Ethanol extract of *Chrysanthemum zawadskii* inhibits the NLRP3 inflammasome by suppressing ASC oligomerization in macrophages

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Abstract. *Chrysanthemum zawadskii* (*C. zawadskii*) is used in traditional East Asian medicine for the treatment of various diseases, including inflammatory disease. However, it has remained unclear whether extracts of *C. zawadskii* inhibit inflammasome activation in macrophages. The present study assessed the inhibitory effect of an ethanol extract of *C. zawadskii* (CZE) on the activation of the inflammasome in macrophages and the underlying mechanism. Bone marrow-derived macrophages were obtained from wild-type C57BL/6 mice. The release of IL-1β and lactate dehydrogenase in response to nucleotide-binding oligomerization domain-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome activators, such as ATP, nigericin and monosodium urate (MSU) crystals, was significantly decreased by CZE in lipopolysaccharide (LPS)-primed BMDMs. Western blotting revealed that CZE inhibited ATP-induced caspase-1 cleavage and IL-1β maturation. To investigate whether CZE inhibits the priming step of the NLRP3 inflammasome, we confirmed the role of CZE at the gene level using RT-qPCR. CZE also downregulated the gene expression of NLRP3 and pro-IL-1β as well as NF-κB activation in BMDMs in response to LPS. Apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD) oligomerization and speck formation by NLRP3 inflammasome activators were suppressed by CZE. By contrast, CZE did not affect NLR family CARD domain-containing protein 4 or absent in melanoma 2 inflammasome activation in response to *Salmonella typhimurium* and poly(dA:dT) in LPS-primed BMDMs, respectively. The results revealed that three key components of CZE, namely linarin, 3,5-dicaffeoylquinic acid and chlorogenic acid, decreased IL-1β secretion in response to ATP, nigericin and MSU. These findings suggest that CZE effectively inhibited activation of the NLRP3 inflammasome.

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Introduction

The inflammasome is formed by cytosolic multi-protein complexes that consist of pattern recognition receptors (PRRs), including nucleotide-binding oligomerization domain-like receptors (NLRs) or absent in melanoma 2 (AIM2), adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD; ASC) and effector protein procaspase-1 (1). Activation of the inflammasome by specific stimuli, such as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), induces ASC oligomerization and caspase-1 cleavage. Active caspase-1 proteolytically processes pro-IL-1β and pro-IL-18 into IL-1β and IL-18, respectively. Inflammasome-mediated caspase-1 activity also induces a specific form of inflammatory cell death known as pyroptosis (2). These activations serve important roles in the immune defense against pathogens, whereas excessive production of IL-1β causes inflammatory diseases (3).

The NLR family pyrin domain containing 3 (NLRP3) inflammasome has been extensively studied and is known to mediate the inflammatory response (4-9). This inflammasome is activated by pathogen-derived ligands, such as components of bacterial cell walls, pore-forming toxins and DAMPs, including uric acid crystals, ATP and β-amyloid (4,10). Two steps, namely signal 1 and signal 2, control NLRP3 inflammasome activation. Signal 1 is a priming step that leads to gene expression of NLRP3 and pro-IL-1β. Molecular sensing by PRRs activates signaling cascades, such as the NF-κB

signaling pathway, which is responsible for priming (2). Signal 2 is an activation step triggered by PAMPs and DAMPs, which results in formation of the NLRP3 inflammasome assembly, caspase-1-mediated IL-1β cleavage and pyroptosis (2). Excessive and persistent activation of the NLRP3 inflammasome results in inflammatory and metabolic disease, as well as neurological disorders, including inflammatory bowel disease, rheumatoid arthritis, type 2 diabetes and Alzheimer's disease (4-9). Therefore, regulation of the NLRP3 inflammasome may be a new therapeutic strategy for the treatment of metabolic disorders, and novel compounds to regulate the activity of the NLRP3 inflammasome are being actively explored at present (11,12).

Chrysanthemum zawadskii (*C. zawadskii*), also known as 'Gu-jeol-cho' in Korea, is a perennial plant belonging to the genus *Chrysanthemum* in the family Asteraceae. This plant has been used in traditional Chinese medicine for the treatment of various diseases, such as bone disease (13,14) and lung injury (15). Previous studies have suggested that an ethanol extract of *C. zawadskii* (CZE) has pharmacological properties, including antioxidant, anti-inflammatory and anticancer effects (16-18). Although CZE has potential pharmacological effects, the mechanism of action underlying its biological effects remains unclear. Therefore, the present study investigated the effect of CZE on activation of the NLRP3 inflammasome in macrophages and the underlying mechanism.

Materials and methods

Plant extraction. The dried aerial parts of *C. zawadskii* were purchased from Canaanherb and used for extractions. The water extract (CZW) was prepared by boiling the dried plant (150 g) in 1 liter of sterilized water at 90˚C for 4 h, while CZE was prepared by refluxing *C. zawadskii* (50 g) with 1 liter ethanol at room temperature for 24 h. These extracts were filtered, evaporated in a rotary vacuum evaporator and lyophilized with a freeze-dryer. The powder extracts were dissolved in PBS or DMSO and diluted to 30-300 μg/ml (CZW) or 25-100 μg/ml (CZE) using Iscove's Modified Dulbecco's medium (IMDM; Gibco; Thermo Fisher Scientific, Inc.).

Animals. A total of 20 8-week-old male wild-type C57BL/6 mice weighing 20-25 g were purchased from Jackson Laboratory. Mice were housed in standard plastic cages in controlled conditions at 23±2˚C with a humidity of 55±10% under a 12/12-h light/dark cycle, with *ad libitum* access to food and water. For tissue collection, mice were anesthetized with 3% isoflurane (for induction) and euthanized by cervical dislocation. Death was pronounced by ascertaining cardiac and respiratory arrest. All animal studies were performed using protocols approved by the Institutional Animal Care and Use Committee of Chonnam National University (Gwangju, Korea; approval no. CNU IACUC-YB-2018-02).

Reagents and bacteria culture. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was purchased from InvivoGen (cat. no. tlrl-eblps). ATP (cat. no. A2383), nigericin (cat. no. N7143) and poly(dA:dT; cat. no. P0883) were purchased from Sigma-Aldrich (Merck KGaA). Monosodium urate (MSU) crystals (cat. no. tlrl-msu) were purchased from InvivoGen. S*almonella enterica serovar typhimurium* (*S. typhimurium*) was cultured on Luria-Bertani (LB) agar or broth (both BD Biosciences) at 37˚C. To prime the inflammasome, LPS was used to treat bone marrow-derived macrophages (BMDMs) in a 5% CO_2 incubator at 37˚C for 6 h. To activate the NLRP3 inflammasome, LPS-primed BMDMs were treated with ATP (2 mM), or nigericin (10 μM) for 40 min or MSU (200 μg/ml) for 4 h in a 5% CO_2 incubator at 37˚C. For AIM2 inflammasome activation, LPS-primed BMDMs were treated with poly(dA:dT) (2 μg/ml) in a 5% CO_2 incubator at 37˚C for 4 h. To activate the NLR family CARD domain-containing protein 4 (NLRC4) inflammasome, *S. typhimurium* was grown on LB agar at 37˚C for 24 h to obtain single colonies, which were inoculated into LB broth and cultured at 37˚C with shaking for 16 h. A 1:10 dilution of culture suspension was grown in fresh medium at 37˚C with shaking for an additional 2 h. The bacteria were concentrated to $1x10^9$ colony-forming units/ml in PBS and diluted to $1x10^7$ colony-forming units/ml in cell culture media (IMDM, Gibco; Thermo Fisher Scientific, Inc.). LPS-primed BMDMs were infected with *S. typhimurium* (multiplicity of infection=10) in the presence or absence of CZE in a 5% CO_2 incubator at 37˚C for 1 h, and the medium was replaced with media containing gentamicin and incubated at 37˚C for 3 h. As major components of CZE, linarin (Merck KGaA; cat. no. PHL80822) and chlorogenic acid (Merck KGaA; cat. no. PHR2202) were purchased from Sigma-Aldrich, while 3,5-di-caffeoylquinic acid (3,5-di-CQA; cat. no. ALX-350-320-M001) was purchased from Enzo Life Sciences, Inc.

