

# Green lipped mussel oil complex suppresses lipopolysaccharide stimulated inflammation via regulating nuclear factor- $\kappa$ B and mitogen activated protein kinases signaling in RAW264.7 murine macrophages

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**Abstract** Here we investigated the effect of green lipped mussel oil complex (GLMOC) on inflammation and underlying mechanism in lipopolysaccharide stimulated RAW264.7 murine macrophage cells. GLMOC containing green lipped mussel oil (GLMO), olive oil, and vitamin E (10:20:1) can induce significant suppression of iNOS, leading to reduced nitric oxide synthesis, and cyclooxygenase-2, leading to reduced prostaglandin E2 synthesis. In addition, it down-regulated the release of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$ , interleukin (IL)-6, and IL-1 $\beta$ . Similar to upstream signaling mediators, GLMOC inhibited the degradation of inhibitory  $\kappa$ B, nuclear translocation of NF- $\kappa$ B, and phosphorylation of mitogen activated protein kinases (MAPKs) in a dose-dependent manner. Among the components of GLMOC, GLMO was responsible for anti-inflammatory efficacy. Taken together, GLMOC induces anti-inflammatory activity via regulating NF- $\kappa$ B and MAPK signaling in lipopolysaccharide-induced RAW264.7 cells, providing underlying mechanisms that elucidate the anti-inflammatory efficacy of GLMOC.

**Keywords** Green lipped mussel oil · Inflammation · Nuclear factor  $\kappa$ B · Mitogen activated protein kinase · RAW264.7

## Introduction

Green lipped mussel (*Perna canaliculus*, GLM) is a bivalve mussel mainly cultivated in New Zealand. Traditionally, the health beneficial effect of GLM has been known based on the finding that people living in the coastal area of New Zealand and consuming GLM in their diet developed less severe arthritis than those living in the inland area, wherein the oil fraction of GLM (GLMO) has been considered as a key fraction responsible for the physiological activity of GLM [1].

Many studies have demonstrated the green lipped mussel oil complex (GLMOC) containing GLMO, vitamin E, and olive oil shows health beneficial effect for treating arthritis, including osteoarthritis and rheumatoid arthritis [2–5], inflammation [6–8], and asthma [9, 10]. Lee et al. [5] reported that GLMOC relieved the pain and regulated cytokines in adjuvant-induced arthritis rat model. In clinical studies, GLMOC significantly improved the symptoms of osteoarthritis, including pain relief and joint function, in patients with osteoarthritis [2] and combination of GLMOC and fish oil ameliorated the joint pain in rheumatoid arthritis patients [3]. In Korea, GLMOC was approved as a raw material for functional foods that can improve joint health by the Korea Ministry of Food and Drug Safety in 2004. In inflammation, GLMOC was reported to significantly inhibit the enzymatic activity of cyclooxygenase (COX)-1 and -2 in vitro [7], as well as suppress the dextran sulfate sodium-induced symptoms of inflammatory bowel disease, including body weight loss, crypt area loss, and colon weight in C57BL/6 mice [8]. In addition, GLMOC reduced the expression of CD23 not only in leukotriene B4 (LTB4)-stimulated human monocytes but also in monocytes from allergic patients producing high level of LTB4 [11].

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However, the mechanistic study underlying the anti-inflammatory efficacy of GLMOC was not clearly investigated.

Inflammation is a defense mechanism caused by physical or chemical injury and/or an infectious agent. Acute inflammation is an early beneficial response that helps eliminate pathogens and necrotic cells as well as initiates the healing process at the site of tissue injury [12]. Chronic inflammation refers to prolonged and/or repeated physiological processes of inflammation, which can develop into different chronic diseases such as cardiovascular diseases, diabetes, inflammatory bowel disease, and cancer [13, 14]. Inflammation can be mediated through toll-like receptors (TLRs) after recognizing the specific molecular patterns such as lipopolysaccharide (LPS) present in the gram-negative microbial compartment [15]. Downstream signaling induced by activation of TLRs is mediated through nuclear factor (NF)- $\kappa$ B, which can be activated through degradation of inhibitory- $\kappa$ B (I $\kappa$ B) by I $\kappa$ B kinase (IKK), and/or phosphorylation of histone H3 by p38 mitogen activated protein kinase (MAPK) [16]. The well-known target genes regulated by NF- $\kappa$ B-mediated inflammatory signal are inducible nitric oxide synthase (iNOS), cyclooxygenase (COX), and different cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-6, and IL-1 $\beta$  [17], and many studies were performed to investigate the effect of natural compounds on them to elucidate the molecular mechanism of their anti-inflammatory efficacy [18].

