Master's Thesis

Inhibitory effect of Sea Lettuce (*Ulva fasciata*) Extract on Immune cells

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February, 2014

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(Supervised by professor **Young-Sang Koh)**

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Medicine

Date Approved:

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ABSTRACT

This report was designed to study inhibitory effect of sea lettuce (*Ulva fasciata*) extract (UFE) on pro-inflammatory cytokine production in bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC). Inflammation is primary localized and protective response of host against microbial infection and autoimmune disorders. Macrophages and dendritic cells are major part of mammalian immune system and play important role in productions of pro-inflammatory cytokines. Thus, here we study the anti-inflammatory effect of UFE on CpG-stimulated BMDMs and BMDCs and human embryonic kidney cell line 293T(HEK293T) cells. The UFE (0-50 μg/mL) pre-treatment showed a dose dependant inhibitory effect on interlukin (IL)-12 p40, IL-6, and tumor necrosis factor (TNF)-α productions in CpG-stimulated BMDMs and BMDCs as compared to non-treated controls. The UFE pre-treatment exhibited strong inhibitory effect on the phosphorylation of p38 mitogen-activated protein kinase (MAPK) while it showed moderate inhibition on nuclear factor (NF)-κB activation as indirectly evaluated by degradation of IκBα. In activator protein (AP)-1 and NF-κB reporter gene assay, the UFE pre-treatment showed moderate inhibitory effect on both AP-1 and NF-κB dependent reporter gene activities. Thus, these results suggest that inhibitory effect of UFE on pro-inflammatory cytokine production may correlate with partial inhibition of both AP-1 and NF-κB pathways. Extracts from the *U. fasciata* has been previously reported to exhibit broad spectrum anti-bacterial activity and antioxidant activity. The present study suggests that UFE has an inhibitory effect on productions of pro-inflammatory cytokines, TLR9-dependent NF-κB and AP-1 activation and thus warrant further studies concerning potential uses of UFE. Hence, our data warrant further studies concerning potentials of sea lettuce for medicinal food.

Keywords : *Ulva fasciata*, p38 mitogen-activated protein kinase, pro-inflammatory cytokine, inflammation

CONTENTS

LIST OF ABBREVIATIONS

BMDMs	Bone marrow-derived macrophages
BMDCs	Bone marrow-derived dendritic cells
UFE	*Ulva fasciata* extract
PAMPs	Pathogen-associated molecular pattherns
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
JNK	c-Jun NH2-terminal kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ELISA	Enzyme-linked Immunosorbent Assay
SD	Standard deviation

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Inhibitory effect of Sea Lettuce (*Ulva fasciata*) Extract on Immune cells

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1. Introduction

Inflammation is primary localized and protective response of host against microbial infection and autoimmune disorders. Hence, inflammatory response is vital for proper function of innate immune system which ultimately results in protection of host against pathogen and tissue injury (1). Pathogen associated molecular patterns are highly conserved in microorganism and are recognized by pattern recognition receptors including Toll-like receptors (2). Toll-like receptors are major type of pattern recognition receptors which play essential role in detecting several types of pathogen associated molecular patterns (3, 4).

Macrophages and dendritic cells are major cellular components of mammalian immune system. These cells are mainly responsible for detection of pathogen associated molecular patterns and activation of innate immune response. Dendritic cells are called as antigen presenting cells as their primary function is to process the antigen and present it on the surface to other cells of the immune system (5). The main function of macrophages is to phagocytose and then digest the harmful stimuli. Stimulation of macrophage and dendritic cells results in productions of interleukin (IL-12 p40), IL-6 and TNF-α (6, 7).

Bacterial DNA consists of unmethylated CpG dinucleotides which can stimulate mammalian immune cells by interacting with TLR9 (8). Detection of CpG by TLR9 results in activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathway, leading to synthesis of pro-inflammatory cytokines (9, 10). Stimulation of TLR9 can trigger phosphorylation and subsequent ubiquitination and degradation of IκB leading to activation of NF-κB (7). The activated NF-κB then translocates to the nucleus and thus results in transcription of NF-κB-regulated genes (7). MAPK is one of the main signal transduction pathways which belong to a large family of serine/threonine kinases. MAPKs have three well characterized subfamilies including extracellular signal-regulated kinases (ERK), the c-Jun NH2-terminal kinases

(JNK), and the p38 family of kinases (p38 MAPKs) (11). The p38 MAPKs are involved in expression of multiple pro-inflammatory cytokines which in turn play important role in innate immunity (12).

Marine algae contain a diverse range of bioactive compounds and being used in pharmaceutical, cosmetic, food and nutraceutical industries (13). *Ulva fasciata* is bright green to dark green marine alga and also called as limu palahalaha or sea lettuce and commonly consumed by humans in many parts of the world. *U. fasciata* could be used to control the body weight and in prevention of gastrointestinal diseases as it contains high dietary fiber content (14). Extracts from the *U. fasciata* has been previously reported to exhibit broad spectrum anti-bacterial activity (15, 16). In addition to antibacterial activity, *U. fasciata* also possess antioxidant activity (17). However, effect of *U. fasciata* ethanol extract (UFE) on innate immune response has been barely studied in terms of its influence on primary murine bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs). Thus, here we study the anti-inflammatory effect of UFE on CpG-stimulated BMDMs, BMDCs and human embryonic kidney cell line 293T (HEK293T) cells.

