3-Hydroxy-4,7-megastigmadien-9-one, Isolated from Ulva pertusa Kjellman, Inhibits LPS-Induced Inflammatory Response by Down-Regulating Mitogen-Activated Protein Kinase and NF-κB Pathways

Irshad Ali1,2, Zahid Manzoor1,2 and Young-Sang Koh1,2*

1Department of Microbiology and Immunology, School of Medicine and Brain Korea 21 PLUS Program, Jeju National University, Jeju; 2Institute of Medical Science, Jeju National University, Jeju, Korea

In the present study we evaluated the anti-inflammatory potential of 3-hydroxy-4,7-megastigmadien-9-one (Comp) isolated from Ulva pertusa Kjellman, in LPS-stimulated bone marrow-derived dendritic cells (BMDCs). Comp treatment exhibited strong dose dependent inhibition of IL-12 p40 and IL-6 cytokine production with IC50 values of 7.85 ± 0.32 and 7.86 ± 0.18, respectively in LPS-stimulated BMDCs. Treatment of Comp inhibited MAPKs and NF-κB pathways in LPS-stimulated BMDCs by inhibiting the phosphorylation of ERK1/2, JNK1/2, p38 and IκB. Thus, these results suggest that Comp have a significant anti-inflammatory property and affirm further studies concerning the potentials of Comp for medicinal use.

Key Words: Ulva pertusa Kjellman; Anti-inflammatory activity; 3-Hydroxy-4,7-megastigmadien-9-one

INTRODUCTION

Chronic inflammation results from persistent exaggerated immune reaction to injury or exposure to foreign pathogens (1, 2). Recognition of microbial pathogen by innate immune system is important for activation of microbicidal effectors and progression of adaptive immunity (3). Toll-like receptors (TLRs) consist of a family of pattern recognition receptor that sense conserved molecular pattern of microbes. They are crucial for recognition of microbial infection and initiating inflammatory and immune responses (4, 5). LPS, product of Gram negative bacteria, stimulates CD14/TLR4/MD2 receptor complex, particularly in dendritic cells, macrophages, monocytes and B cells (6). Stimulation of immune cell with microbial product causes the production of pro-inflammatory cytokine such as TNF-α and various interleukins and is maintained for the duration of inflammatory response (7, 8).

TLRs stimulation leads to the activation of mitogen activated protein kinases (MAPKs) and NF-κB pathways. MAPKs including extracellular signal regulated kinase (ERK), p38 and c-Jun NH2-terminal kinase (JNK) are highly conserved protein serine/threonine kinases which get activated through phosphorylation (9). Activation of NF-κB pathway requires phosphorylation and proteosomal degradation of IκB which results in the release and nuclear translocation of NF-κB (10). Activation of MAPKs and NF-κB pathways leads to high expression of pro-inflammatory cytokines in—
cluding IL-12, IL-6 and TNF-α (11).

Assaying folk remedies for active ingredients is an important approach in drug development, and numerous seaweed species are utilized as traditional medicines, food and health care products in different regions of the world (12). *Ulva pertusa* (*U. pertusa*) is edible green seaweed (13). Dietary supplementation of *U. pertusa* Kjellman improved superoxide dismutase-like activity and affects the immune system by inhibiting inflammatory response in broiler chicks (14). 3-hydroxy-4,7-megastigmadien-9-one (Comp) is one of the norisoprenoid degradation products of carotenoids (15). In the present study, for the first time we investigated the anti-inflammatory potential of Comp, isolated from *U. pertusa* Kjellman, particularly on TLR4-induced inflammatory response in bone marrow-derived dendritic cells (BMDCs).

**MATERIALS AND METHODS**

**Isolation of 3-hydroxy-4,7-megastigmadien-9-one from Ulva pertusa Kjellman**

*U. pertusa* was collected on Jeju Island, South Korea. Ethyl acetate-soluble fraction obtained from 70% aqueous ethanol extract of dried *U. pertusa* was subjected to MPLC (Medium Pressure Liquid Chromatography) by reversed-phase silica gel through water-methanol gradients to give several fractions. One fraction was further purified by silica gel with *n*-Hex/EtOAc = 1/1 to give 3-hydroxy-4,7-megastigmadien-9-one (Comp).