To overcome the inconsistent efficacy of green lipped mussel extract on inflammation, GLMO was prepared by freeze-drying GLM (obtained from New Zealand) and extracted using CO<sub>2</sub>-supercritical fluid extraction (SFE), and GLMOC was formulated with vitamin E and olive oil as an antioxidant, which can achieve a consistently high inflammation regulatory activity [19]. In this study, we investigated the effect of freeze-dried and CO<sub>2</sub>-SFE extracted GLMOC on inflammation and the underlying mechanism of the action in LPS-stimulated RAW264.7 murine macrophage cells.

## Materials and methods

### Materials

Freeze-dried and CO<sub>2</sub>-SFE extracted GLMOC was from Gwanjeolpalpal (Syspang, Seoul). GLMO, vitamin E, and olive oil were also provided by Syspang (Seoul). LPS, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and sulfuraphane were purchased from Sigma (St. Louis, MO, USA).

### Cell culture

RAW264.7 cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM media (Life Technologies, Grand Island, USA) supplemented with 10% fetal bovine serum (Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 1% HEPES (Life Technologies).

### NO assay

The amount of NO released by RAW264.7 cells was determined using an NO assay kit (Promega, USA) following the manufacturer's protocol. Briefly, 50  $\mu$ L supernatant from different treatments was collected, and the enzymatic conversion of the supernatant nitrate to nitrite by nitrate reductase were determined by colorimetric assay at 540 nm.

### Quantitative real time-PCR

Total mRNA was isolated using Trizol (Life Technologies) after 12 h treatments in RAW264.7 cells. cDNA was synthesized using a RevertAid strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The Taqman primers were used and the expression of the target RNA was determined with ABI PRISM 7900 (Life Technology). As an internal control, GAPDH was used.

### PGE2 and cytokines assay

The supernatants after treatments for 24 h in RAW264.7 cells were used for the determination of the released PGE2 and cytokines. The concentrations of PGE2 were measured using the PGE2 competitive ELISA kit (Enzo Life Sciences Inc. America) and those of TNF $\alpha$ , IL-6, and IL-1 $\beta$  were by Sandwich ELISA kit (Ray Biotech, Inc. America) following the manufacturer's protocol.

### Western blot analysis assay

Cell lysates were isolated with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% phenylmethylsulfonyl fluoride, and 1% protease inhibitor). The proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Milipore, Billerica, MA, USA). The primary antibodies against iNOS, p-p65, p-ERK, p-p38, p-JNK (Cell signaling Inc., USA), COX-2, I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and  $\beta$ -actin (Sigma) were probed.

Secondary antibodies from Santa Cruz Biotechnology Inc. were used. Protein expression levels were visualized by a chemiluminescence reagent (GE healthcare, Little Chalfont, UK) in EZ capture MG (ATTO, Tokyo, Japan). The blots were quantified using EZ west Lumi plus (ATTO).

### Cell proliferation assay

RAW264.7 cells were seeded into a 96 well plate and treated with different components after 24 h. Four hours before cell harvest, MTT solution (5 mg/mL) was added to each well. The dark blue formazan crystals formed were dissolved in DMSO and measured at 570 nm.

### Statistical analysis

Statistical differences were determined using one-way ANOVA. Differences were considered as statistically significant at  $p < 0.05$ .

## Results and discussion

### GLMOC suppressed iNOS expression and NO synthesis in LPS-induced RAW264.7 cells

As mediators for inflammatory responses, NO synthesized by iNOS enzyme possesses cytotoxic property against not only exogenous substances but also host tissues and are considered as the targets for anti-inflammatory efficacy [20, 21]. Here we first investigated the effect of GLMOC on NO synthesis and expression of iNOS. As shown in Fig. 1(A), GLMOC at the concentration of 50, 100, and 300  $\mu\text{g/mL}$  exerted a dose-dependent inhibition of NO synthesis induced by LPS in RAW 264.7 cells. Sulforaphane is a well-known phytochemical used as a positive control. The mRNA and protein expressions of iNOS were determined by quantitative real time PCR and western blot, respectively. GLMOC significantly suppressed the expression of iNOS stimulated by LPS, which were  $28.4 \pm 0.9$  and  $53.2 \pm 0.1\%$  inhibition of iNOS mRNA and protein at 50  $\mu\text{g/mL}$ ,  $40.9 \pm 2.8$  and  $61.0 \pm 0.2\%$  inhibition at 100  $\mu\text{g/mL}$ , and  $67.4 \pm 0.3$  and  $78.9 \pm 0.7\%$  inhibition at 300  $\mu\text{g/mL}$ , respectively [Fig. 1(B), (C)]. These results indicate that GLMOC possesses the significant regulatory efficacy on NO synthesis cascades during LPS-induced inflammatory process in RAW264.7 cells.