2. Materials and Methods

2.1. Preparation of *U. fasciata* extract

Thalli of *Ulva fasciata* Delile were collectedon Jeju Island, Korea, on October 2008. A voucher specimen (JBRI20025) has been deposited at the herbarium of Jeju Bio diversity Research Institute. The materials for extraction were cleaned, dried at room temperature for one weekand ground into a fine powder. The dried alga (100g) was extracted with 80% ethanol (EtOH; 2L) at room temperature for 24 h and then evaporated under a vacuum. The *U.fasciata* ethanol extract (UFE, 21g) was suspended in water (4L).

2.2. Mice

Six-week-old female C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam Si, South Korea) and maintained under specific pathogen-free conditions. All mice were maintained and used in accordance with institutional and National Institutes of Health guidelines. All animal procedures were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of Jeju National University (#2010-0028).

2.3. Cell cultures and measurement of cytokine production

To grow BMDMs and BMDCs, wild-type six-week-old female C57BL/6 mice were used (18). The bone marrow cells from wild-type and mutant mice were obtained from tibia and femur of mice by flushing with DMEM (Invitrogen, CA, USA) containing 10% heat-inactivated FBS, 100 U of penicillin G and streptomycin. The 1×10^7 bone marrow cells were cultured in 10 ml of DMEM medium containing glutamine, 20% heat-inactivated FBS, 100 U of penicillin G, streptomycin and 30% L929 cell supernatant containing M-CSF in 100 mm petri dish (BD Falcon, NJ, USA) at 37℃ in humidified 5% CO_2 for 6 days. At day 6 of

culture, cells were harvested with cold PBS, washed, resuspended in DMEM supplemented with 10% FBS and used at a density of 2×10^5 cells/well in a 24 well plate for experiments. Bone Marrow-derived Dendritic Cells (BMDCs) were grown from wild-type and various knockout mice. Briefly, bone marrow from tibia and femur was obtained as described above, and bone marrow cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) medium containing 10% heat-inactivated FBS, 50 μM of 2-ME, and 2 mM of glutamine supplemented with 3% J558L hybridoma cell culture supernatant containing GM-CSF. The culture medium containing GM-CSF was replaced every other day. At day 6 of culture, nonadherent cells and loosely adherent DC aggregates were harvested, washed, resuspended in RPMI 1640 supplemented with 5% FBS and used at a density of 2×10^5 cells/ml for experiments unless mentioned otherwise (18). Briefly, bone marrow cells were differentiated in RPMI 1640 medium containing granulocyte-macrophage colony-stimulating factor for dendritic cells generation. For BMDMs, bone marrow cells were differentiated in DMEM medium containing macrophage colony-stimulating factor. For BMDMs and BMDCs, on day 6 of incubation the cells were harvested and seeded in 48-well plates, and then treated with the UFE for 1 h before stimulation with CpG (1 μM). 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 ELISA (BD PharMingen, San Jose, CA, R&D system, Minneapolis, MN).

2.4. Cell viability assay

To assess cell viability standard procedure of 3-(4,5-dimethyl-2,5thiazolyl)-2,5 diphenyl tetrazolium bromide (MTT) assay was used. Briefly, the cells at a concentration of 1×10^6 cells were seeded on 96-well culture plate. After incubation for 1h at 37℃, cells were treated with extracts at various concentrations for 18 h. Cells were added 0.2mg MTT (Sigma, MO, USA) and then incubated for 4 h at 37℃. The plate was centrifuged and the

supernatants were aspirated. The formazan crystals in each well were dissolved in 250 μl dimethyl sulfoxide (DMSO) (AMresco, OH, USA). Absorbance was measured at wavelength of 540 nm.

2.5. Western blot analysis

This was performed using standard techniques as previously described (19). Briefly, BMDMs were dispensed to 60-mm culture dishes (Nunc, Roskilde, Denmark) at 4×10^6cells per dish and cultured for 24 h at 37^{o}C. The cells were pre-treated with or without UFE (25 μg/mL) for 1 h before treatment with CpG for the indicated time points. The cells were collected and then lysed in lysis buffer (PRO-PREP lysis buffer, iNtRON Biotechnology, Seongnam, 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). The membrane was incubated with 1/1,000-diluted rabbit polyclonal antibodies that specifically recognize phospho-p44/42 (P-ERK1/2), p44/42 MAPK, phospho-p38, p38 MAPK and phospho-SAPK/JNK, SAPK/JNK, phospho-IκBα or IκBα (Cell Signaling Technology, MA, USA), β-actin (Santacruz biotechnology, INC, USA). After washing, the membrane was incubated with a horseradish peroxidase-linked goat anti-rabbit IgG (Cell Signaling Technology) and immune active bands were detected as previously described (Yun *et al*., 2009). Densitometric analyses of images were performed using image J software, version 1.46r (Http://rsb.info.nih.gov/nih-image/).

2.6. Luciferase assay

For AP-1 and NF-κB reporter assays, HEK293T cells were plated in 24-well plates and grown overnight. Cells were transfected using Fugene 6 (Roche, Indianapolis, USA) with a AP-1 or NF-κB reporter gene, pRLnull (Promega, Madison, WI) and pcDNA3 (empty vector) or murine TLR9-encoding pcDNA3 provided as a kind gift by Prof. R. Medzhitov

(Yale University, CT, USA). After incubation of 24 h, cells were pre-treated with UFE for 1 h before stimulation with CpG (1 μM). After further incubation for 18 h, cells were lysed in a passive lysis buffer (Promega, Madison, WI), and firefly luciferase versus renilla activities were measured using a dual luciferase reporter assay system (Promega, Madison, WI).

2.7. Data analysis

All experiments were performed at least three times, and the data are presented as mean±standard deviation (SD) of three independent experiments. One-way analysis of variance (ANOVA) was used to evaluate the data at significance levels $*p<0.05$ and $**p<0.01$.