Cell culture. BMDMs were isolated from mice and differentiated as previously described (19). Briefly, BMDMs were incubated in IMDM (Gibco; Thermo Fisher Scientific, Inc.) containing 30% L929 cell culture supernatant, 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.), 1% MEM non-essential amino acids (Gibco; Thermo Fisher Scientific, Inc.), 1% sodium pyruvate (Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (Costar; Corning, Inc.) in a 5% CO_2 incubator at 37˚C for 6 days. Fresh medium was added 3 days later, and the cells were cultured for 3 days. Next, cells were seeded into 48- or 6-well plates at a density of $2x10^6$ cells/ml and incubated in a 5% CO_2 incubator at 37˚C.

Measurement of cytokines. The concentrations of IL-1β (Mouse IL-1β/IL-1F2 DuoSet ELISA kit; cat. no. DY401), IL-6 (Mouse IL-6 DuoSet ELISA kit; cat. no. DY406) and TNF-α (Mouse TNF-α DuoSet ELISA kit; cat. no. DY410) in the culture supernatants were determined using commercial ELISA kits (all R&D Systems, Inc.) according to the manufacturer's instructions.

Measurement of lactate dehydrogenase (LDH). The level of LDH in culture supernatants was determined using a commercial kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay; cat. no. G1780; Promega Corporation) according to the manufacturer's instructions.

Western blotting. For detection of the pro- and cleaved forms of IL-1β and caspase-1, LPS-primed BMDMs were treated with ATP (2 mM) in the presence or absence of CZE in a

5% CO_2 incubator at 37˚C for 40 min. The attached cells and culture supernatant were lysed with 1% Triton-X 100 solution (Sigma-Aldrich; Merck KGaA) containing cOmplete™ Protease Inhibitor Cocktail (Roche Applied Science). The total protein concentration of cell lysates was measured using the Bradford protein assay kit II (cat no. 5000002; Bio-Rad Laboratories, Inc.). The supernatant was mixed with sample loading buffer (5X), separated by 15% SDS-PAGE with 20 μg protein loaded per lane and transferred to nitrocellulose (NC) membranes.

To confirm the involvement of CZE in the priming step of the NLRP3 inflammasome, BMDMs were stimulated with LPS (100 ng/ml) for 4 or 24 h in the presence or absence of CZE (100 μg/ml) in a 5% CO_2 incubator at 37˚C. BMDMs were lysed in lysis buffer containing 1% NP-40, 50 mM Tris (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.02% NaN_3 and 1 mM Na_3VO_4 supplemented with phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2; Sigma-Aldrich; Merck KGaA), protease inhibitors (Complete Mini EDTA-free Protease Inhibitor; Roche Applied Science) and 2 mM dithiothreitol. The total protein concentration of cell lysate was measured using the Bio-Rad Protein Assay kit II (cat no. 5000002; Bio-Rad Laboratories, Inc.). A standard curve was generated using bovine serum albumin (BSA, cat no. 10735086001; Sigma-Aldrich; Merck KGaA) in the 1-10 μg/μl range. The cell lysates were separated by 10% SDS-PAGE with 20 μg protein/lane and transferred to NC membranes. Following blocking for 2 h at room temperature in 5% skimmed milk, all membranes were probed with primary antibodies against IL-1β (cat. no. AF-401-NA; R&D Systems, Inc.), caspase-1 (cat no. AG-20B-0042; Adipogen Life Sciences), IκB-α (cat no. 9242; Cell Signaling Technology, Inc.), phosphorylated p65 (cat. no. 3031; Cell Signaling Technology, Inc.), p65 (cat. no. 8242; Cell Signaling Technology, Inc.), NLRP3 (cat. no. 15101; Cell Signaling Technology, Inc.) and β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. The primary antibodies were diluted 1:1,000 in TBST (TBS containing 0.05% Tween-20). After immunoblotting with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:10,000 in 5% skim milk; cat. no. 31460; Invitrogen; Thermo Fisher Scientific, Inc.) or anti-mouse IgG (H+L) secondary antibody (1:10,000 in 5% skim milk; cat. no. 31430; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 2 h, the proteins were detected with Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.). β-actin was used as the loading control. Quantification of protein bands was performed using ImageJ Software version 1.53t (National Institutes of Health).

ASC oligomerization assay. Following inflammasome activation in the absence or presence of CZE, the cells were harvested, resuspended in cold lysis buffer containing 0.2% Triton X-100 (cat no. T9284; Sigma-Aldrich; Merck KGaA) and Complete Protease Inhibitor Cocktail (Roche Applied Science) and passed 10 times through a 26-gauge syringe. Following centrifugation at 330 x g for 10 min at 4˚C, the supernatants (Triton-X-100 soluble fraction) were mixed with sample loading buffer (5X). The remaining cell pellets were resuspended in PBS containing 2 mM disuccinimidyl suberate cross-linker (cat. no. S1885; Sigma-Aldrich; Merck KGaA) and incubated at room temperature for 30 min, followed by centrifugation at 330 x g for 10 min at 4˚C. The total protein concentration of cell lysates was measured using the Bradford protein assay kit II (cat no. 5000002; Bio-Rad Laboratories, Inc.). A standard curve was generated using bovine serum albumin (BSA, cat no. 10735086001; Sigma-Aldrich; Merck KGaA) in the 1-10 μg/μl range. To detect ASC oligomerization, cross-linked pellets (Triton-X-100-insoluble fraction) were separated by 12% SDS-PAGE with 20 μg protein/lane and transferred to NC membranes. The membranes were blocked with 5% skimmed milk at room temperature for 2 h. Following this, membranes were incubated with ASC antibody (cat. no. 67824S; Cell Signaling Technology, Inc.) diluted 1:1,000 in TBST (TBS containing 0.05% Tween-20) overnight at 4˚C. The Triton-X-100-soluble fraction was used to detect the total form of ASC (1:1,000 in TBST; cat. no. 67824S; Cell Signaling Technology, Inc.) and β-actin (1:1,000 in TBST; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). After immunoblotting with HRP-conjugated goat anti-rabbit (1:10,000 in 5% skimmed milk; cat. no. 31460; Invitrogen; Thermo Fisher Scientific, Inc.) or anti-mouse IgG (H+L) secondary antibodies (1:10,000 in 5% skim milk; cat. no. 31430; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 2 h, the proteins were detected using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.). β-actin was used as the loading control.

ASC speck assay. For ASC speck assay, BMDMs were cultured in IMDM (Gibco; Thermo Fisher Scientific, Inc.) containing 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (Costar; Corning, Inc.) overnight in round glass dishes in a 5% CO_2 incubator at 37˚C. The cells were stimulated with LPS (100 ng/ml) for 6 h and subsequently treated with CZE (100 μg/ml) for 30 min. The cells were incubated with ATP (2 mM) for 40 min, nigericin (10 μM) for 40 min and MSU (200 μg/ml) for 4 h at 37˚C, then fixed with 2% formaldehyde solution for 5 min at room temperature and permeabilized with 0.2% Triton X-100, followed by staining with rabbit ASC antibody (1:100 in 2% BSA; cat. no. 67824S; Cell Signaling Technology, Inc.) overnight at 4˚C. The cells were incubated with FITC-conjugated anti-rabbit IgG (1:100 in 2% BSA; cat. no. F0382; Sigma-Aldrich; Merck KGaA) at room temperature for 2 h and mounted on slides. Nuclei were stained with DAPI (ProLong™ Gold Antifade Mountant; Invitrogen; Thermo Fisher Scientific, Inc.) at 4˚C for 16 h. ASC speck images were acquired using a Leica TCS SP5/AOBS/Tandem laser confocal scanning microscope (x200 magnification, Leica Microsystems) at Gwangju Center of the Korea Basic Science Institute.