### GLMOC inhibited the expression of COX-2 and prostaglandin E2 level in LPS-induced RAW264.7 cells

COX is the enzyme catalyzing the oxygenation of fatty acids, preferably arachidonic acid and producing different cell

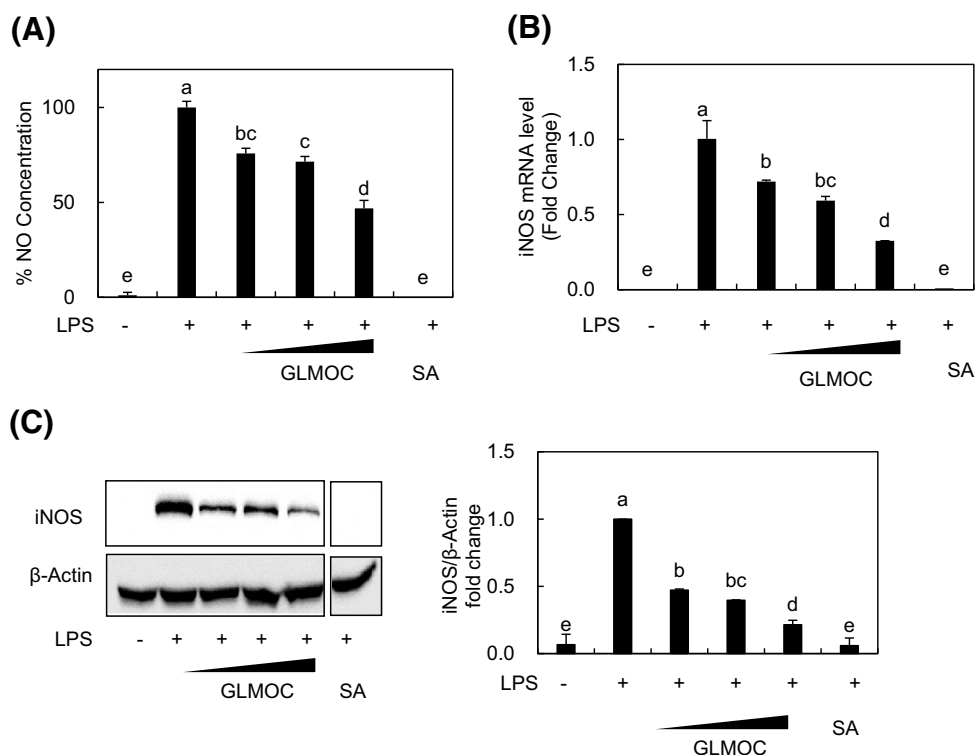
signaling mediators involved in the inflammatory processes [22], and many natural and synthetic agents have been studied to inhibit the specific COX, particularly COX-2 to suppress the inflammation and/or prevent cancer [18, 22, 23]. GLMOC significantly reduced the expression of COX-2 stimulated by LPS, which were  $35.2 \pm 2.6$  and  $38.0 \pm 11\%$  inhibition of mRNA and protein at 50  $\mu\text{g/mL}$ ,  $43.8 \pm 0.2$  and  $42.2 \pm 10\%$  inhibition at 100  $\mu\text{g/mL}$ , and  $54.2 \pm 3.2$  and  $51.7 \pm 1.4\%$  inhibition at 300  $\mu\text{g/mL}$ , respectively [Fig. 2(B), (C)]. In addition, the level of prostaglandin E2 (PGE2) produced by activated COX-2 was significantly decreased from  $29.6 \pm 0.5$  ng/mL in LPS-stimulated cells to  $19.5 \pm 0.9$  ng/mL after GLMOC treatment [Fig. 2(A)]. McPhee et al. [7] reported that GLMO inhibited the in vitro enzymatic activities of COX-1 and COX-2, wherein the activity was enhanced by hydrolysis of GLMO, supporting our results showing the significant regulation of COX-2 and related mediator of inflammation by GMLOC in RAW264.7 cells.