Table 1. Antibodies used in Western blot analysis on BMDMs

Antibody	Origin	Company
Phospho-ERK1/2	rabbit polyclonal	Cell signaling Technology
p38/MAPK	rabbit polyclonal	Cell signaling Technology
Phospho-p38/MAPK	rabbit polyclonal	Cell signaling Technology
Phospho-JNK1/2	rabbit polyclonal	Cell signaling Technology
IκBα	rabbit polyclonal	Cell signaling Technology
Phospho-IκBα	rabbit polyclonal	Cell signaling Technology
β-actin	rabbit polyclonal	Santa Cruz Biotechnology

3. Results

3.1. Effects of UFE on cell viability in BMDCs and BMDMs

To evaluate the effect of Seaweed extracts on cell viability was assessed using MTT assay. We observed little or no effect of Seaweed extracts on cell viability of BMDCs (Fig. 1) and BMDMs (Fig. 5) at the concentration range.

3.2. Inhibitory effects of *Ulva fasciata* extracton IL-12 p40, IL-6, and TNF-α production in CpG-stimulated BMDCs and BMDMs

Macrophages and dendritic cells have a major role in productions of key pro-inflammatory cytokines (7). Hence, we investigated the ability of UFE to inhibit CpG-stimulated IL-12 p40, IL-6 and TNF-α productions in BMDCs and BMDMs. CpG induced significantly increased IL-12 p40, IL-6 and TNF-α productions in BMDCs (Fig. 2, 3, 4) and BMDMs (Fig. 6, 7, 8). The UFE treatment alone showed no productions of cytokines. The UFE treated cells exhibited strong dose-dependent inhibition of IL-12 p40 and TNF-α productions in BMDMs (Fig. 6, 8). However, its inhibitory effect was moderate on IL-6 production in CpG-stimulated BMDMs. Pre-treatment of UFE also resulted in a dose dependent inhibition of IL-12 p40, IL-6 and TNF-α productions in CpG-stimulated BMDCs as compared to non-treated control (Fig. 2, 3, 4). SB203580, an inhibitor of cytokine suppressive binding protein/p38 mitogen-activated protein kinase, was used as a positive control (20). To confirm the anti-inflammatory activity of UFE, cell viability was simultaneously determined by using colorimetric MTT assay and as a result, the UFE had little or no effect on BMDCs and BMDMs at the indicated concentrations. Taken together, these data show that UFE has an inhibitory effect on pro-inflammatory cytokine productions in CpG-stimulated BMDCs and BMDMs.

3.3. Effects of *Ulva fasciata* extract on MAPK phosphorylation by CpG-stimulated BMDMs

Engagement of TLR9 by CpG triggers the activation of NF-κB and MAPK pathways (7). Thus, we investigated the effect of UFE treatment on MAPK phosphorylation and NF-κB activation in CpG-stimulated BMDMs by western blot analysis (Fig. 9, 10). NF-κB activation was indirectly evaluated by IκBα degradation. All three MAPKs became phosphorylated in CpG-stimulated BMDMs (Fig. 9). The UFE pre-treatment exhibited strong inhibitory effect on the phosphorylation of p38 MAPK while it showed no significant inhibition on ERK1/2 and JNK1/2 phosphorylation (Fig. 9).

3.4. Effects of *Ulva fasciata* extract on IκBα degradation by CpG-stimulated BMDMs

CpG stimulation was able to induce IκBα degradation within 30 min of stimulation (Fig. 10). The amount of IκBα protein returned to baseline levels after 60 min of post-stimulation. However, UFE pre-treatment partially blocked the IκBα degradation, and hence the activation of NF-κB in CpG-stimulated BMDMs (Fig. 10). Taken together, these data suggest that UFE strongly inhibited the phosphorylation of p38 MAPK while it marginally inhibited NF-κB activation.

3.5. Effects of *Ulva fasciata* extract on NF-κB and AP-1 reporter activity in HEK-293T cells

Activation of NF-κB pathway results in translocation of NF-κB into the nucleus where it activates the expression of NF-κB target genes (3). To assess the inhibitory effect of UFE on CpG-stimulated NF-κB transcriptional activity, the NF-κB reporter gene assay was performed (Fig. 11). The HEK293T cells transfected with empty pcDNA3 showed no NF-κB-dependent luciferase activity upon CpG-stimulation. In contrast, the HEK293T cells transfected with TLR9-expressing plasmid exhibited strong NF-κB-dependent luciferase

activity upon CpG-stimulation. However, UFE pre-treatment exhibited moderate dose-dependent inhibition of NF-κB-dependent luciferase activity (Fig. 11, *p<0.05).

3.6. Effects of *Ulva fasciata* extract on AP-1 reporter activity in HEK-293T cells

We can conclude that UFE has moderate inhibitory effect on TLR9-dependent NF-κB activation upon CpG-stimulation. Activation of MAPK induces increased AP-1 transcriptional activity which in turn leads to expression of multiple AP-1-associated genes including pro-inflammatory cytokines (3). To investigate whether the UFE had inhibitory effect on CpG-stimulated AP-1 transcriptional activity, the AP-1 reporter gene assay was performed (Fig. 12). The HEK293T cells transfected with empty pcDNA3 showed little AP-1-dependent luciferase activity upon CpG-stimulation. However, the HEK293T cells transfected with TLR9-expressing plasmid exhibited robust AP-1-dependent luciferase activity upon CpG-stimulation. In contrast, UFE pre-treatment showed moderate inhibition of AP-1-dependent luciferase activity (Fig. 12, *p<0.05). Taken together, our data suggest that UFE has a moderate inhibitory effect on TLR9-dependent NF-κB and AP-1 activation upon CpG-stimulation.

