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Research Article

# Methyl 9-Oxo-(10*E*,12*E*)-octadecadienoate Isolated from *Fomes fomentarius* Attenuates Lipopolysaccharide-Induced Inflammatory Response by Blocking Phosphorylation of STAT3 in Murine Macrophages

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**Abstract** *Fomes fomentarius* is a fungus of the Polyporaceae family and is used in traditional oriental therapies. Although the anti-inflammatory activities of this species have been previously reported, the identity of the bioactive compounds responsible for this activity remains unknown. Here, we investigated whether methyl 9-oxo-(10*E*,12*E*)-octadecadienoate (FF-8) purified from *F. fomentarius* exerts anti-inflammatory activity in murine macrophages stimulated with lipopolysaccharide (LPS). FF-8 suppressed secretion of nitric oxide (NO) and prostaglandin $E_2$ through downregulation of inducible NO synthase and cyclooxygenase-2 expression induced by LPS. In addition, pretreatment of cells with FF-8 led to a reduction in levels of secreted inflammatory cytokines such as tumor necrosis factor-α and interleukin-6 in macrophages stimulated with LPS. Conversely, FF-8 did not affect nuclear factor κB, p38, c-Jun NH2-terminal kinase, and extracellular signal-regulated kinase pathways. Instead, FF-8 specifically interfered with signal transducer and activator of transcription 3 (STAT3) phosphorylation induced by LPS. Collectively, this study demonstrated that FF-8 purified from *F. fomentarius* suppresses inflammatory responses in macrophages stimulated with LPS by inhibiting STAT3 activation. Further studies will be required to elucidate the anti-inflammatory effect of FF-8 *in vivo*.

**Keywords** Anti-inflammatory effect, *Fomes fomentarius*, Macrophages, STAT3

Lipopolysaccharide (LPS) is a major component of Gram-negative bacteria cell walls and activates immune cells including macrophages, which are one of the most potent types of inflammatory cells. Macrophages activated by LPS produce pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), and prostaglandin $E_2$ ($PGE_2$), all of which play important roles in innate immune responses [1]. However, excessive or dysregulated production of pro-inflammatory mediators can lead to systemic inflammatory response syndrome, severe tissue damage, and septic shock, which are also associated with autoimmune disorders such as type 1 diabetes, rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, asthma, and psoriasis [1-3]. Therefore, compounds that interfere with macrophage activation and inhibit pro-inflammatory mediators may exert a therapeutic effect on various inflammatory diseases.

Binding of LPS to Toll-like receptor 4 (TLR4) results in activation of signal transduction pathways such as nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) [1, 2]. In unstimulated cells, NF-κB is bound by inhibitory κBα (IκBα) protein in the cytoplasm. LPS stimulation causes phosphorylation of IκBα, which leads to its degradation and is followed by nuclear translocation of NF-κB and induction of cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), and increases expression of pro-inflammatory cytokines genes. MAPKs such as

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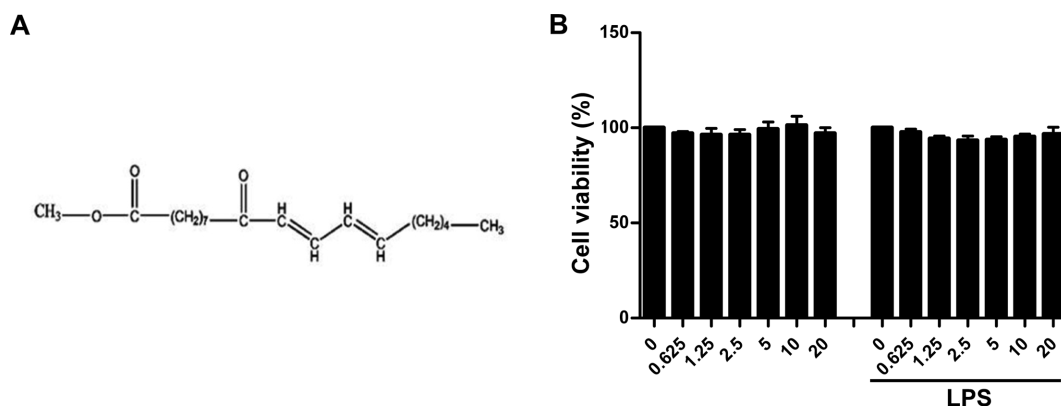