### GLMOC reduced the production of pro-inflammatory cytokines in LPS-stimulated RAW264.7 Cells

Pro-inflammatory cytokines, including TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , play important role in the inflammatory process [24]. Previously, GLMO suppressed the expression of TNF- $\alpha$  and interferon (IFN)- $\gamma$  in adjuvant-induced arthritis model in rats [5] and TNF- $\alpha$  and IL-12p40 in LPS-induced human THP-1 monocytes [25]. Therefore, we tested the effect of GLMOC on the expression levels of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in LPS-stimulated RAW264.7 cells. After the induction of mRNA expression of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  with LPS, GLMOC treatment significantly inhibited up to  $50.8 \pm 0.2$ ,  $51 \pm 10$ ,  $30 \pm 11\%$ , respectively, compared to the LPS-treated control [Fig. 3(A)]. The amount of cytokines released into the media was also measured by ELISA assay. As shown in Fig. 3(B), the concentrations of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  were decreased from  $2.58 \pm 0.5$ ,  $1.02 \pm 0.1$  ng/mL, and  $113 \pm 15$  pg/mL in LPS-treated control to  $0.84 \pm 0.1$ ,  $0.73 \pm 0.0$  ng/mL, and  $24.9 \pm 5.8$  pg/mL in GLMOC treated groups, respectively, which showed similar pattern with mRNA regulation by GLMOC treatment. In line with previous studies, GLMOC strongly down-regulated the expression of pro-inflammatory cytokines in RAW264.7 cells.

### GLMOC blocked the NF- $\kappa$ B signaling pathway in LPS-stimulated RAW264.7 cells

NF- $\kappa$ B composed of p65 and p50 subunits is a well-known transcription factor that plays a key role in mediating inflammatory responses and is activated by inflammatory cytokines such as TNF- $\alpha$  and/or pathogen-associated molecular patterns [26]. After LPS treatment, we found that



**Fig. 1** GLMOC suppressed iNOS expression and NO synthesis in LPS-induced RAW264.7 cells. **(A)** The produced NO was measured with NO assay. The cells ( $4 \times 10^4$ /well in 96-well plate) were pretreated with GLMOC or SA for 2 h, and then LPS (100 ng/mL) for 22 h. **(B)** The relative amount of iNOS mRNA was measured with quantitative RT-PCR. The cells ( $8 \times 10^5$  cells in a 60 mm dish) were pretreated with GLMOC or SA for 2 h, and then LPS for 10 h.

inhibitor  $\kappa$ B that can bind and sequester NF- $\kappa$ B in the cytoplasm was degraded, and then significantly recovered in the presence of GLMOC in a dose-dependent manner [Fig. 4(A)]. The phosphorylation of NF- $\kappa$ B subunit p65 activated by LPS stimulation was also suppressed by GLMOC maintaining the total form of p65 at certain level in RAW264.7 cells [Fig. 4(A)]. To confirm the effect of GLMOC on NF- $\kappa$ B signaling, we collected the nuclear fractions and checked the activated NF- $\kappa$ B localized in the nucleus. As shown in Fig. 4(B), upon exposure of RAW264.7 cells to LPS, both p65 and phosphorylated p65 were accumulated in the nuclear fraction, which were significantly inhibited by GLMOC treatment at 300  $\mu$ g/mL. These results clearly indicate that GLMOC can modulate the NF- $\kappa$ B signaling activated by LPS, which might lead to down-regulation of iNOS, COX-2, and cytokines in RAW264.7 cells.

#### GLMOC regulated the MAPKs signaling pathway in LPS-induced RAW264.7 cells

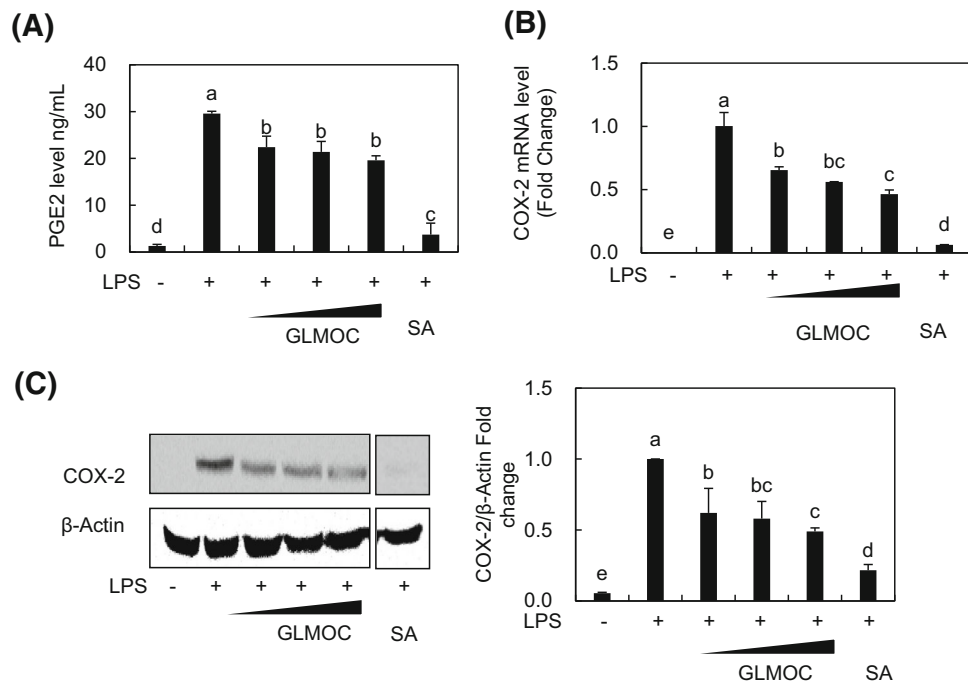
In addition to NF- $\kappa$ B, MAPKs are also critical in mediating inflammation. For example, extracellular signal-regulated