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted from LPS-treated BMDMs with or without CZE pretreatment using the Easy-BLUE™ Total RNA Extraction kit (Intron Biotechnology, Inc.) and cDNA was synthesized using ReverTra Ace™ qPCR RT Master Mix (Toyobo Life Science) according to the manufacturer's instructions. qPCR was performed to detect gene expression of NLRP3 and pro-IL-1β using the CFX Connect™ Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) and QGreen™ 2X SybrGreen qPCR Master Mix (cat.

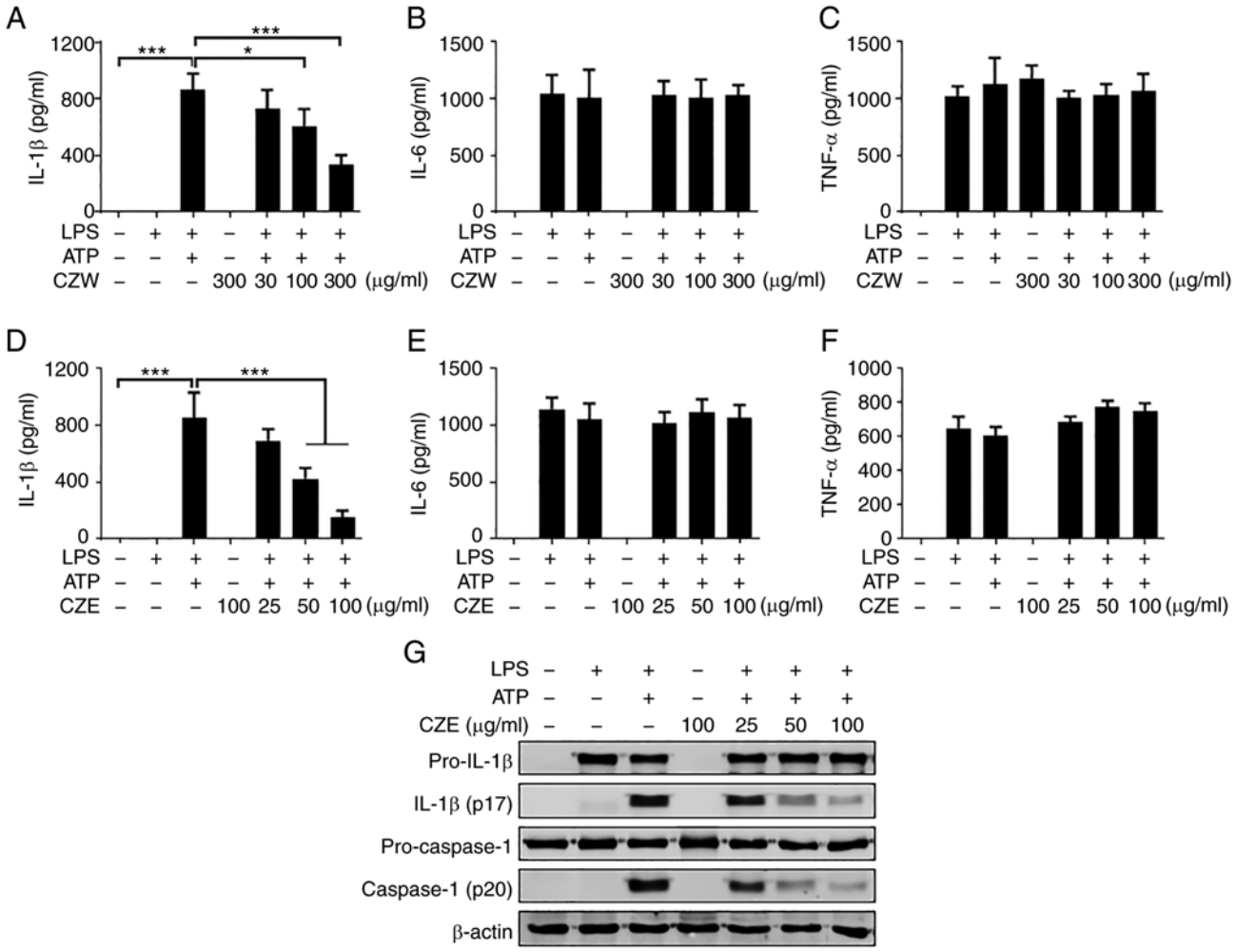

Figure 1. CZE inhibits ATP-induced IL-1β secretion in LPS-primed BMDMs. BMDMs were primed with LPS (100 ng/ml) for 6 h and subsequently treated with ATP (2 mM) for 40 min in the presence or absence of CZW for 30 min. Levels of (A) IL-1β, (B) IL-6 and (C) TNF-α in culture supernatants were measured by ELISA. Next, LPS-primed BMDMs were treated with ATP (2 mM) for 40 min, with or without CZE. The levels of (D) IL-1β, (E) IL-6 and (F) TNF-α in culture supernatants were measured by ELISA. The results are presented as the mean ± SD. *P<0.05, ***P<0.001. (G) Culture supernatant and cell lysates were used to detect the immature and cleaved forms of IL-1β and caspase-1 by western blotting. β-actin was used as a loading control. LPS, lipopolysaccharide; BMDM, bone marrow-derived macrophage; CZE, ethanol extract of *Chrysanthemum zawadskii*; CZW, water extract of *Chrysanthemum zawadskii*; CASP1, caspase-1.

no. QGHR-05; CellSafe). Thermocycling was performed using a two-step protocol of 95˚C for 10 sec followed by 40 cycles at 58˚C for 45 sec. The primers used for qPCR were as follows: Mouse NLRP3 forward, 5'-ATGGTATGCCAG GAGGACAG-3' and reverse, 5'-ATGCTCCTTGACCAG TTGGA-3'; mouse IL-1β forward, 5'-GATCCACACTCT CCAGCTGCA-3' and reverse, 5'-CAACCAACAAGTGAT ATTCTCCATG-3' and mouse GAPDH forward, 5'-CGACTT CAACAGCAACTCCCACTCTTCC-3' and reverse, 5'-TGG GTGGTCCAGGGTTTCTTACTCCTT-3'. GAPDH was used as an internal control. The method used to analyze the relative quantification of mRNA expression was $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq=(Cq_{\text{target gene}}-Cq_{GAPDH})_{\text{target sample}}-(Cq_{\text{target gene}}-Cq_{GAPDH})_{\text{reference sample}}$ (20).

Statistical analysis. Data are presented as the mean ± SD and of three independent experiments. The statistical significance of differences between groups was evaluated using one-way ANOVA followed by Tukey's post-hoc test or two-way ANOVA followed by Bonferroni's post-hoc test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

CZE has a stronger inhibitory activity than CZW on ATP-induced NLRP3 inflammasome activation in LPS-primed macrophages. To investigate whether *C. zawadskii* extract inhibits activation of the NLRP3 inflammasome, LPS-primed BMDMs were treated with CZW or CZE for 30 min and stimulated with ATP to activate the NLRP3 inflammasome. Treatment with ATP led to large secretion of IL-1β from the LPS-primed BMDMs (Fig. 1A and D). Both CZW and CZE suppressed ATP-induced IL-1β secretion in a dose-dependent manner (Fig. 1A and D) but did not affect production of IL-6 or TNF-α (Fig. 1B, C, E and F). Since CZE was more effective at lower concentrations than CZW, only CZE was selected for further experiments. Next, the effect of CZE on cleavage of IL-1β and caspase-1 to a mature form was examined. Treatment with ATP led to notable cleavage of IL-1β and caspase-1 but this was suppressed by