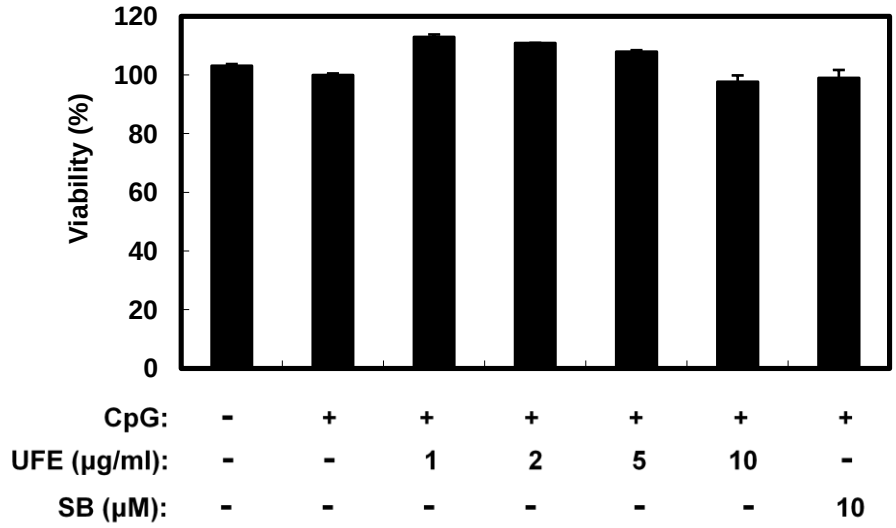


Fig 1. Cell viability in CpG-stimulated bone marrow-derived dendritic cells

BMDCs were treated with UFE extract for the indicated doses. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *$p<0.05$, **$p<0.01$ vs. UFE-untreated cells in the presence of CpG

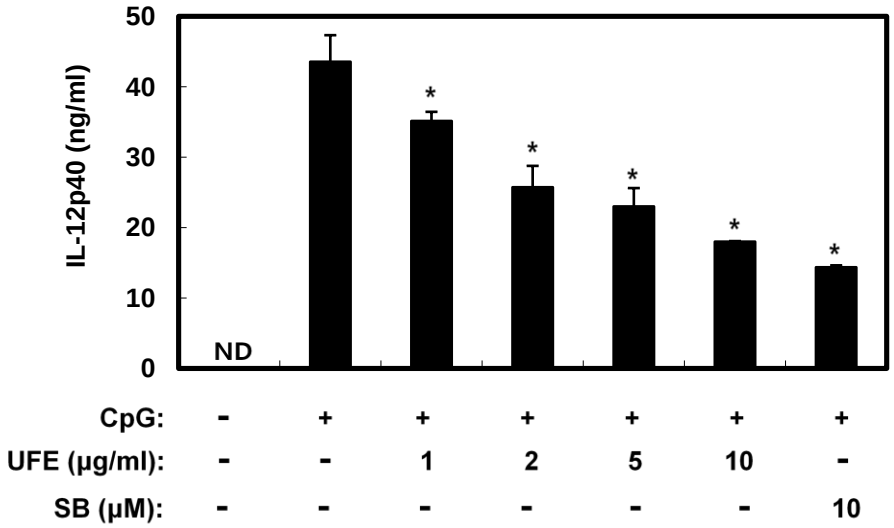


Fig 2. Inhibitory effects of UFE on IL-12 p40 productions in CpG-stimulated bone marrow-derived dendritic cells

BMDCs were treated with UFE extract for the indicated doses and cytokine level was assessed by ELISA. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *$p<0.05$, **$p<0.01$ vs. UFE-untreated cells in the presence of CpG

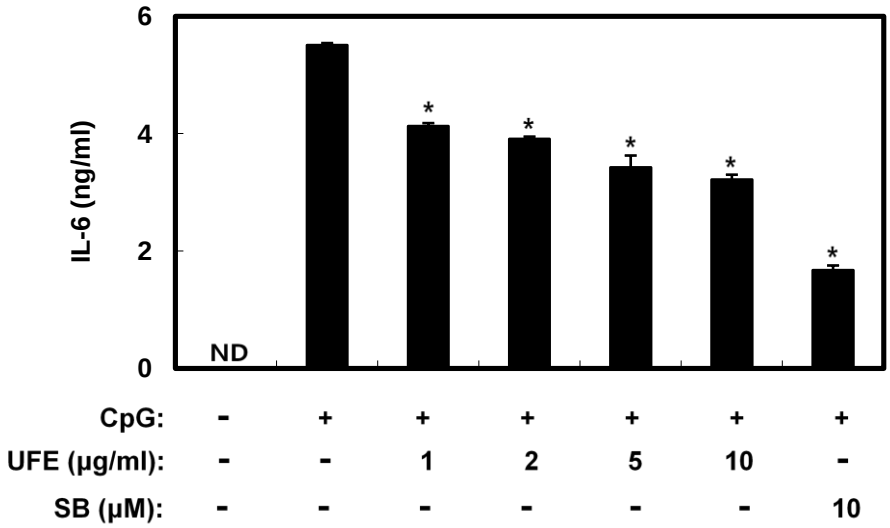


Fig 3. Inhibitory effects of UFE on IL-6 productions in CpG-stimulated bone marrow-derived dendritic cells