**Cell cultures and measurement of cytokine production**

To grow BMDCs, wild-type 6-week-old female C57BL/6 mice were used as previously described (16). Briefly, bone marrow cells were differentiated in RPMI 1640 (BD, Grand Island, NY, USA) medium containing granulocyte-macrophage colony-stimulating factor for dendritic cells generation. For BMDCs, on day 6 of incubation the cells were harvested and seeded in 48-well plates at a density of $1 \times 10^5$ cells/0.5 ml, and then treated with the Comp for 1 h before stimulation with LPS. Supernatants were harvested 18 h after stimulation. Concentrations of murine IL-12 p40, IL-6, and TNF-α in the culture supernatants were measured by enzyme linked immunosorbent assay (ELISA) (BD PharMingen, San Jose, CA, USA). SB203580 (Calbiochem, Darmstadt, Hesse, Germany) was used as positive control.

**Cell viability assay**

The cell viability was measured by standard procedure of MTT assay (17).

**Western blot analysis**

Bone marrow-derived dendritic cells (BMDCs) were dispensed to 35 mm culture dishes at a concentration of $2 \times 10^6$ cells/2 ml and incubated for 1 h at 37°C. The cells were pre-treated with or without Comp for 1 h before treatment with LPS at the indicated time points. The cells were collected and then lysed in lysis buffer (PRO-PREP lysis buffer, iNtRON Biotechnology, South Korea). A protein sample (30 μg) was subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated with 1/1,000-diluted rabbit polyclonal antibodies that specifically recognize phospho-ERK1/2, phospho-p38, phospho-JNK1/2, phosphor-IκBα (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membrane was incubated with a horse-radish peroxidase-linked goat anti-rabbit IgG (Cell Signaling Technology), and immunoactive bands were detected as previously described (18).

**Data analysis**

All experiments were performed at least three times, and the data are presented as the mean ± standard deviation (SD) of three independent experiments. One-way ANOVA was used for comparison between the treated and the control groups. $p < 0.05$ was considered statistically significant.

**RESULTS AND DISCUSSION**

LPS is recognized by TLR4, especially in dendritic cells, macrophages, monocytes and B cells. TLR4 stimulation subsequently causes the activation and translocation of NF-
κB and AP-1 into the nucleus, where it causes the excessive production of pro-inflammatory cytokines such as IL-6 and TNF-α (6). To determine the anti-inflammatory activity, Comp was investigated for inhibition of IL-12 p40, IL-6 and TNF-α production in LPS-stimulated BMDCs. The maximum concentration of Comp that does not affect cell viability for treating the cells was determined by MTT assay (Fig. 1). BMDCs treated with Comp alone showed no production of pro-inflammatory cytokines. Stimulation of BMDCs with LPS caused significant increase in the production of IL-12 p40, IL-6 and TNF-α (Fig. 2). Comp treatment exhibited inhibition of IL-12 p40 and IL-6 production

Table 1. Anti-inflammatory effects of Comp on LPS-stimulated BMDCs

| Compound | IC_{50} values (μM) |  
|-----------|----------------------|---|
|           | IL-12 p40           | IL-6 | TNF-α |
| Comp a   | 7.85 ± 0.32         | 7.86 ± 0.18 | > 100 |
| SB203580 b | 5.00 ± 0.21         | 3.50 ± 0.13 | 7.20 ± 0.26 |

aIC_{50} values for Comp are given in column IL-6, IL-12 p40, and TNF-α. Values >100 μM are considered to be inactive.

bPositive control.

Figure 1. Effects of Comp on cell viability of BMDCs. BMDCs were treated with Comp (1–50 μM) for 18 h and viability was measured using MTT assay. Data are representative of three independent experiments.