**Fig. 1.** Effect of methyl 9-oxo-(10*E*,12*E*)-octadecadienoate (FF-8) on cell viability. A, Structure of FF-8; B, Peritoneal macrophages were pretreated with different concentration of FF-8 for 2 hr and stimulated with lipopolysaccharide (LPS; 500 ng/mL) for an additional 24 hr. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results of three independent experiments were averaged and are expressed as the percent cell viability relative to the untreated control group.

extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) are also activated by LPS stimulation and coordinate the production of pro-inflammatory mediators. LPS binding to TLR4 induces the Janus kinase-signal transducers and activators of transcription (JAK-STATs) cascade, which is one of the essential inflammatory signaling pathways that mediate immune responses triggered by LPS [4]. Therefore, most compounds that possess anti-inflammatory activity are known to suppress these signaling pathways [5-7].

*Fomes fomentarius* belongs to a fungus of the polyporaceae family, and is a broadleaf tree parasite [8]. *F. fomentarius* has been used in China and Korea as a traditional herbal medicine. It has been reported that *F. fomentarius* has antioxidant, anti-inflammatory, anti-diabetic, and anti-tumor effects [8-12]. Red-brown purpurogallin derivative fomentariol, ergosterol, fungisterol, isoergosterone, and ergosta-7,22-dien-3-one have all been reported to be chemical constituents of *F. fomentarius* [13-15]. During our investigation of the anti-inflammatory constituents of wild mushrooms, methyl 9-oxo-*10E,12E*-octadecadienoate (FF-8) was isolated from the methanolic extract of the fruiting body of *F. fomentarius*. Here, we investigated whether FF-8 purified from *F. fomentarius* exerts anti-inflammatory activity in murine macrophages stimulated with LPS. In addition, we also investigated the molecular mechanisms by which this compound inhibits inflammatory responses.

## MATERIALS AND METHODS

**Isolation and purification of FF-8.** The fruiting body of *F. fomentarius* (fresh weight 7.1 kg) was ground and extracted twice with methanol at room temperature. The methanolic extract was partitioned consecutively between hexane, chloroform, ethyl acetate, butanol, and water. The hexane-soluble portion concentrated under reduced pressure was subjected to silica gel column chromatography and eluted stepwise with a hexane : ethyl acetate gradient (100 : 1 to 1 : 1, v/v). An active fraction was chromatographed on a column of Sephadex LH-20 eluted with a chloroform : methanol solution (1 : 1, v/v), followed by preparative high-performance liquid chromatography with a reverse-phase column and elution with 75% aqueous methanol to afford the active compound. The chemical structure of the active compound was determined as FF-8 by extensive one- and two-dimensional nuclear magnetic resonance spectroscopy and mass measurements (Fig. 1A). The spectroscopic data were well matched to those previously reported in the literature [16, 17].

**Preparation of peritoneal macrophages and cell culture.** BALB/c mice (8~10-wk-old) were injected intraperitoneally with 2 mL of sterile 4% thioglycollate (Difco, Detroit, MI, USA). After 2~3 days, peritoneal macrophages were lavaged with phosphate buffered saline (PBS). Next, cells were suspended in Dulbecco's Modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL) and streptomycin sulfate (100 μg/mL) and incubated at 37°C in a 5% $CO_2$ atmosphere. After 2 hr, the cells were washed three times with PBS to remove non-adherent cells and the remaining cells were equilibrated with culture media. Cells were pre-treated with FF-8 at various concentrations or vehicle (dimethyl sulfoxide [DMSO]) for 2 hr, followed by LPS stimulation (500 ng/mL). DMSO alone did not affect cell viability and LPS-induced inflammatory activities in primary peritoneal macrophages.