BMDCs were treated with UFE extract for the indicated doses and cytokine level was assessed by ELISA. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *$p<0.05$, **$p<0.01$ vs. UFE-untreated cells in the presence of CpG

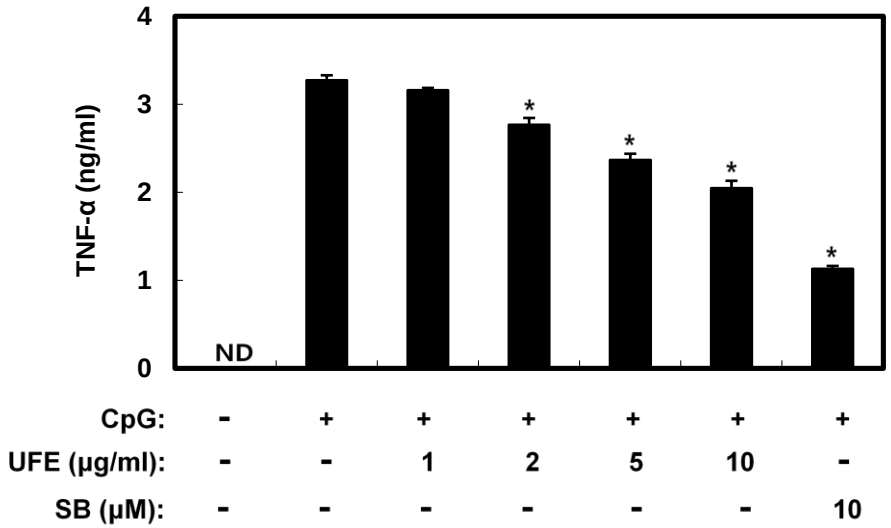


Fig 4. Inhibitory effects of UFE on TNF-α productions in CpG-stimulated bone marrow-derived dendritic cells

BMDCs were treated with UFE extract for the indicated doses and cytokine level was assessed by ELISA. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *$p<0.05$, **$p<0.01$ vs. UFE-untreated cells in the presence of CpG

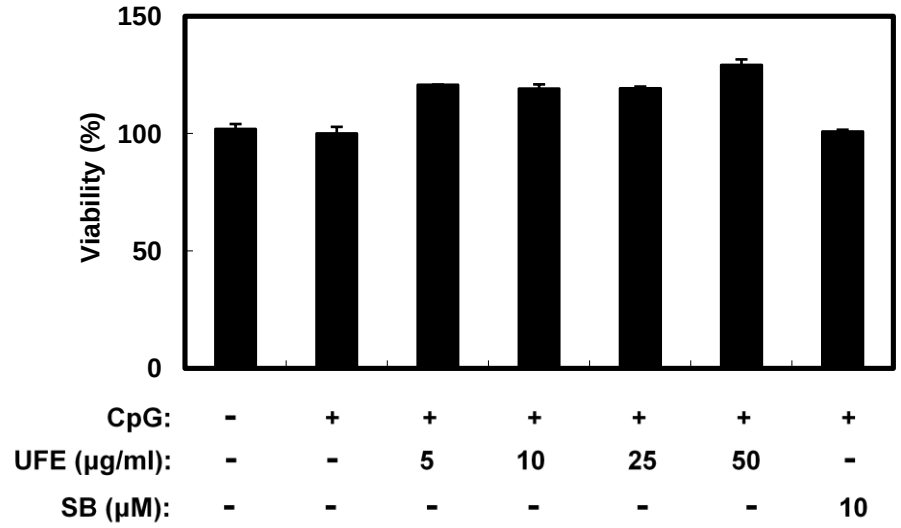


Fig 5. Cell viability in CpG-stimulated bone marrow-derived macrophages

BMDMs were treated with UFE extract for the indicated. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *p<0.05, **p<0.01 vs. UFE-untreated cells in the presence of CpG

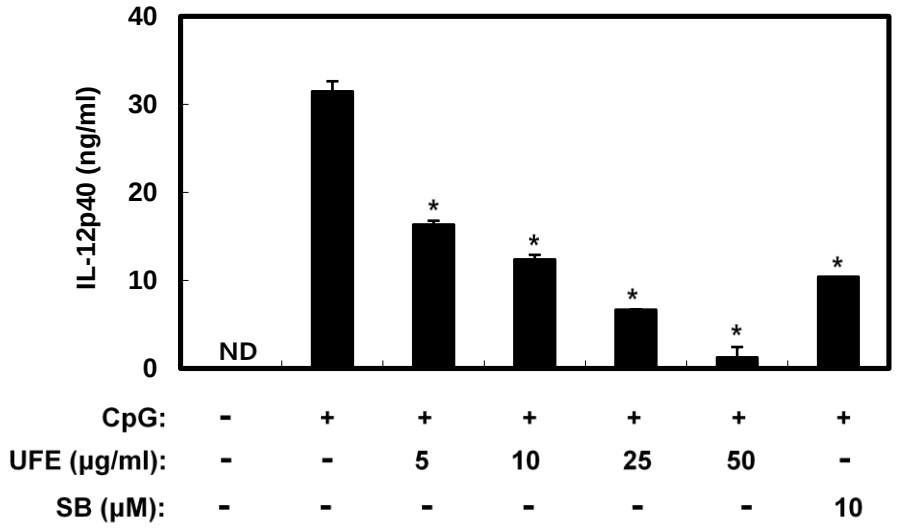


Fig 6. Inhibitory effects of UFE on IL-12 p40 productions in CpG-stimulated bone marrow-derived macrophages

BMDMs were treated with UFE extract for the indicated doses and cytokine level was assessed by ELISA. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *$p<0.05$, **$p<0.01$ vs. UFE-untreated cells in the presence of CpG