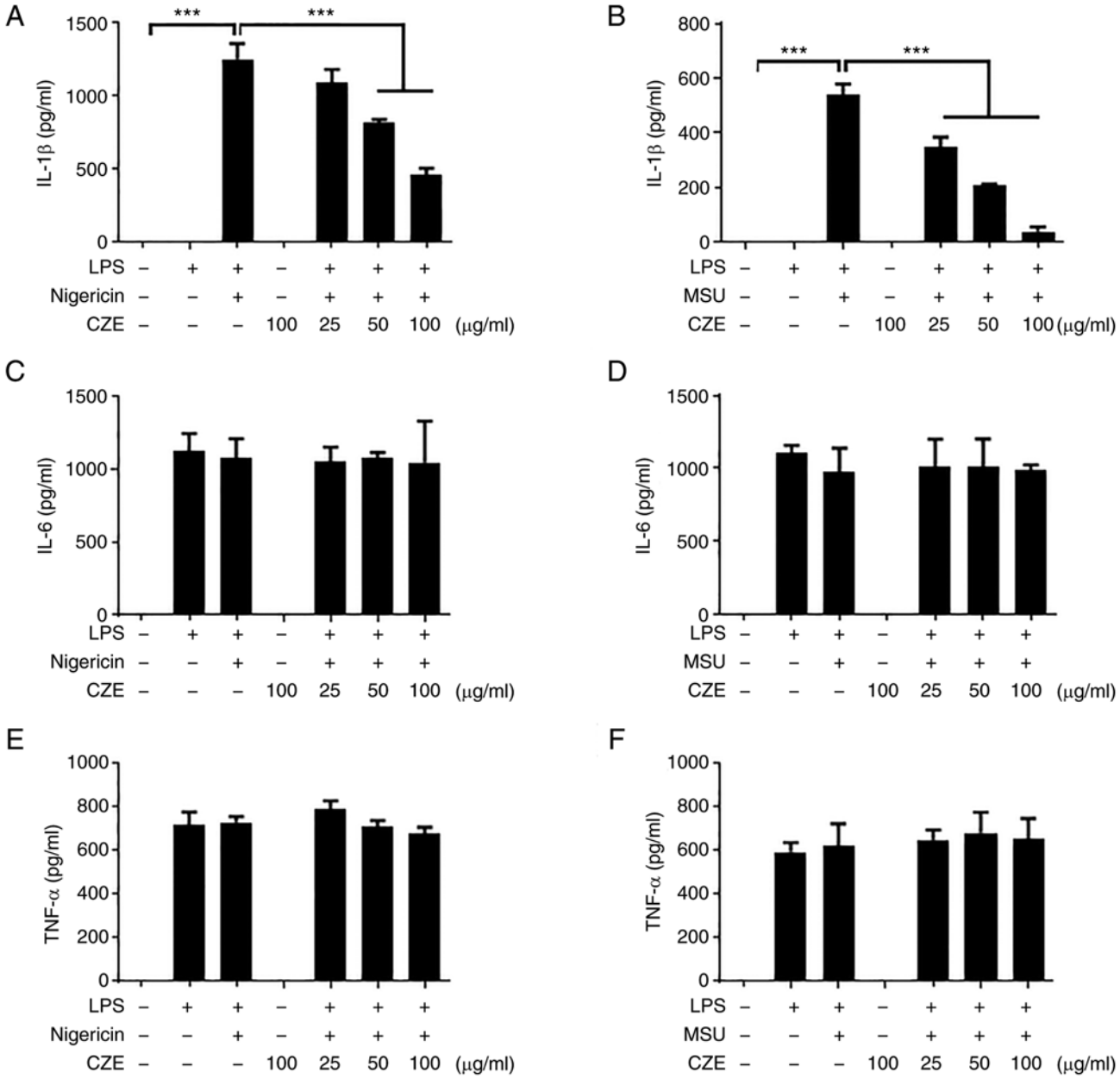

Figure 2. CZE inhibits nigericin and MSU-induced IL-1β secretion in LPS-primed BMDMs. BMDMs were primed with LPS (100 ng/ml) for 6 h and subsequently treated with CZE for 30 min. The cells were additionally incubated with nigericin (10 μM) for 40 min or MSU (200 μg/ml) for 4 h. Levels of (A and B) IL-1β, (C and D) IL-6 and (E and F) TNF-α in culture supernatant were measured by ELISA. The results are presented as the mean ± SD. ***P<0.001. LPS, lipopolysaccharide; BMDM, bone marrow-derived macrophage; MSU, monosodium urate; CZE, ethanol extract of *Chrysanthemum zawadskii*.

CZE in a dose-dependent manner in LPS-primed BMDMs (Fig. 1G).

CZE inhibits IL-1β secretion in response to nigericin and MSU. Various molecules, including nigericin, MSU crystals and ATP, induce the activation of the NLRP3 inflammasome by different mechanisms (21). The present study investigated whether CZE suppressed IL-1β production in macrophages in response to nigericin and MSU treatment. Both nigericin and MSU induced IL-1β secretion in LPS-primed BMDMs (Fig. 2A and B). The level of IL-1β was decreased by CZE treatment in a dose-dependent manner (Fig. 2A and B). CZE did not affect the production of IL-6 or TNF-α (Fig. 2C-F). Taken together, CZE exerted a notable inhibitory effect on the activation of the NLRP3 inflammasome.

CZE inhibits pyroptosis in BMDMs following activation of the NLRP3 inflammasome. Since activation of the NLRP3 inflammasome induces caspase-1-dependent programmed cell death (pyroptosis) (2), the current study investigated whether CZE influenced NLRP3 inflammasome-mediated pyroptosis in LPS-primed BMDMs. Pyroptosis was quantified by measuring the quantity of LDH released into the cell culture supernatant. The levels of LDH released by LPS-primed cells in response to ATP, nigericin and MSU were ~66, 84 and 46%, respectively (Fig. 3A-C). ATP-induced LDH release was slightly decreased by CZE at concentrations

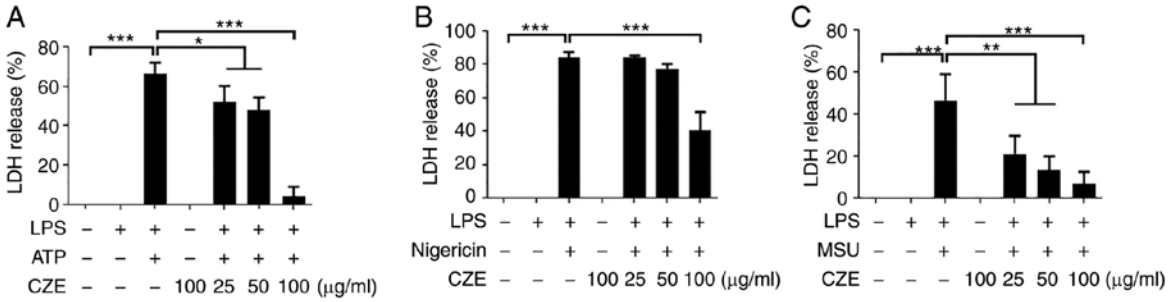

Figure 3. CZE decreases nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 inflammasome-induced release of LDH in BMDMs. BMDMs were primed with LPS (100 ng/ml) for 6 h and subsequently treated with CZE for 30 min. The cells were incubated with (A) ATP (2 mM) and (B) nigericin (10 μM) for 40 min and (C) MSU (200 μg/ml) for 4 h. The level of pyroptosis was measured by LDH release assay. The results are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. LPS, lipopolysaccharide; BMDMs, bone marrow-derived macrophages; LDH, lactate dehydrogenase; CZE, ethanol extract of *Chrysanthemum zawadskii*; MSU, monosodium urate.