**Measurement of NO and $PGE_2$ production and cell viability.** Cells were seeded at $1 \times 10^5$ cells per well in 96-well plates and pre-treated with various concentration of FF-8 for 2 hr followed by treatment with LPS (500 ng/mL) for 24 hr in the presence of FF-8. Cell culture supernatants were collected and mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid, and

0.1% naphthylethylenediamide) at room temperature for 10 min. The absorbance was then read at 540 nm using a microplate reader. NO concentrations in the supernatant were determined by comparison with a standard curve. The level of $PGE_2$ in cell culture media was measured using the $PGE_2$ ELISA kit according to the manufacturer's introductions (R&D Systems, Minneapolis, MN, USA). Cell viability was measured after 24 hr of exposure to FF-8 based on the ability of mitochondria in viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co., St. Louis, MO, USA) [18].

**Measurement of cytokine production.** The levels of TNF-α and IL-6 production in cell culture supernatant were measured using respective ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

**Immunoblotting.** For immunoblotting, cell lysates were prepared using M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA) containing phosphatase/protease cocktail inhibitor (Thermo Scientific). Protein concentrations were determined and 50 µg of protein for each sample were resolved by SDS-PAGE and transferred to a PVDF membrane (Amersham, Bucks, UK). After transfer, membranes were blocked with 5% skim milk (Difco) and then probed with primary antibodies against phosphorylated or total levels of NF-κB/IκBα, MAPK (ERK, p38, and JNK), STAT3 (Cell Signaling, Danvers, MA, USA) at 4°C overnight. Species-appropriate horseradish peroxidase-conjugated IgGs were used as secondary antibodies. Finally, proteins were detected using a SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific). Relative protein levels were quantified using Image J software.

**Real-time PCR.** Total RNA was prepared using RNAiso reagent (TaKaRa, Tokyo, Japan) and cDNA was synthesized using a PrimeScript cDNA synthesis kit (TaKaRa) according to the manufacture's instruction. Quantitative real-time PCR was performed using a SYBR Premix Ex Taq 2× (TaKaRa) on a Qiagen Rotor Gene 6200 system. The primers used for real time PCR were as follows: mTNF-α (5′-AGGTTCTGTCCCTTTCACTCACTG-3′) and (3′-AGAG-AACCTGGGAGTCAAGGT-5′) mIL-6 (5′-CAACGATGA

