values were expressed as the mean ± SD. Quantitative data in paired groups were determined using Student's *t* test. One-way analysis of variance was performed for multiple group comparisons. A value of $P < 0.05$ indicated significant differences.

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## AUTHOR'S CONTRIBUTION

Fan Zhang and Bufu Tang performed the experiments. Fan Zhang analyzed the data. Zijiao Zhang and Di Xu contributed reagents, materials, and analysis tools. Fan Zhang and Bufu Tang wrote the paper. Fan Zhang edited the paper.

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest.** The authors declare that they have no conflict of interest.



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