(C) The protein levels of iNOS and  $\beta$ -actin were determined by Western blot. The cells ( $8 \times 10^5$  cells in a 60 mm dish) were pretreated with GLMOC or SA for 2 h, and then LPS (100 ng/mL) for 22 h. Values represent the mean  $\pm$  SD. Different lowercase letters indicate significant differences at  $p < 0.05$ . GLMOC (50, 100, and 300  $\mu$ g/mL), SA (sulforaphane, 10  $\mu$ M), LPS (100 ng/mL)

kinase (ERK) and p38 are known to be phosphorylated by LPS and involved in posttranscriptional regulation of TNF- $\alpha$ , which can result in inflammation driven by massive TNF- $\alpha$  [27, 28]. Here we also repeated the significant activation of p38, ERK, and c-Jun N-terminal kinase (JNK) by LPS stimulation and found that GLMOC at 50, 100, and 300  $\mu$ g/mL showed the dose-dependent suppressions of phosphorylation on p38, ERK, and JNK of up to  $56.5 \pm 3.6$ ,  $52.2 \pm 3.1$ , and  $57.3 \pm 7.8\%$ , respectively, compared to LPS-treated control (Fig. 5). These results suggest that GLMOC provides the additional efficacy on inhibiting inflammation through MAPKs in macrophages.

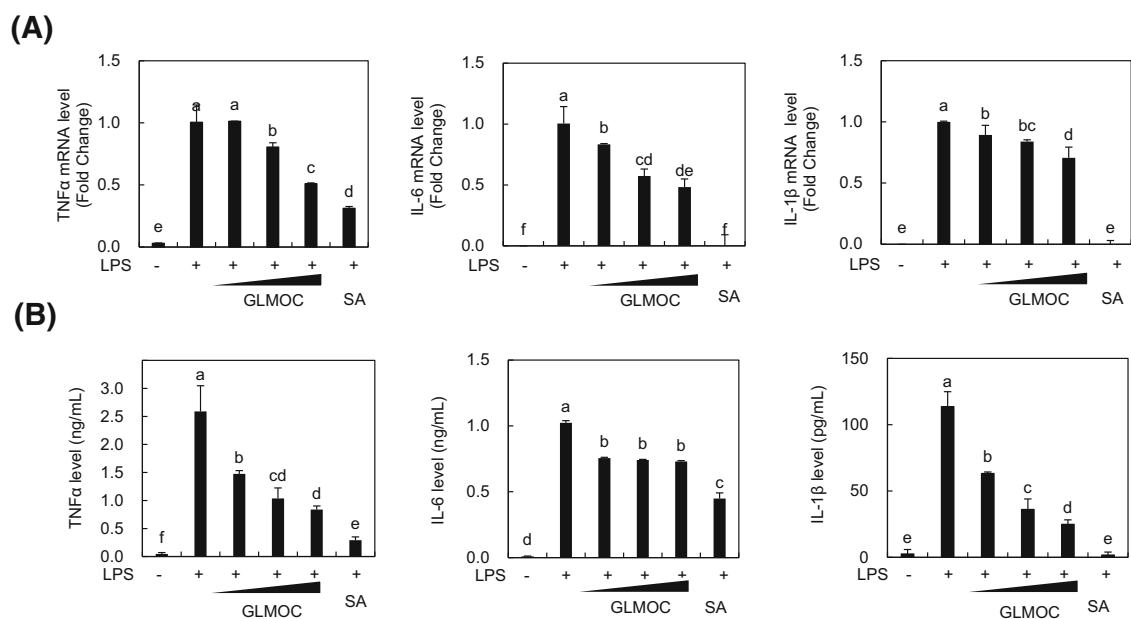
#### GLMO is the major components exerting anti-inflammatory effect without cytotoxicity in RAW264.7 cells