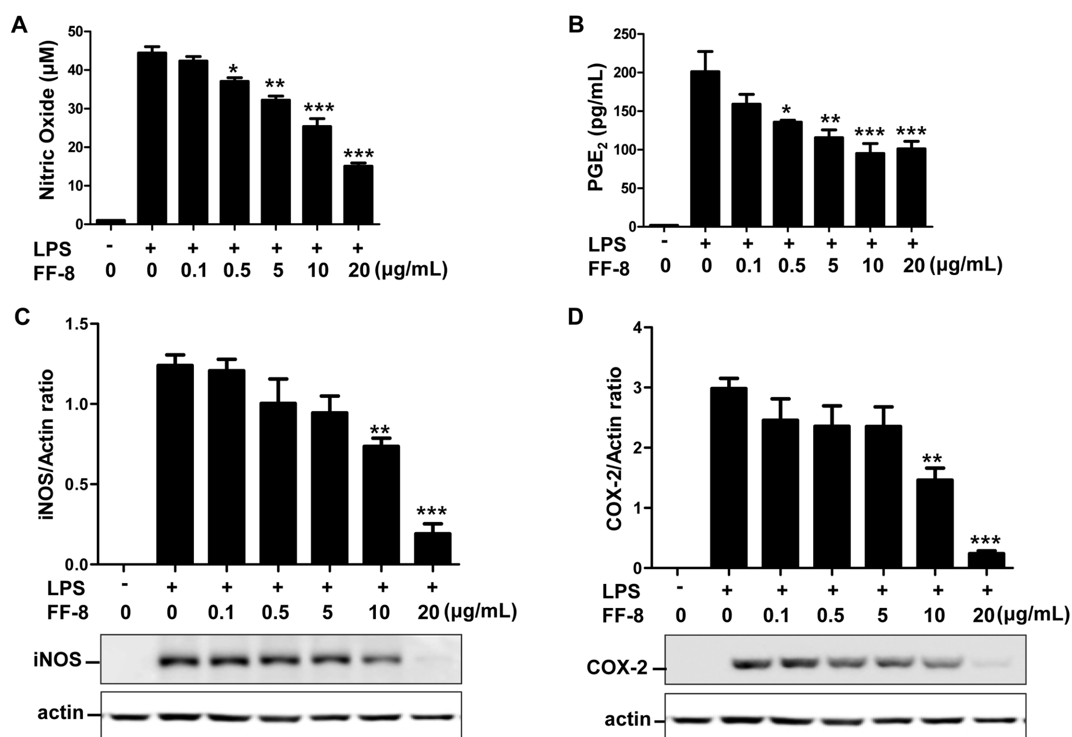


**Fig. 2.** Effect of methyl 9-oxo-(10*E*,12*E*)-octadecadienoate (FF-8) on nitrite accumulation and prostaglandin $E_2$ ($PGE_2$) secretion in murine peritoneal macrophages. Peritoneal macrophages were pretreated with different concentration of FF-8 for 2 hr and stimulated with lipopolysaccharide (LPS; 500 ng/mL) for an additional 24 hr. A, The levels of nitric oxide (NO) in culture supernatants were measured by Griess reaction assay; B, The level of $PGE_2$ in culture supernatants was measured by ELISA; C, D, Cells pretreated with FF-8 for 2 hr were stimulated with LPS (500 ng/mL) for 24 hr. Cell lysates were then prepared for Western blot analysis using specific inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) antibodies as described in the Materials and Methods. β-Actin was used as internal control. Bar graphs represent relative density normalized to β-actin. Data are presented as the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate significant differences compared to LPS alone. A representative blot from three independent experiments is shown.

TGCACTTGCAGA-3′) and (3′-TCTCTCTGAAGGACTC-TGGCT-5′).

**Statistical analysis.** Values are expressed as the mean ± standard error of the mean (SEM). Data analysis was carried out using Graphpad Prism (GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA followed by Dunnett's *post hoc* test was used to compare means between groups. Differences with a *p*-value of $< 0.05$ were considered statistically significant.

## RESULTS

**The effects of FF-8 on LPS-stimulated NO and $PGE_2$ production in macrophages.** Prior to testing whether FF-8 modulates macrophage activation, the cytotoxic effects of FF-8 were evaluated in macrophages in the absence or presence of LPS (500 ng/mL) using MTT assay. As shown in Fig. 1B, FF-8 was not cytotoxic to peritoneal macrophages at concentrations of up to 20 μg/mL regardless of LPS stimulation. To investigate the effects of FF-8 on LPS-induced NO and $PGE_2$ production in macrophages, culture media were harvested after 24 hr, and nitrite and $PGE_2$ levels were determined. As shown in Fig. 2A and 2B, when macrophages were pretreated with FF-8, production of NO and $PGE_2$ triggered by LPS was suppressed in a dose dependent manner. At the highest concentration tested (20 μg/mL), FF-8 reduced the level of NO and $PGE_2$ by 65% and 50%, respectively. Next, we investigated whether the inhibitory effects FF-8 on NO and $PGE_2$ production were related to the downregulation of the expression of their respective synthesis enzymes, iNOS and COX-2. When macrophages were treated only with FF-8, iNOS and COX-2 protein levels were undetectable (data not shown). However, iNOS and COX-2 protein levels were markedly up-regulated by LPS treatment and FF-8 showed potent inhibitory effects on the expression of iNOS and COX-2 protein in a dose-dependent manner (Fig. 2C and 2D). Together, these data indicated that FF-8 suppressed LPS-induced NO and $PGE_2$ production by inhibiting iNOS and COX-2 protein expression.

**The effects of FF-8 on LPS-stimulated TNF-α and IL-6 production in macrophages.** FF-8 interferes with inflammatory mediator production induced by LPS, and thus we next examined whether FF-8 also modulates inflammatory cytokines production in macrophages stimulated with LPS. Pretreatment with FF-8 markedly decreased the production of TNF-α and IL-6 initiated by LPS (Fig. 3A and 3B). These inhibitory effects were associated with lower mRNA levels of TNF-α and IL-6 in macrophages pre-treated with FF-8 followed by stimulation with LPS (Fig. 3C and 3D), suggesting that FF-8 inhibits the production of pro-inflammatory cytokines by regulating the expression of cytokines at the level of transcription.