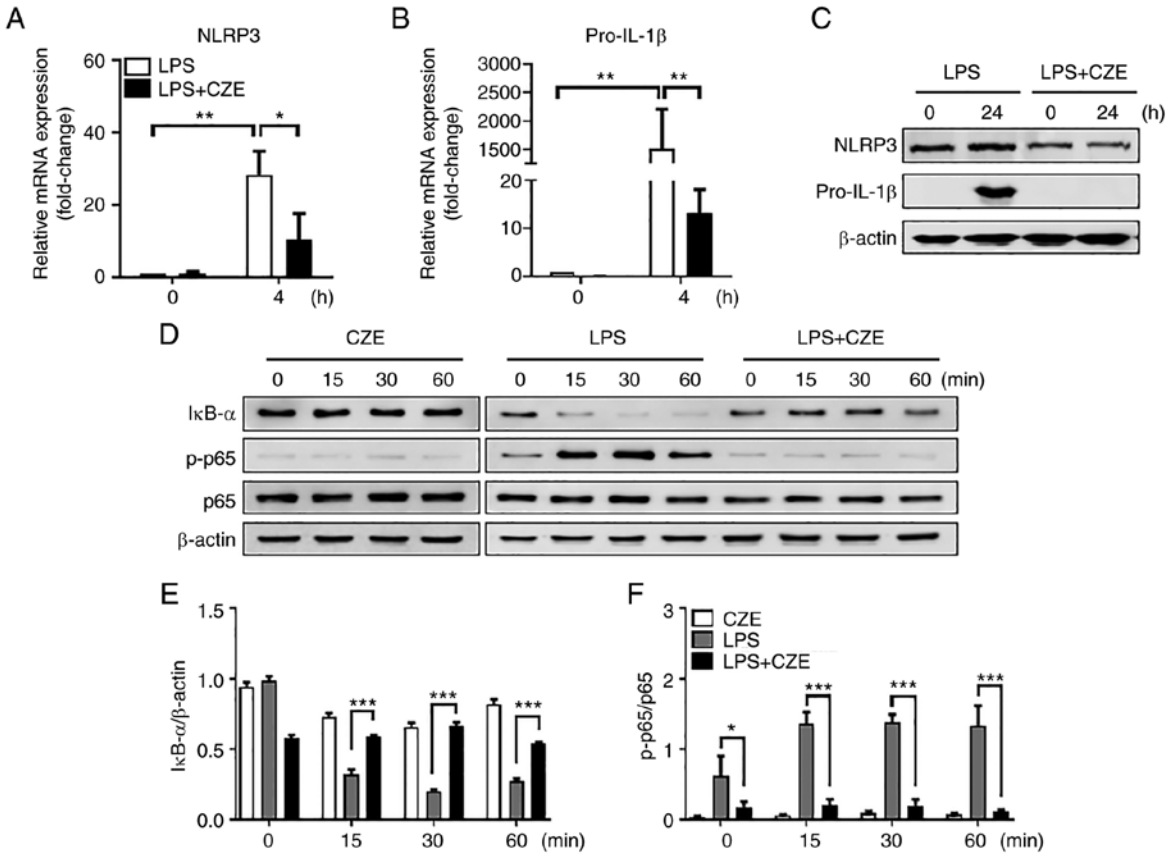

Figure 4. CZE decreases LPS-induced gene expression of NLRP3 and pro-IL-1β via inhibition of NF-κB signaling. BMDMs were pretreated with CZE (100 μg/ml) for 2 h and subsequently treated with LPS (100 ng/ml) for 4 h (for gene expression) or 24 h (for protein expression). Gene expression of (A) NLRP3 and (B) pro-IL-1b was determined by reverse transcription-quantitative PCR. The results are presented as the mean ± SD. *P<0.05, **P<0.01. (C) Western blotting was performed to detect NLRP3 and pro-IL-1β. (D) BMDMs were treated with CZE and stimulated with LPS. Western blotting was performed to detect the degradation of IκB-α and the phosphorylation of p65 using specific primary antibodies. β-actin was used as a loading control. Quantification of the protein levels of (E) IκB-α/β-actin and (F) p-p65/p65 ratio was performed. The results are presented as the mean ± SD. *P<0.05, ***P<0.001. LPS, lipopolysaccharide; BMDM, bone marrow-derived macrophage; NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3; CZE, ethanol extract of *Chrysanthemum zawadskii*; p, phosphorylated.

of 25 and 50 μg/ml; CZE at 100 μg/ml inhibited LDH release by >90% (Fig. 3A). In addition, nigericin-induced LDH release was decreased by CZE at 100 μg/ml (Fig. 3B). CZE treatment also inhibited LDH release in response to MSU in a dose-dependent manner (Fig. 3C). These findings indicate that CZE inhibited NLRP3 inflammasome-induced pyroptotic cell death.

CZE decreases gene expression of NLRP3 and pro-IL-1β and the activation of NF-κB in BMDMs in response to LPS. To examine whether CZE inhibits the priming step of the NLRP3 inflammasome, BMDMs were treated with LPS for 4 and 24 h in the presence or absence of CZE (Fig. 4A-C). LPS-induced gene expression of NLRP3 and pro-IL-1β was decreased in CZE-treated cells compared

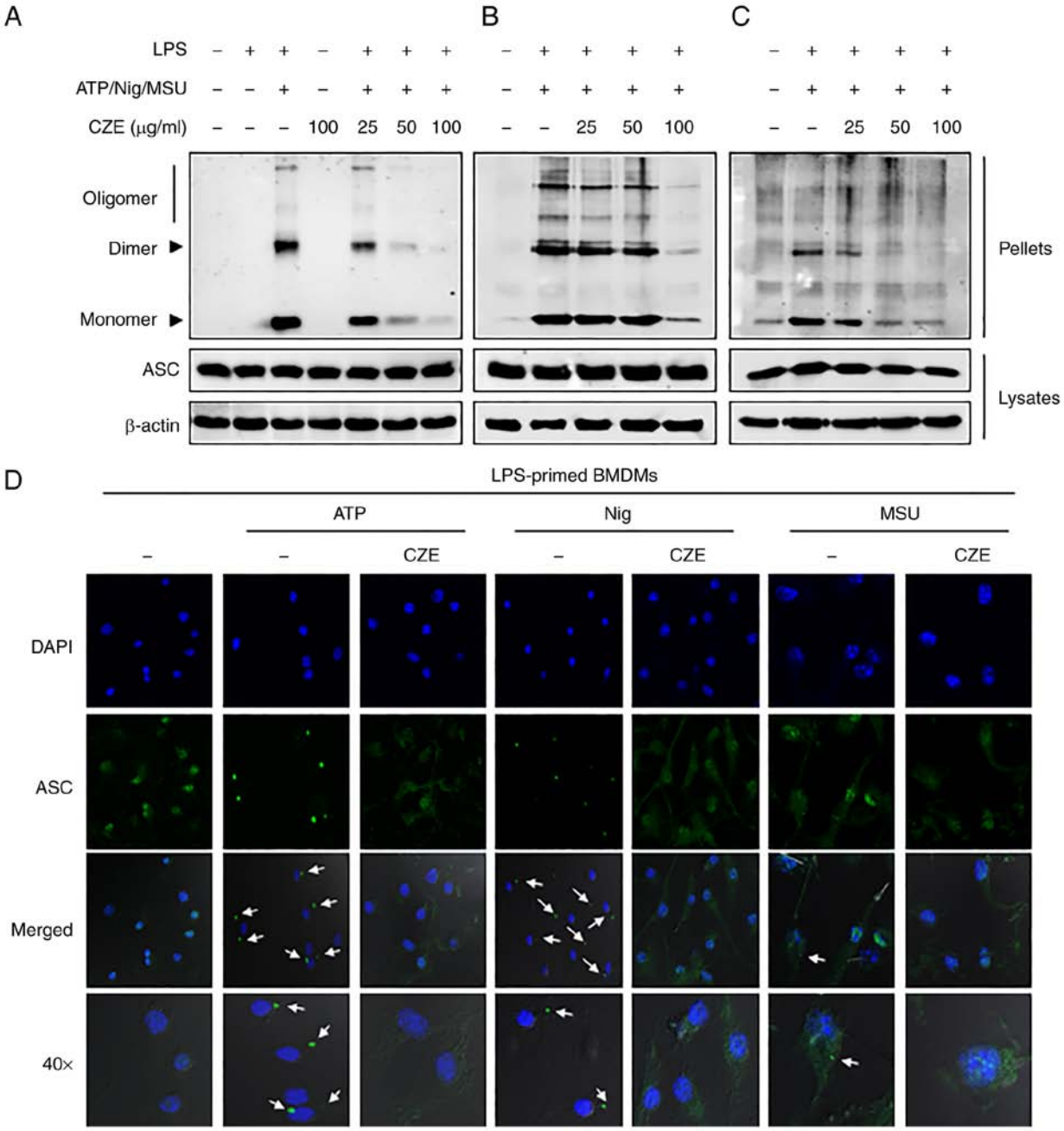

Figure 5. CZE inhibits nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 inflammasome-induced oligomerization and speck formation of ASC in BMDMs. BMDMs were primed with LPS (100 ng/ml) for 6 h and treated with CZE for 30 min. The cells were incubated with (A) ATP (2 mM) or (B) Nig (10 μM) for 40 min or (C) MSU (200 μg/ml) for 4 h. The oligomerization of ASC in Triton X-100-insoluble pellets was determined by western blotting. ASC and β-actin were used as loading controls. (D) LPS-primed BMDMs were treated with ATP (2 mM) or Nig (10 μM) for 40 min or MSU (200 μg/ml) for 4 h and stained with an anti-ASC antibody (green). The nuclei (blue) were stained with DAPI. 200x magnification. White arrows indicate ASC specks. ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; LPS, lipopolysaccharide; BMDMs bone marrow-derived macrophage; MSU, monosodium urate; CZE, ethanol extract of *Chrysanthemum zawadskii*; Nig, nigericin.