GLMOC is a mixture of GLMO with olive oil and vitamin E at a ratio of 10:20:1. To confirm that GLMO is the main constituents regulating inflammation, we compared GLMO (96.7  $\mu$ g/mL), olive oil (193.5  $\mu$ g/mL), and vitamin E (9.8  $\mu$ g/mL) at the same concentration present in GLMOC



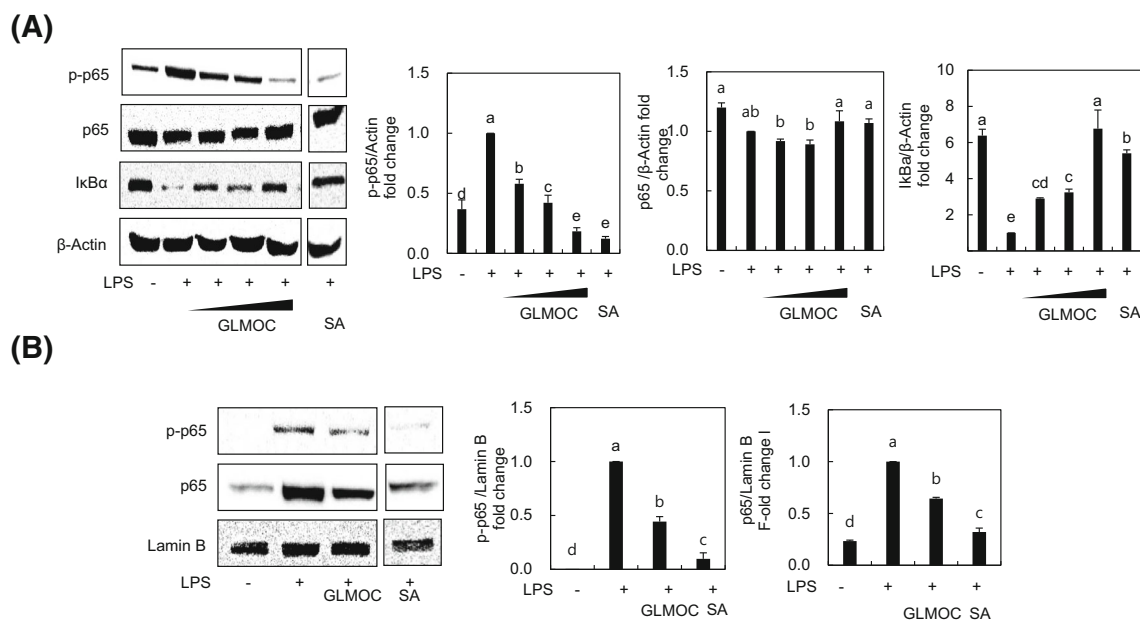
**Fig. 2** GLMOC inhibited the expression of COX-2 and prostaglandin E2 level in LPS-induced RAW264.7 cells. **(A)** The PGE2 amount in the media was determined by competitive ELISA. The cells ( $8 \times 10^5$  cells in a 60 mm dish) were pretreated with GLMOC or SA for 2 h, and then LPS for 22 h. **(B)** The relative amount of COX-2 mRNA was measured with quantitative RT-PCR. The experimental condition is

same with Fig. 1(B). **(C)** The protein levels of COX-2 and  $\beta$ -actin were determined by Western blot. The experimental condition is same with Fig. 1(C). Values represent the mean  $\pm$  SD. Different lowercase letters indicate significant differences at  $p < 0.05$ . GLMOC (50, 100, and 300  $\mu$ g/mL), SA (10  $\mu$ M), LPS (100 ng/mL)