**The effects of FF-8 on LPS-stimulated NF-κB and MAPK signaling pathway activation in macrophages.** To investigate the mechanisms responsible for FF-8-mediated suppression of iNOS, COX-2, and inflammatory cytokines,

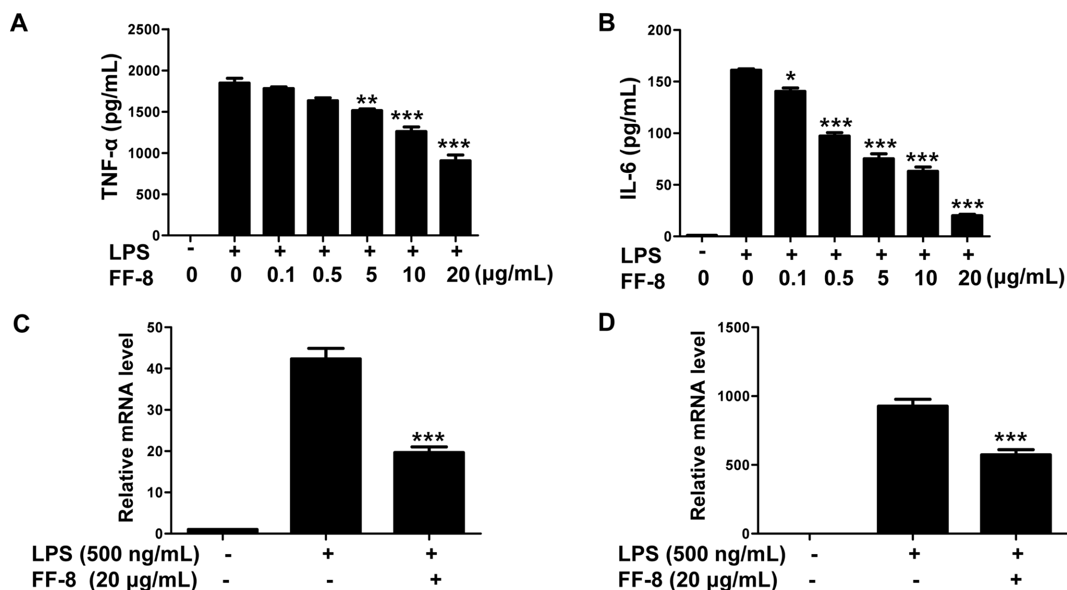


**Fig. 3.** Effect of methyl 9-oxo-(10*E*,12*E*)-octadecadienoate (FF-8) on lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in murine peritoneal macrophages**.** Peritoneal macrophages were pretreated with the indicated concentrations of FF-8 for 2 hr followed by stimulation with LPS (500 ng/mL) for 24 hr. A, B, The amounts of TNF-α and IL-6 in the culture supernatants were measured by ELISA; C, D, The mRNA levels of TNF-α and IL-6 were determined by real-time reverse transcriptase polymerase chain reaction analysis. Data are presented as the mean ± SEM of independent three experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate significant differences compared to LPS alone.