with LPS-stimulated cells (Fig. 4A and B). Protein expression of NLRP3 and pro-IL-1β in response to LPS was decreased by CZE (Fig. 4C). Western blot analysis showed that IκB-α degradation was prevented by CZE treatment in BMDMs in response to LPS; p65 phosphorylation was also decreased by CZE (Fig. 4D-F). CZE alone did not affect IκB-α degradation or p65 phosphorylation in BMDMs (Fig. 4D-F). Accordingly, it is likely that CZE regulated NLRP3 inflammasome activation by inhibiting both the priming and activation steps.

CZE inhibits NLRP3 inflammasome-mediated oligomerization and speck formation of ASC. ASC oligomerization is a hallmark of inflammasome activation (2,22). Therefore, the present study sought to determine whether CZE inhibits ASC oligomerization in response to ATP, nigericin and MSU in LPS-primed BMDMs. ATP, nigericin and MSU treatment led to ASC oligomerization in LPS-primed BMDMs, which was decreased by CZE in a dose-dependent manner (Fig. 5A-C). The present study further examined the effect of CZE on ASC speck formation, which contributes to signal

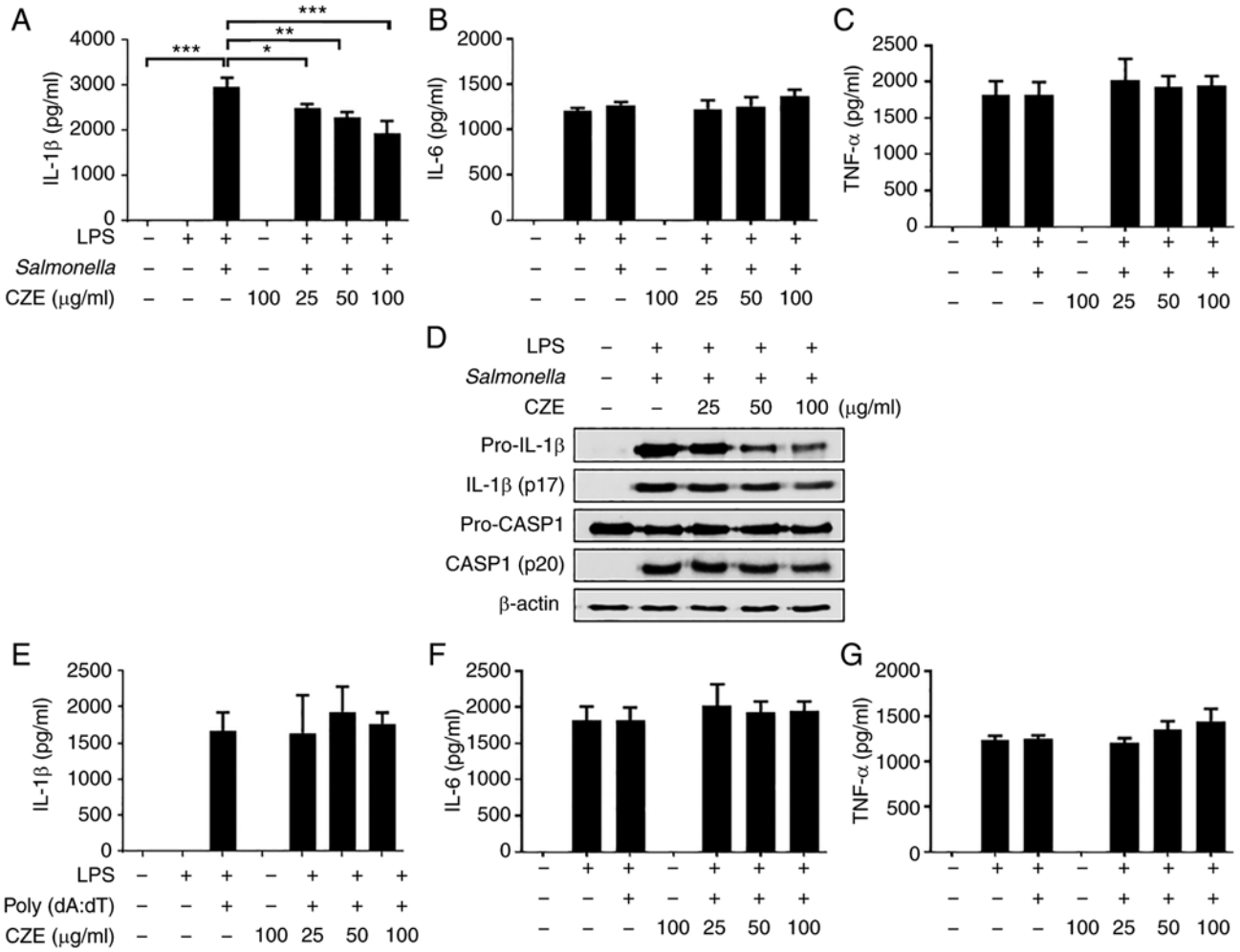

Figure 6. CZE inhibits activation of the NLR family CARD domain-containing protein 4 inflammasome but not absent in melanoma 2 inflammasome in BMDMs. BMDMs were primed with LPS (100 ng/ml) for 6 h and subsequently treated with CZE for 30 min. The cells were infected with *Salmonella typhimurium* (multiplicity of infection=10) for 1 h, and the medium was replaced with medium containing gentamicin and incubated for 3 h. Levels of (A) IL-1β, (B) IL-6 and (C) TNF-α in the culture supernatants were measured by ELISA. (D) Culture supernatant and cell lysates were used to detect the immature and cleaved forms of IL-1β and CASP1 by western blotting. β-actin was used as a loading control. LPS-primed BMDMs were treated with CZE. The cells were additionally incubated with poly(dA:dT) (2 μg/ml) for 4 h. Levels of (E) IL-1β, (F) IL-6, (G) TNF-α in the culture supernatants were measured by ELISA. The results are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. LPS, lipopolysaccharide; BMDM, bone marrow-derived macrophage; CZE, ethanol extract of *Chrysanthemum zawadskii*; CASP1, caspase-1.

amplification (22,23). ATP, nigericin and MSU treatment induced ASC speck formation in LPS-primed macrophages, which was prevented by CZE treatment (Fig. 5D). These results indicated that CZE may regulate the NLRP3 inflammasome by inhibiting ASC oligomerization and speck formation.

CZE does not inhibit inflammasome activation of NLRC4 or AIM2. In addition to NLRP3, NLRC4 and AIM2 are also involved in the formation of the inflammasome (24). Therefore, the present study examined whether CZE also regulates NLRC4 or AIM2 inflammasome activation. LPS-primed BMDMs were infected with *S. typhimurium* to induce NLRC4 inflammasome activation and treated with poly(dA:dT) to activate the AIM2 inflammasome. CZE treatment slightly reduced the *S. typhimurium*-induced IL-1β secretion, whereas the production of IL-6 or TNF-α was not affected (Fig. 6A-C). Western blot analysis revealed that expression of both pro- and cleaved IL-1β was decreased by CZE (Fig. 6D). By contrast, the expression of pro-caspase-1 was not affected by CZE and cleaved caspase-1 expression was slightly decreased only at high concentrations (Fig. 6D), suggesting that the decrease in IL-1β induced by CZE in *S. typhimurium*-infected BMDMs may have been due to a decrease in pro-IL-1β rather than inflammasome activation. In addition, CZE did not affect the poly(dA:dT)-induced production of IL-1β, IL-6 or TNF-α (Fig. 6E-G). These findings suggested that CZE did not inhibit NLRC4 or AIM2 inflammasome activation, although it reduced the protein expression of pro-IL-1β in response to *S. typhimurium*.