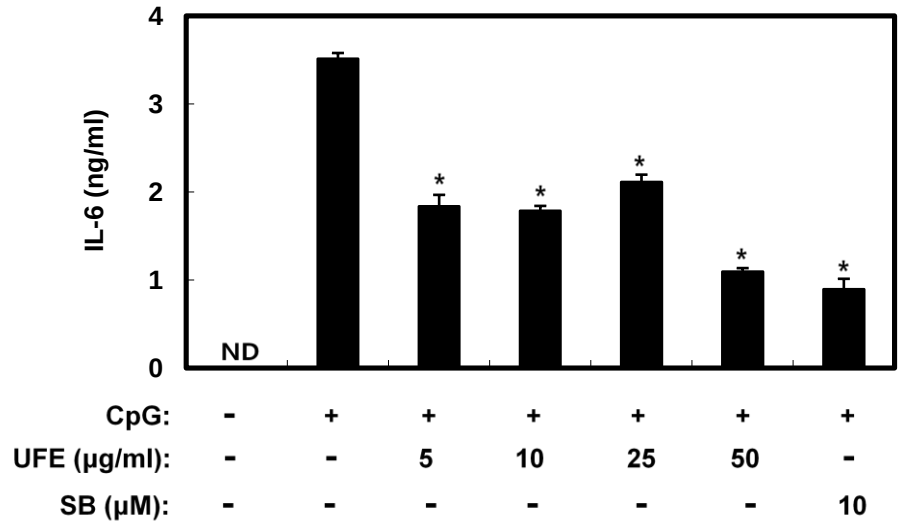


Fig 7. Inhibitory effects of UFE on IL-6 productions in CpG-stimulated bone marrow-derived macrophages

BMDMs were treated with UFE extract for the indicated doses and cytokine level was assessed by ELISA. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *$p<0.05$, **$p<0.01$ vs. UFE-untreated cells in the presence of CpG

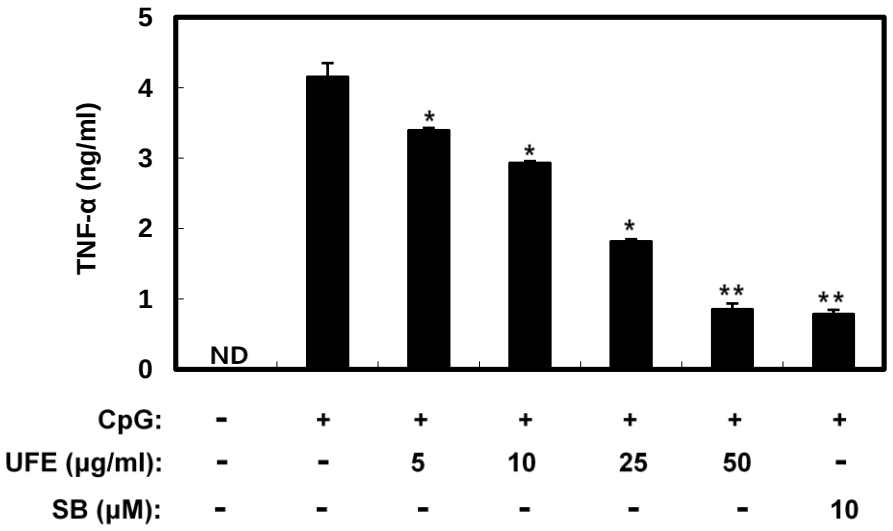
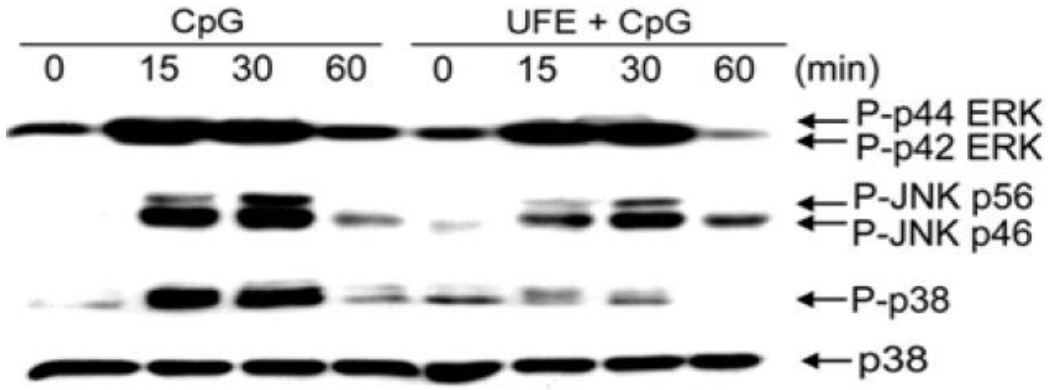


Fig 8. Inhibitory effects of UFE on TNF-α productions in CpG-stimulated bone marrow-derived macrophages

BMDMs were treated with UFE extract for the indicated doses and cytokine level was assessed by ELISA. SB203580 (SB) was used as positive control. Results shown are the mean±SD of an experiment done in triplicate and are representative of 3 independent experiments; ND, not detected; *$p<0.05$, **$p<0.01$ vs. UFE-untreated cells in the presence of CpG