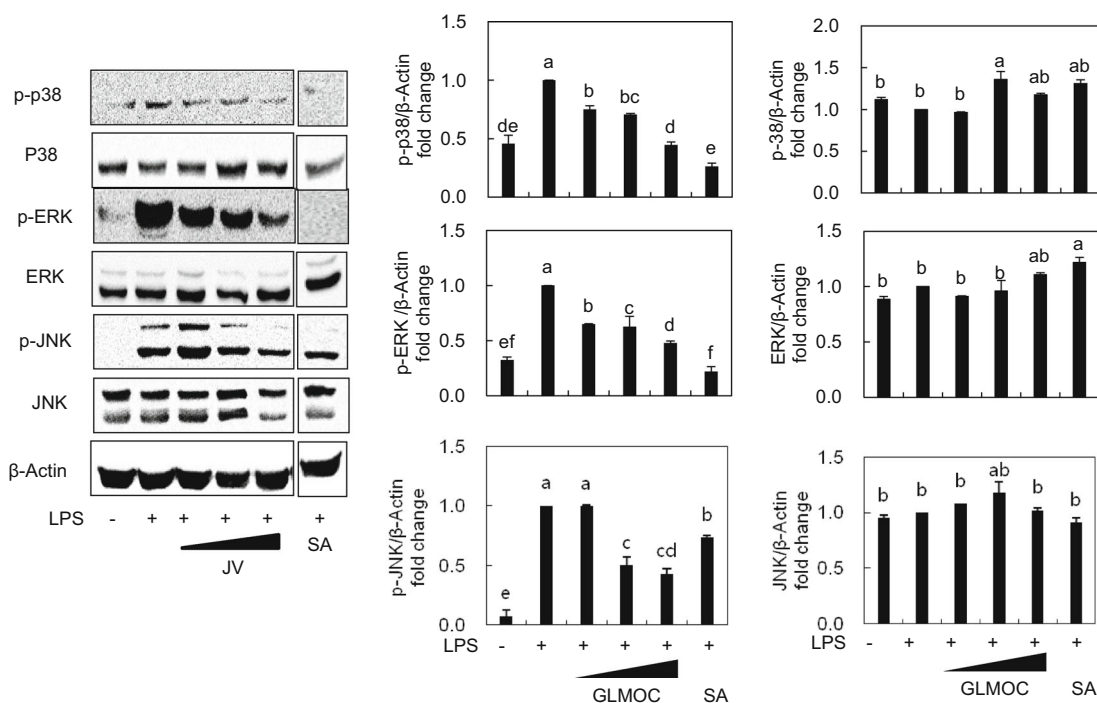
**Fig. 3** GLMOC reduced the production of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. The cells ( $8 \times 10^5$  cells in a 60 mm dish) were pretreated with GLMOC or SA for 2 h. LPS was then treated for 10 h for mRNA measurement by quantitative real time PCR **(A)**, and for 22 h for protein measurement of cytokines in

the media by ELISA assay **(B)**. Values represent the mean  $\pm$  SD. Different lowercase letters indicate significant differences at  $p < 0.05$ . GLMOC (50, 100, and 300  $\mu$ g/mL), SA (10  $\mu$ M), LPS (100 ng/mL)



**Fig. 4** GLMOC blocked the NF- $\kappa$ B signaling pathway in LPS-stimulated RAW264.7 cells. The cells ( $8 \times 10^5$  cells in a 60 mm dish) were pretreated 2 h with GLMOC or SA, and then LPS was treated for 30 min. The protein levels of phosphorylated p65 (p-p65) and p-65, I $\kappa$ B, and  $\beta$ -actin (**A**), and the nuclear protein levels of

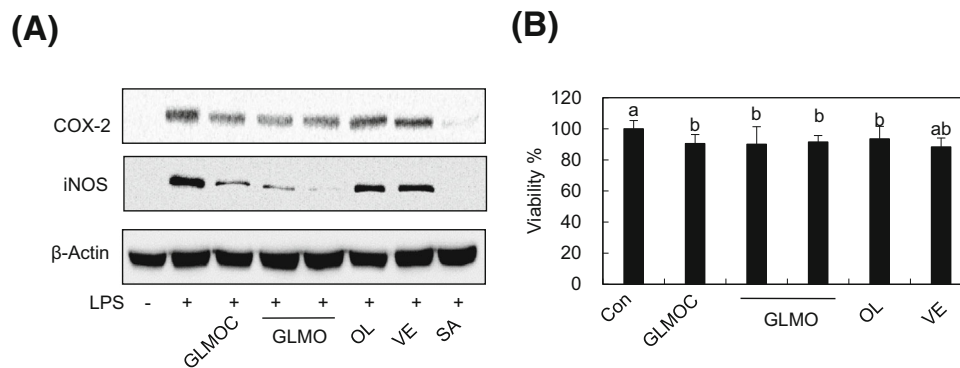
p-p65, p-65, and lamin B (**B**) were determined by Western blot. Values represent mean  $\pm$  SD. Different lowercase letters indicate significant differences at  $p < 0.05$ . GLMOC [50, 100, and 300  $\mu$ g/mL (**A**) or 300  $\mu$ g/mL (**B**)], SA (10  $\mu$ M), LPS (100 ng/mL)