Key components of CZE inhibit IL-1β production in response to NLRP3 inflammasome activators in LPS-primed BMDMs. CZE is known to contain three key components: Linarin, 3,5-di-CQA and CGA (13). These components have been reported to have anti-inflammatory effects (25-27). Therefore, the present study investigated whether these components regulate NLRP3 inflammasome-induced IL-1β production in LPS-primed BMDMs. LPS-primed BMDMs were treated with 100 μM linarin, 3,5-di-CQ and CGA and stimulated

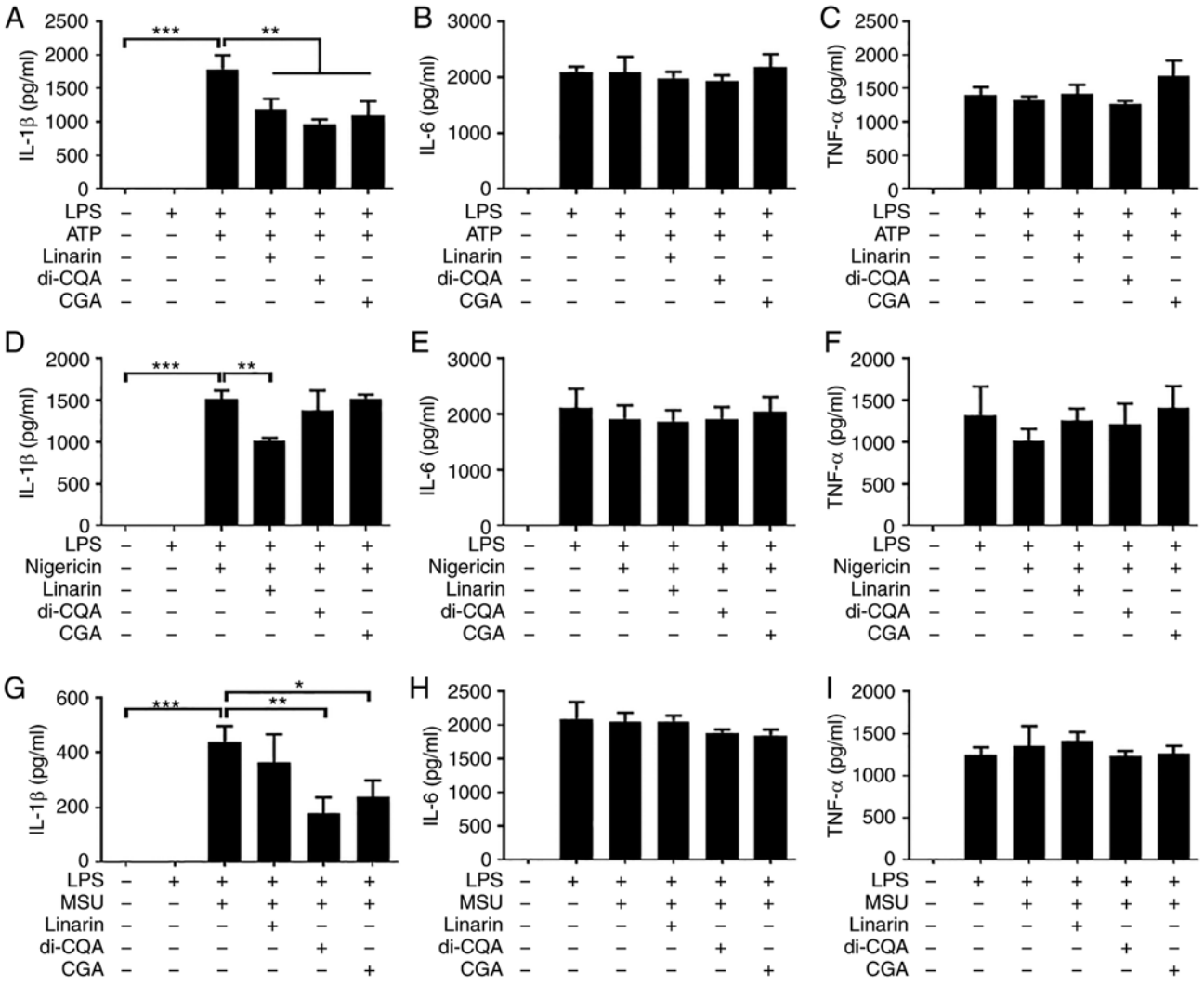

Figure 7. Linarin, di-CQA and CGA decrease nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 inflammasome-induced IL-1β secretion from LPS-primed BMDMs. BMDMs were primed with LPS (100 ng/ml) for 6 h and subsequently treated with 100 μM linarin, di-CQA or CGA for 30 min. Cells were incubated with ATP (2 mM) or nigericin (10 μM) for 40 min or MSU (200 μg/ml) for 4 h. The levels of (A) IL-1β, (B) IL-6 and (C) TNF-α in culture supernatant from BMDMs treated with or without LPS and ATP and linarin, di-CQA and CGA were measured by ELISA. LPS-primed BMDMs were treated with nigericin (10 μM) for 40 min in the presence or absence of linarin, di-CQA and CGA. The levels of (D) IL-1β, (E) IL-6 and (F) TNF-α in culture supernatants were measured by ELISA. LPS-primed BMDMs were treated with MSU (200 μg/ml) for 4 h with or without linarin, di-CQA and CGA. Levels of (G) IL-1β, (H) IL-6 and (I) TNF-α in culture supernatant were measured by ELISA. The results are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. di-CQA, di-caffeoylquinic acid; CGA, chlorogenic acid; LPS, lipopolysaccharide; BMDM, bone marrow-derived macrophage; MSU, monosodium urate.

with ATP, nigericin and MSU. All components inhibited the ATP-induced secretion of IL-1β, but not IL-6 or TNF-α, in LPS-primed BMDMs (Fig. 7A-C). Linarin, but not 3,5-di-CQA or CGA, decreased nigericin-induced IL-1b secretion (Fig. 7D). By contrast, IL-1β induced by MSU was decreased by 3,5-di-CQA and CGA, but not by linarin (Fig. 7G). IL-6 secretion in response to nigericin (Fig. 7E and F) and MSU (Fig. 7H and I) was not affected by any of the components. These results indicated that the three major components of CZE may inhibit the NLRP3 inflammasome via different mechanisms.

Discussion

The uncontrolled activation of the NLRP3 inflammasome is associated with various metabolic and inflammatory diseases (4-9). Therefore, efforts have been made to find new compounds that inhibit the NLRP3 inflammasome. Specifically, numerous compounds in plants, such as curcumin, epigallocatechin gallate, quercetin and resveratrol have been shown to inhibit the NLRP3 inflammasome (28). *C. zawadskii* var. *latilobum* (CZ) is widely used in traditional Chinese medicine. Its extract has been reported to inhibit nitric oxide (NO) production via induction of heme oxygenase-1 (16). CZ extract also increases transactivation of peroxisome proliferator-activated receptors-responsive element, suppresses TNF-α and IL-6-induced NF-κB activation and NO production and plays a role in the homeostasis of the skin barrier (29). However, to the best of our knowledge, whether CZ extract regulates NLRP3 inflammasome activation has not been reported. The present study found that CZE efficiently inhibited NLRP3 activator (ATP, nigericin and MSU)-induced IL-1β secretion, cleavage