A



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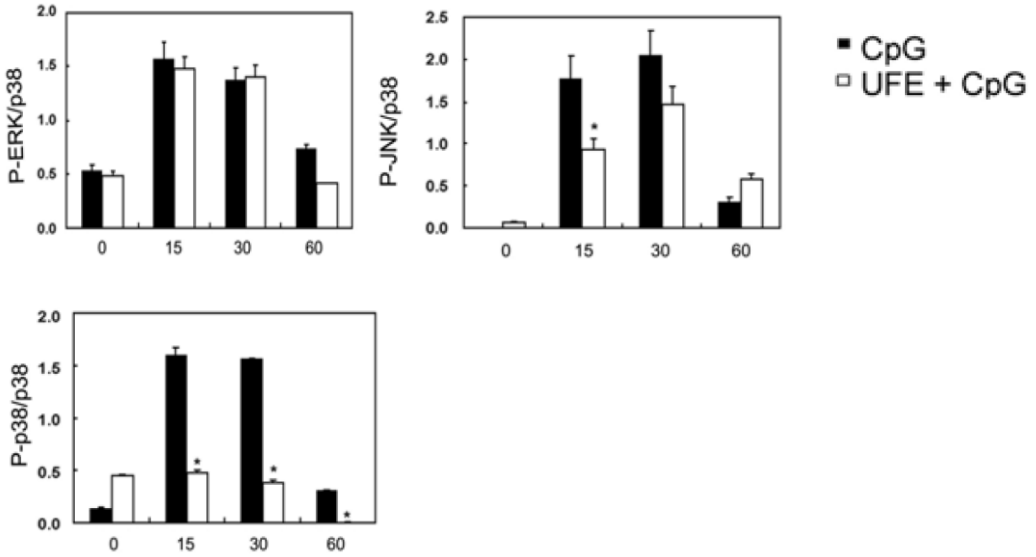
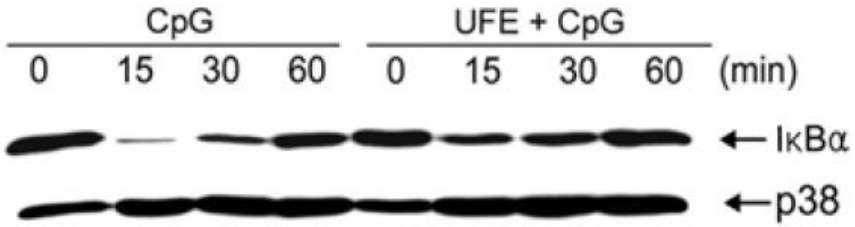


Fig 9. Effect of UFE on MAPK phosphorylation by CpG-stimulated bone marrow-derived macrophages

Results shown are the representative of independent experiments in Phosphorylation of ERK, JNK, and p38 protein expression (A) was quantified using scanning densitometry and the band intensities were normalized by that of total p38 protein (B). ND, not detected; *p<0.05 vs. CpG- treated group

A



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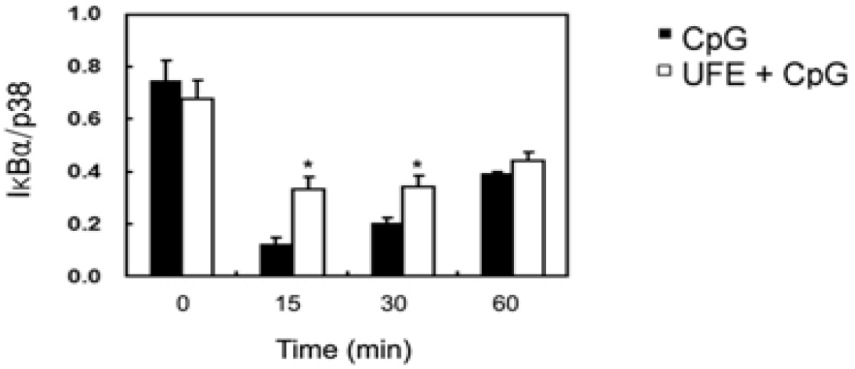

Fig 10. Effect of UFE on degradation of IκBα by CpG-stimulated bone marrow-derived macrophages

Results shown are the representative of independent experiments in IκBα protein expression (A) was quantified using scanning densitometry and the band intensities were normalized by that of total p38 protein (B). ND, not detected; *p<0.05 vs. CpG- treated group

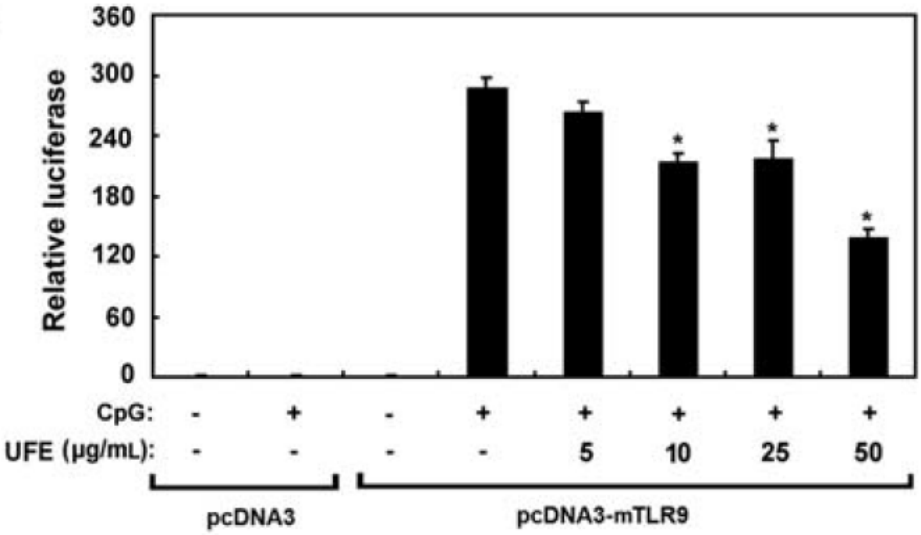

Fig 11. Effect of *Ulva fasciata* extract on NF-κB reporter activity in HEK293T cells