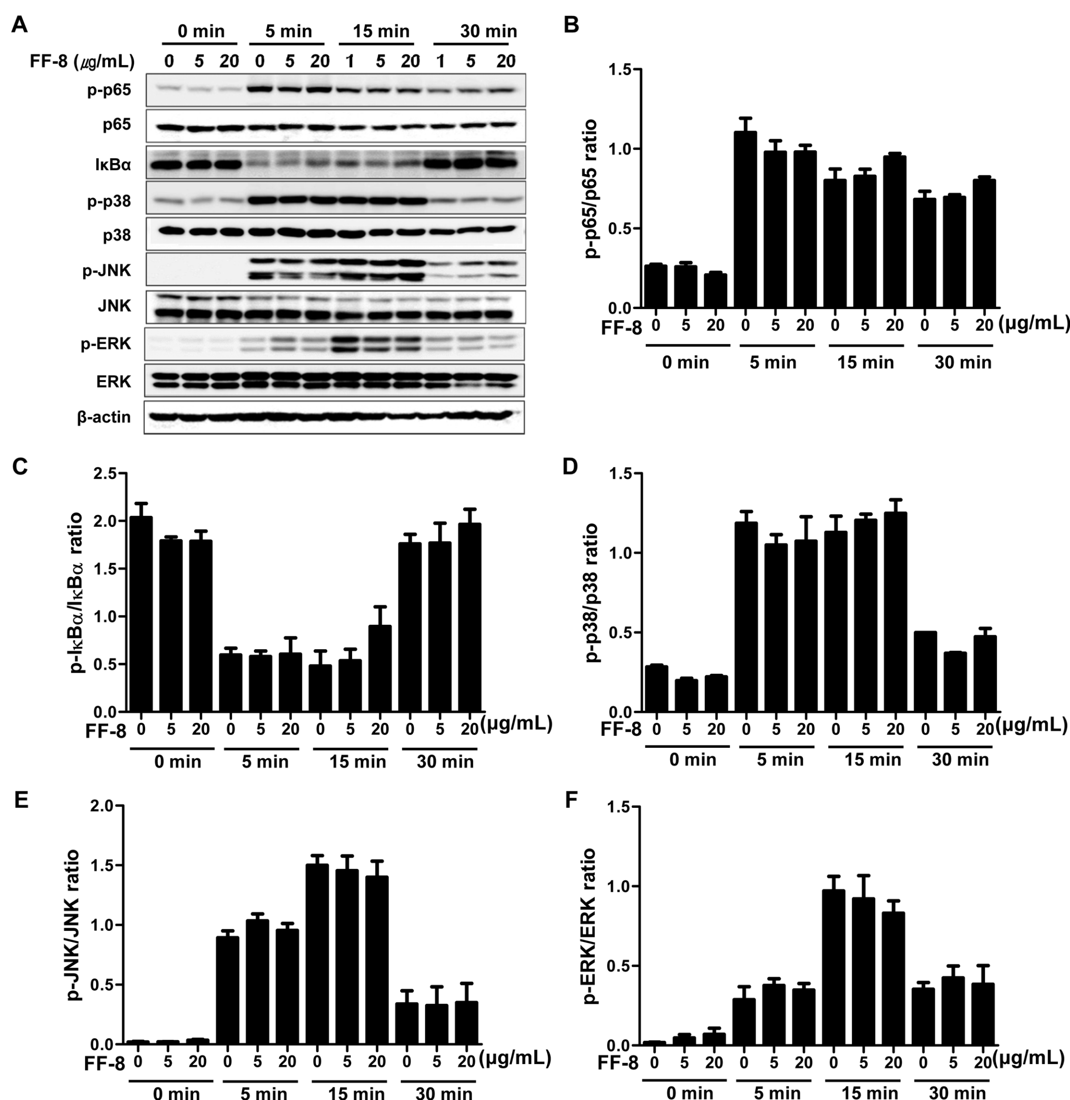


**Fig. 4.** Effect of methyl 9-oxo-(10*E*,12*E*)-octadecadienoate (FF-8) on lipopolysaccharide (LPS)-induced nuclear factor κB and mitogen-activated protein kinase signaling pathway in murine peritoneal macrophages. A, Peritoneal macrophages were pretreated with the indicated concentrations of FF-8 for 2 hr followed by stimulation with LPS (500 ng/mL) for 5, 15, or 30 min; B~F. The levels of phospho- and total proteins of p65 (B), inhibitory kBα (IκBα) (C), p38 (D), c-Jun NH2-terminal kinase (JNK) (E), extracellular signal-regulated kinase (ERK) (F), and β-actin in cell lysates were determined using specific antibodies. Bar graphs represent relative densities normalized to β-actin. Data are presented as the mean ± SEM of independent three experiments.