**Fig. 5** GLMOC regulated the MAPKs signaling pathway in LPS-induced RAW264.7 cells. The cells ( $8 \times 10^5$  cells in a 60 mm dish) were pretreated 2 h with GLMOC or SA, and then LPS was treated for 30 min. The protein levels of p-p38, p38, p-ERK, ERK, p-JNK,

JNK, and  $\beta$ -actin were determined by Western blot. Values represent mean  $\pm$  SD. Different lowercase letters indicate significant differences at  $p < 0.05$ . GLMOC (50, 100, and 300  $\mu$ g/mL), SA (10  $\mu$ M), LPS (100 ng/mL)





**Fig. 6** GLMO is the major components exerting anti-inflammatory effect without cytotoxicity in RAW264.7 cells. **(A)** The cells ( $8 \times 10^5$  cells in a 60 mm dish) were pretreated with GLMOC (300  $\mu\text{g}/\text{mL}$ ), GLMO (96.7  $\mu\text{g}/\text{mL}$ ), olive oil (OL, 193.5  $\mu\text{g}/\text{mL}$ ), and vitamin E (VE, 9.8  $\mu\text{g}/\text{mL}$ ) for 2 h, and then LPS (100 ng/mL) for 22 h. The protein levels of iNOS, COX-2, and  $\beta$ -actin were

determined by Western blot. **(B)** The cells ( $1 \times 10^4$  cells/well in 96-well plate) were treated with GLMOC (300  $\mu\text{g}/\text{mL}$ ), GLMO (96.7  $\mu\text{g}/\text{mL}$ ), olive oil (OL, 193.5  $\mu\text{g}/\text{mL}$ ), and vitamin E (VE, 9.8  $\mu\text{g}/\text{mL}$ ) for 24 h, and the cell viabilities were determined by MTT assay. Values represent the mean  $\pm$  SD. *Different lowercase letters indicate significant differences at  $p < 0.05$*

(300  $\mu\text{g}/\text{mL}$ ). As shown in Fig. 6, two separate treatments of GLMO showed similar activity in regulating iNOS and COX-2 expression with GLMOC, whereas vitamin E and olive oil exerted marginal effect. To determine whether the effect of GLMOC and its constituents on inflammation induced by LPS is derived from cytotoxicity in RAW264.7 cells, we performed MTT assay and found that the viability of the cells was maintained above 90% after the treatment of GLMOC, GLMO, olive oil, or vitamin E. These results indicate that the anti-inflammatory effect of GLMOC is mainly derived from GLMO without inducing cytotoxicity.

GLMO is known to contain different lipid components, including sterol esters, triglycerides, and free fatty acids, wherein  $\omega$ -3 polyunsaturated fatty acids (PUFAs) are the major components, particularly eicosapentaenoic acid (EPA, 13%) and docosahexaenoic acid (DHA, 21%) [29]. McPhee et al. [7] reported that the increased amount of fatty acids by hydrolysis of triglycerides from GLMO and fish oils significantly increased the activity in suppressing the COX-1 and -2 activity, suggesting that the role of GLMO on inflammation is mainly due to the significant amounts of PUFAs. Interestingly, however, the administration of fish oil did not show beneficial efficacy in preventing the development of arthritis and ameliorating the symptoms of inflammatory bowel disease, whereas GLMO was found to be effective in both animal models [8, 30]. Although furan fatty acid that is unstable during oil extraction and analysis process was identified and suggested as a key component responsible for the anti-inflammatory activity of GLMO in adjuvant-induced arthritis rat model [31], more studies are necessary to identify the active ingredients and clarify the underlying mechanism of GLMO on inflammation related chronic diseases. In conclusion, GLMOC containing GLMO as the major active component showed the inhibition of iNOS expression,

leading to the reduced production of NO, and COX-2 expression, leading to the reduced production of PGE2 as well as suppression of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$  expression in LPS-stimulated RAW264.7 cells. The upstream signaling mediating the anti-inflammatory efficacy of GLMOC was suggested to be through the regulation of NF- $\kappa$ B signaling by recovering I $\kappa$ B and blocking nuclear translocation of NF- $\kappa$ B, and the MAPK signaling by inhibiting phosphorylation of p38, ERK, and JNK. Our findings in this study can provide the underlying mechanisms elucidating the anti-inflammatory efficacy of GLMOC in macrophage cells.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

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