UFE treatment inhibited NF-κB reporter activity in HEK293T cells. HEK293T cells were transfected and then treated with UFE at the indicated doses. Cell lysates were prepared and assayed for firefly luciferase vs. renilla activities, and results were expressed as relative luciferase. *$p<0.05$ vs. UFE-untreated cells in the presence of CpG

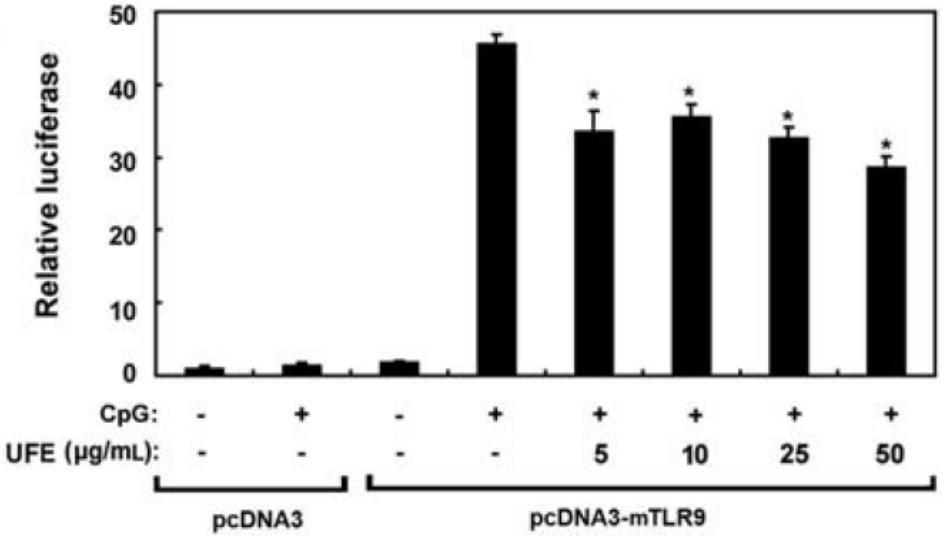

Fig 12. Effect of *Ulva fasciata* extract on AP-1 reporter activity in HEK293T cells

UFE treatment inhibited AP-1 reporter activity in HEK293T cells. HEK293T cells were transfected and then treated with UFE at the indicated doses. Cell lysates were prepared and assayed for firefly luciferase vs. renilla activities, and results were expressed as relative luciferase. *$p<0.05$ vs. UFE-untreated cells in the presence of CpG

4. Discussion

This report was designed to study inhibitory effect of sea lettuce (*Ulva fasciata*) extract (UFE) on pro-inflammatory cytokine production in bone marrow-derived macrophages and dendritic cells. The UFE (0-50 μg/mL) pre-treatment showed a dose dependant inhibitory effect on IL-12 p40, IL-6, and TNF-α productions in CpG-stimulated BMDMs and BMDCs as compared to non-treated controls.

Macrophages and dendritic cells have a major role in productions of key pro-inflammatory cytokines (7). Macrophages and dendritic cells are major part of mammalian immune system and play important role in productions of pro-inflammatory cytokines (6). IL-12 is a key cytokine in Th1-mediated autoimmune responses, so down regulation of IL-12 production by the UFE may be helpful in combating IL-12-associated autoimmune diseases (21). Controlled TNF-α has critical immunoregulatory roles while its overproduction is injurious as it results in inflammatory diseases such as rheumatoid arthritis and Crohn's disease (22). In the present study, pre-treatment of UFE exhibited a strong dose-dependent inhibition of TNF-α production in CpG-stimulated BMDMs, so it may hold therapeutic potential for combating TNF-α associated diseases. IL-12 p40 and TNF-α production in CpG-stimulated BMDMs and BMDCs showed dose dependant inhibition while IL-6 does not show a dose-dependent inhibition. This apparent discrepancy between cytokines may be due to differences of the regulatory factors associated with the upstream promoters of corresponding pro-inflammatory cytokines.

This study indicates that treatment of UFE resulted in strong inhibition on productions of pro-inflammatory cytokines such as IL-12 p40 and TNF-α. However, unexpectedly we observed partial inhibitory effect of UFE treatment on TLR9-dependant NF-κB and AP-1 activity even though it showed strong inhibition on phosphorylation of p38 MAPK. There are two possible explanations of this apparent disparity. First, the p38 pathway is involved in

transcriptional control of pro-inflammatory cytokines (23). The p38 MAPK phosphorylate different transcriptional factors including AP-1, ATF-2, MEF2A, Elk-1, and Ets-1 (24). The strong expression of IL-12 p40 and TNF-α genes could be due to cooperative effect of these multiple transcriptional factors regulated by p38 MAPK. Second, the p38 MAPK has been reported to regulate the pro-inflammatory cytokine production at the post-transcriptional level by stabilizing the cytokine mRNAs containing common AU-rich elements (23, 25). Hence, blockage of p38 MAPK strongly inhibits productions of IL-12 p40, IL-6, and TNF-α.

The present study suggests that UFE has an inhibitory effect on productions of pro-inflammatory cytokines, TLR9-dependent NF-κB and AP-1 activation and thus warrant further studies concerning potential uses of UFE for medicinal food. Further studies are required regarding in-depth investigation and detail mode of actions of the pure biologically active compounds from the *U. fasciata.*

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