we next investigated whether FF-8 regulates LPS-induced signal transduction pathways in peritoneal macrophages. Since NF-κB and MAPK are important transcription factors that control the expression of iNOS and COX-2, as well as inflammatory cytokines such as TNF-α and IL-6, we first tested whether FF-8 treatment affected NF-κB activation. As shown in Fig. 4, phosphorylation of p65 and degradation of IκBα were dramatically enhanced upon exposure to LPS, while FF-8 alone had no effect on either process. Furthermore, neither JNK nor p38 activation was affected by FF-8 in LPS-stimulated macrophages. However, phosphorylation of ERK1/2 was slightly reduced in FF-8 treated cells after LPS stimulation for 15 min, but there were no statistically significant difference between untreated and FF-8 treated samples. Taken together, these data suggested that the inhibition of inflammatory responses by FF-8 may be mediated by suppression of ERK1/2 activation but not suppression of NF-κB, JNK, and p38 activation in macrophages.

**The effects of FF-8 on LPS-stimulated STAT3 signaling pathways activation in macrophages.** The above results did not support the hypothesis of FF-8 as an inhibitor of either NF-κB or MAPK signaling pathways. Thus, we next investigated whether FF-8 modulates the JAK-STAT pathway, which is another inflammatory signaling

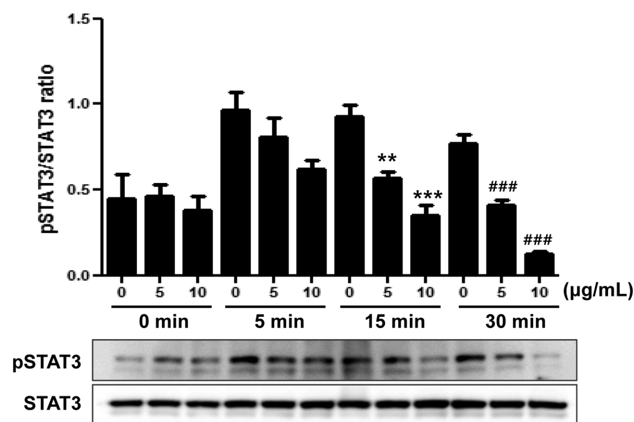


**Fig. 5.** Effect of methyl 9-oxo-(10*E*,12*E*)-octadecadienoate (FF-8) on lipopolysaccharide (LPS)-induced signal transducers and activators of transcription 3 (STAT3) activation in murine peritoneal macrophages. Peritoneal macrophages were pretreated with the indicated concentrations of FF-8 for 2 hr followed by stimulation with LPS (500 ng/mL) for 5, 15, or 30 min. The levels of phosphorylated STAT3 and total proteins of STAT3 in cell lysates were determined using specific antibodies. Bar graphs represent relative densities normalized to β-actin. Data are presented as the mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant difference compared to LPS alone at 15 min. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ indicate significant difference compared to LPS alone at 30 min. A representative blot from three independent experiments is shown.

cascade activated by LPS. Pretreatment of macrophages with FF-8 led to a marked suppression of LPS-induced STAT3 tyrosine phosphorylation without impairing total STAT3 protein levels, suggesting that the anti-inflammatory effect of FF-8 are likely mediated by suppression of STAT3 activation (Fig. 5).

## DISCUSSION

Macrophages play an important role in the innate immune response against pathogens. Activation of macrophages leads to production of pro-inflammatory molecules including NO, $PGE_2$, TNF-α, and IL-6, and these molecules directly or indirectly remove pathogens. However, dysregulated activation of these cells results in massive production of NO, $PGE_2$, TNF-α, and IL-6, which have been linked to the development of various pathological conditions including autoimmune diseases and metabolic diseases. Furthermore, although it has been suggested that *F. fomentarius* has anti-inflammatory effects, the bioactive constituents and mechanisms responsible for this physiological functions are not yet well defined. To the best of our knowledge, this is the first study showing that FF-8 isolated from *F. fomentarius* exerts anti-inflammatory activities. Specifically, FF-8 significantly inhibited the production of the pro-inflammatory mediators such as NO and $PGE_2$, as well as cytokines, including IL-6 and TNF-α in macrophages stimulated with LPS. These effects were accompanied by the reduced level of STAT3 activation in FF-8 treated cells.