Figure 2. Inhibitory effects of Comp on pro-inflammatory cytokine production in LPS-stimulated BMDCs. BMDCs were treated with Comp at the indicated doses for 1 h before stimulation with LPS (10 ng/ml). Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of murine IL-12 p40 (A), IL-6 (B), and TNF-α (C) in the culture supernatants. Data are representative of three independent experiments. Comp, 3-hydroxy-4,7-megastigmadien-9-one. *p < 0.05, **p < 0.01 vs. Comp-untreated cells in the presence of LPS.
in the LPS-stimulated BMDCs (Fig. 2) with IC50 values of 7.85 ± 0.32 and 7.86 ± 0.18 (μM), respectively (Table 1). However TNF-α production was not significantly inhibited by Comp (Table 1). Synthesis of TNF-α by LPS is under complex control and regulation occur at both transcription and post-transcriptional level (20). The 3’untranslated region of TNF-α mRNA has been defined as playing a major role in post-transcriptional control of TNF-α expression (20). It is

![Figure 3. Effects of Comp on the phosphorylation of MAPK and IκBα in LPS-stimulated BMDCs. (A) Cells were pre-treated with or without Comp (50 μM) for 1 h before stimulation with LPS (10 ng/ml). Total cell lysate was obtained at the indicated time intervals. Western blot analysis was performed on the cell lysate to assess phosphorylation of ERK, JNK, p38 and IκBα. β-actin was taken as the loading control. Data are representative of three independent experiments. (B) Phosphorylation of ERK, JNK, p38 and IκBα was quantified using scanning densitometry, and the band intensities were normalized by that of β-actin. Comp, 3-hydroxy-4,7-megastigmadien-9-one. *p < 0.05 vs. Comp-untreated cells in the presence of LPS.](image-url)
likely that Comp may exert different effect on TNF-α protein production at post-transcriptional level, that might explain for no inhibition of TNF-α production. These data demonstrate that Comp had an inhibitory effect on production of cytokines particularly IL-6 and IL-12 p40 in LPS-stimulated BMDCs.

LPS stimulation causes the phosphorylation of ERK1/2, JNK and p38, which leads to LPS-induced pro-inflammatory cytokine production (8). Hence, through western blot analysis, we investigated the effects on MAPK phosphorylation in LPS-stimulated BMDCs, with and without Comp treatment (Fig. 3). All three MAPKs get phosphorylated in BMDCs stimulated with LPS. ERK1/2, JNK1/2 and p38 phosphorylation was detected between 15 and 30 min of LPS stimulation. Comp treatment inhibited LPS-induced phosphorylation of ERK1/2, JNK1/2 and p38 (Fig. 3A, 3B). Taken together, these results indicate that Comp can inhibit LPS-stimulated phosphorylation of ERK1/2, JNK1/2 and p38 in BMDCs.

LPS is a potent activator of NF-κB pathway. IκB keeps NF-κB in inactive cytoplasmic form by masking its nuclear localization signal. Through upstream kinases, IκBα is subjected to phosphorylation and subsequent proteasomal degradation, allowing the nuclear localization of NF-κB where it causes the expression of target genes (19). Activation of NF-κB pathway was analyzed indirectly through phosphorylation of IκBα. Phosphorylation of IκBα was detected between 15 and 30 of LPS stimulation (Fig. 2A, 2B). Comp treatment inhibited phosphorylation of IκBα in LPS-stimulated BMDCs (Fig. 2A, 2B). Taken together these results demonstrate that Comp inhibits LPS-stimulated phosphorylation of IκBα and consequently inhibits NF-κB pathway.

Dysregulated production of pro-inflammatory cytokines like IL-12, IL-6 and TNF-α leads to different inflammatory diseases such as arthritis, atherosclerosis and cancer (9). In the present study, Comp treatment showed inhibition of IL-12 p40 and IL-6 production in LPS-stimulated BMDCs. Here, Comp treatment inhibited MAPKs and NF-κB pathways. Thus, Comp that suppresses the level of pro-inflammatory cytokines and inhibits MAPKs and NF-κB signaling pathways may help for relieving chronic inflammatory diseases.

Our study shows that Comp significantly suppressed the level of pro-inflammatory cytokines. It has also been demonstrated that Comp strongly inhibited MAPK and NF-κB pathways. As LPS-induced inflammatory response is inhibited by Comp. Thus, Comp-mediated anti-inflammatory activity represents a potential therapeutic use of the Comp for inflammatory diseases.

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