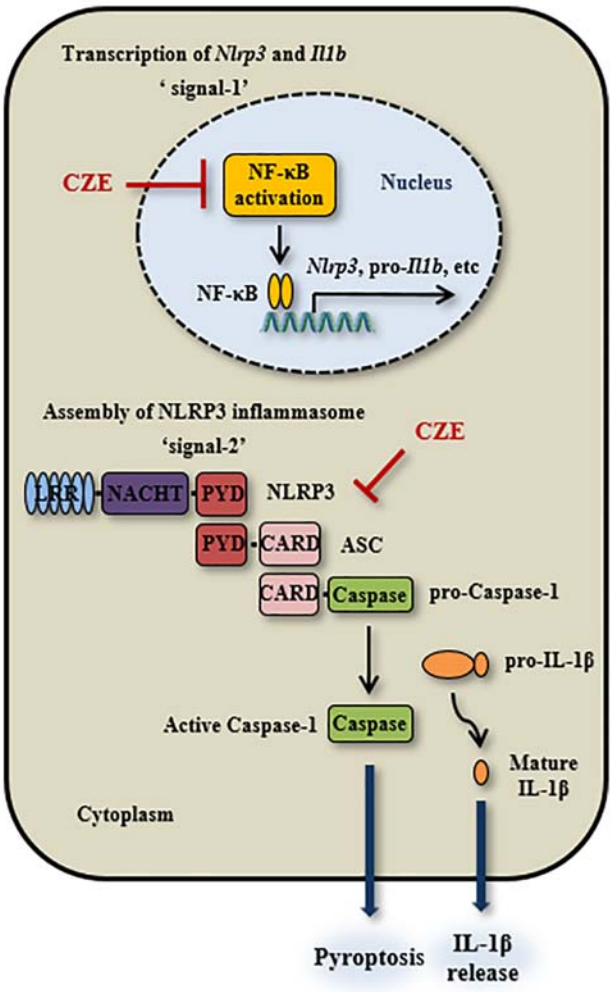

Figure 8. Schematic of the inhibitory effect of CZE on activation of the NLRP3 inflammasome in macrophages. CZE downregulates expression of NLRP3 and pro-IL-1β in response to LPS by inhibiting NF-κB activation in macrophages. It also suppresses ATP/nigericin/monosodium urate-induced activation of the NLRP3 inflammasome in LPS-primed macrophages via inhibition of ASC oligomerization and speck formation, resulting in decreased IL-1β secretion and pyroptosis. NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3; LPS, lipopolysaccharide; CZE, ethanol extract of *Chrysanthemum zawadskii*; LRR, leucine-rich repeat; NACHT, NAIP, CIITA, HET-E and TEP1; PYD, pyrin domain; CARD, caspase-recruitment domain; ASC, apoptosis-associated speck-like protein containing a CARD.

of caspase-1 and IL-1β and pyroptotic cell death. CZE also inhibited the LPS-induced gene expression of NLRP3 and pro-IL-1β and the activation of NF-κB, suggesting that CZE regulated activation of the NLRP3 inflammasome at both signals 1 and 2.

To the best of our knowledge, little is known about the role of the *Chrysanthemum* extract in inflammasome activation. Methanol extract of *Chrysanthemum indicum* suppresses both NLRP3 and AIM2 inflammasome activation by regulating ASC phosphorylation (30). In high-performance liquid chromatography fingerprinting analysis of *C. indicum*, one major peak corresponding to 1,5-di-CQA and minor peaks corresponding to luteolin and CGA were observed (30). Kim *et al* (13) identified that CGA, 3,5-di-CQA and linarin are three key components of CZE. Linarin, a natural flavonoid, has been reported to have anti-inflammatory effects by downregulating phagocytosis and induce pro-inflammatory cytokine production, such as IL-6, TNF and IL-1β and antigen presentation in LPS-stimulated macrophages (27). Kim *et al* (18) showed that ethanol extract of *Chrysanthemum zawadskii* Herbich (ECZ) induces reactive oxygen species-mediated apoptosis and autophagy in mouse colon cancer CT-26 cells. In addition, high performance liquid chromatography analysis was performed to identify and quantify the components (CGA, 3,5-di-CQA and luteolin) in ECZ. However, in the aforementioned study, the physiological effects of the components contained in CZE were not confirmed. In the present study, it was confirmed that three components (linarin, 3,5-di-CQA and CGA) of CZE inhibited production of IL-1β by using various molecules that induce NLRP3 inflammasome activation. CZE and its components (CGA, 3,5-di-CQA and linarin) contribute to inhibition of NLRP3 inflammasome activation by regulating both step 1 and 2, but not NLRC4 and AIM2 inflammasome activation. 3,5-Di-CQA has been shown to inhibit pro-inflammatory gene expression and NO production via inducible NO synthase and cyclooxygenase-2 (25). CGA exerts an anti-inflammatory effect in LPS-stimulated macrophages and microglial cells (26) and inhibits fibroblast-like synoviocyte proliferation by inducing apoptosis (31). In addition, CGA administration increases expression of nuclear factor E2-related factor-2 (Nrf2) and antioxidant genes in liver tissue and rescues liver damage induced by CCl_4 in rats (32). The expression of NLRP3 inflammasome-associated proteins, such as caspase-1 and IL-1β (both full length and cleaved forms), is also reduced in liver tissues of CCl_4-treated rats by CGA administration. CGA inhibits the activation of the NLRP3 inflammasome by activating Nrf2 (32). Linarin is known to prevent acute lung injury induced by LPS in mice by suppressing oxidative stress and inflammation (33). Protein expression of NLRP3, ASC and caspase-1 are decreased by linarin in LPS-treated lung epithelial cells. In the present study, linarin, 3,5-di-CQA and CGA differently regulated NLRP3 inflammasome activation, as only linarin inhibited nigericin-induced IL-1β production, whereas 3,5-di-CQA and CGA, but not linarin, decreased IL-1β production induced by MSU. Thus, further studies are required to uncover the underlying mechanism by which each component controls NLRP3 inflammasome activation.

ASC serves a key role in the formation of the NLRP3 inflammasome complex by recruiting pro-caspase-1 via interaction with the pyrin domain of the NLRP3 inflammasome (22,23). Previous studies have demonstrated that NLRP3 activator-mediated phosphorylation and oligomerization of ASC is affected by plant extracts (30,34). In the present study, ASC oligomerization in response to NLRP3 activators was decreased by CZE in LPS-primed BMDMs. ASC oligomerization is a key step in the formation of the AIM2 inflammasome complex. Inhibition of ASC speck formation by plant extracts affects the activation of AIM2, as well as the NLRP3 inflammasome (30,34). In the present study, CZE did not affect AIM2 inflammasome-induced IL-1β secretion. CZE may regulate NLRP3 inflammasome activation upstream of ASC; alternatively, CZE may be specific for NLRP3 inflammasome complex formation. Further studies are therefore needed to clarify the precise mechanism. In addition, unlike NLRP3 and AIM2, the CARD of NLRC4 binds pro-caspase-1 without

ASC (23,35). In the present study, *Salmonella*-induced IL-1β production was slightly decreased by CZE treatment, although this was due to decreased production of pro-IL-1β. The effect of CZE on the cleavage of IL-1β and caspase-1 in response to *Salmonella* infection was limited. Despite it being known that activated NLRC4 recruits and interacts with NLRP3 (36), the effect of CZE on NLRC4 inflammasome activation is not notable. In conclusion, the present study demonstrated that CZE and its components (CGA, 3,5-di-CQA and linarin) effectively inhibited activation of the NLRP3 inflammasome at both steps 1 and 2 (Fig. 8). The present results suggested that CZE and its components may be developed as novel therapeutics against NLRP3-mediated metabolic or inflammatory disease. *Gnaphalium pensylvanicum* extracts containing caffeoylquinic acid derivatives shows anti-gout activity in mice with MSU-induced acute gouty arthritis (37). Therefore, animal experiments are needed to evaluate the potential treatment effect of NLRP3 inflammasome inhibition using CZE and its components.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JP designed the study. AJ and HL performed experiments and collected data. AJ, HL, JH, YK and JP analyzed and interpreted data. AJ and JP wrote, revised and reviewed the manuscript. All authors have read and approved the final manuscript. AJ, HL and JP confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal studies were performed using protocols approved by the Institutional Animal Care and Use Committee of Chonnam National University (Gwangju, Republic of Korea; approval no. CNU IACUC-YB-2018-02).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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