Free radical NO is synthesized from arginine and $O_2$ by iNOS upon cell activation, and acts as an intracellular messenger playing various regulatory functions in blood flow, neural activity, and immune defense mechanisms against pathogens [19, 20]. However, excessive production of NO can contribute to host cell and tissue damage, leading to a number of pathogenic inflammatory conditions such as autoimmune diseases, diabetes, neuroinflammation, inflammatory bowel disease, and cancer. Therefore, compounds that inhibit iNOS expression and NO production could be used to treat diseases caused by excess levels of NO. In the present study, we found that FF-8 reduced NO production via the inhibition of iNOS expression. Previous studies have reported that mushroom extracts or compounds isolated from mushroom effectively suppress iNOS expression, followed by reduced NO production [21-24]. In these studies, inhibitory effects of mushroom extracts or compounds on $PGE_2$ production and COX-2 expression were also observed. Similarly, FF-8 downregulated $PGE_2$ production as a result of reduced COX-2 expression. $PGE_2$ is a potent lipid mediator that exhibits a broad range of regulatory activities. For example, $PGE_2$ is known to control inflammation, fibrosis and tissue regeneration [25], and overproduction of $PGE_2$ is associated with aggravated pathologies related to chronic inflammation and cancer [26]. In this way, agents that block $PGE_2$ production by targeting COX-1/2 or COX-2 function represent the most common and effective anti-inflammatory drugs [25, 26]. Therefore, the results of the present study strongly suggested that FF-8 has the potential to be developed as an anti-inflammatory therapeutic.

We confirmed the anti-inflammatory effects of FF-8 via investigation of inflammatory cytokines in LPS-stimulated macrophages. Similar to NO and $PGE_2$, dysregulated production of inflammatory cytokines such as TNF-α and IL-6 plays a crucial role in many inflammatory diseases. Therefore, targeting of these cytokines is an effective strategy to relieve clinical symptoms of certain inflammatory diseases such as rheumatoid arthritis. We found that FF-8 effectively reduced cytokine production induced by LPS (Fig. 3). Interestingly, the same dose of FF-8 exerted a greater suppressive effect on IL-6 than TNF-α. Specifically, FF-8 reduced TNF-α levels to 35% while it reduced IL-6 levels to 13% at the highest concentrations tested in this study. However, mRNA levels of both cytokines were suppressed by FF-8 treatment to a similar extent. Other studies have also demonstrated that mushroom extracts and compounds isolated from mushrooms possess potent suppressive effects on inflammatory cytokines in LPS stimulated macrophages [21-24].

Once LPS binds to its receptor TLR4, it induces a cascade of protein phosphorylation that mediates various signals and induces expression of various proteins including iNOS, COX-2, TNF-α and IL-6. Among these signal

transduction pathways, NF-κB and MAPKs are the major pathways that mediate expression of inflammatory mediators induced by LPS. Therefore, numerous studies have suggested the importance of compounds that inhibit inflammatory cytokine production through suppression of one or both of these two pathways. For example, Jedinak *et al.* [27] demonstrated that oyster mushroom extracts markedly suppress secretion of TNF-α and IL-6 by inhibiting NF-κB and AP-1 signaling. Likewise, Zha *et al.* [28] reported that soysaponins block inflammation by inhibiting activation of the NF-κB pathway. Different from these studies, our results showed that FF-8 did not affect these pathways. Rather, FF-8 suppressed phosphorylation of STAT3 induced by LPS. A recent study by Zhu *et al.* [29] demonstrated that mollugin, a kind of naphthohydroquinone, blocks the JAK-STAT signaling pathway to inhibit LPS induced inflammatory responses. They also reported that mollugin binds to JAK2, concluding that mollugin may be a JAK2 inhibitor. Here, we suggest that FF-8 exhibited anti-inflammatory activities not by interfering with NF-κB or MAPKs, but through blocking the JAK/STAT pathway. Further studies should be performed to investigate molecular mechanisms by with FF-8 suppresses the phosphorylation of STAT3.

In conclusion, the present study demonstrated that FF-8 purified from *F. fomentarius* suppresses inflammatory responses in macrophages stimulated with LPS by inhibiting STAT3 activation. It will be important going forward to confirm whether FF-8 can be used as a novel therapeutic agent to alleviate inflammation in pathological conditions *in vivo* using animal disease models.

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