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Molecules and Cells

Immunostimulating Activity by Polysaccharides Isolated from Fruiting Body of *Inonotus obliquus*

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In this study, we investigated the immunostimulating activity of polysaccharides isolated from fruiting body of *Inonotus obliquus* (PFIO). Additionally, the signaling pathway of PFIO-mediated macrophage activation was investigated in RAW264.7 macrophage cells. We found that PFIO was capable of promoting NO/ROS production, TNF-α secretion and phagocytic uptake in macrophages, as well as cell proliferation, comitogenic effect and IFN-γ/IL-4 secretion in mouse splenocytes. PFIO was able to induce the phosphorylation of three MAPKs as well as the nuclear translocation of NF-κB, resulting in activation of RAW264.7 macrophages. PFIO also induced the inhibition of TNF-α secretion by anti-TLR2 mAb, consequently, PFIO might be involved in TNF-α secretion via the TLR2 receptor. In addition, our results showed that oral administration of PFIO suppressed *in vivo* growth of melanoma tumor in tumor-bearing mice. In conclusion, our experiments presented that PFIO effectively promotes macrophage activation through the MAPK and NF-κB signaling pathways, suggesting that PFIO may potentially regulate the immune response.

INTRODUCTION

Cancer is one of the major causes of human death worldwide. There are many anti-cancer therapies available, including chemotherapy and anti-cancer drugs, which are known to cause adverse effects such as injury to the immune system. Therefore, it is necessary to investigate novel anti-tumor drugs that improve immunity without harming the host. Mushrooms have attracted much attention recently in the biochemical and medical fields due to their useful therapeutic effects (Balkwill, 2009). Especially, *Inonotus obliquus* (*I. obliquus)*, a traditional medicinal mushroom, has been widely used to promote health and longevity. *I. obliquus* is known as cinder conk and belongs to the *Hymenochaetaceae* family (Zhong et al., 2009).

Recently, it was tried to investigate the active substances of *I. obliquus* involved in the prevention of various diseases such as cancer (Kim et al., 2006) and cardiovascular diseases (Cui et al., 2005). The biological response modifiers derived from mushrooms are increasingly being used to treat a wide variety of clinical conditions, with relatively little knowledge of their modes of action. Especially, polysaccharides are considered to be one of the major active substances in mushrooms (Dalmo and Bøgwald, 2008). Polysaccharides isolated from mushrooms can have significant effects on the innate and adaptive immune response and therefore have the potential as immunostimulators with wide clinical and medicinal applications (Ooi and Liu, 2000; Tsan and Baochong, 2007).

Immunostimulation is regarded as one of the important strategies to improve the body's defense mechanism in elderly people as well as cancer patients (Borchers et al., 2004; 2008). There is a significant amount of experimental evidence suggesting that polysaccharides form mushrooms enhance the most immune system by stimulating natural killer (NK) cells, T cells, B cells and macrophages-dependent immune responses (Kim et al., 2006; Lull et al., 2005). Among them, T cells are characterized as either $CD4^+$ Th1 cells or $CD8^+$ Th2 cells based on their functional capabilities and cytokine profiles. Th1 cells secrete interferon (IFN)-γ and stimulate cell-mediated immunity, whereas Th2 cells secrete IL-4 and IL-10 and activate humoral immunity. Since the cytokines produced by these two sub-types have different immunological activities, balance between them is considered to be important to the immune response (So et al., 2007). When the body is stimulated by pathologic stimuli or injury, phagocytosis is the first step in the macrophage response to pathogens. In addition, macrophages can defend against pathogen invasion by secreting pro-inflammatory cytokines [*e.g.,* tumor necrosis factor (TNF)-α and IL-1] (Balkwill, 2009) and releasing cytotoxic and inflammatory molecules [*e.g.*, nitric oxide (NO) and reactive oxygen species (ROS)] (Carini et al., 2004; Goossens et al., 1995).

In this study, we demonstrated that PFIO can enhance immunostimulating activity such as cell proliferation, the release of toxic molecules, phagocytic uptake, and cytokine secretion in mouse splenocytes and macrophages. And then, mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) signaling pathway by PFIO on macrophage-mediated immune system responses was also demonstrated. Finally, we demonstrated that PFIO suppressed *in vivo* growth of melanoma in an experimental mouse model.

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MATERIALS AND METHODS

Preparation of polysaccharides from *I. obliquus*

Dried fruiting bodies of *I. obliquus* were purchased from the local market and ground in a blender. A milled mushroom (20 g) was extracted with distilled water (600 ml) at 121°C for 2 h. Extracts were centrifuged at 5,000 rpm for 20 min and filtered through 0.45-μm Whatman filter paper (Whatman #4, UK) to remove insoluble matter, and then freeze-dried. Polysaccharides were precipitated from resuspended extracts using 75% ethanol, collected by filtration through 0.45-μm Whatman filter paper, resuspended and dialyzed against distilled water for 5 days to remove low-molecular-weight compounds (Lee et al., 2010).

Materials

Fetal bovine serum (FBS), fetal calf serum (FCS), penicillin G, streptomycin and RPMI 1640 media were obtained from GIBCO (USA). Polymyxin B (PMB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (ConA), lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) and fluorescein isothiocyanate (FITC)-labeled dextran were purchased from Sigma Chemical Co. (USA). Dichlorodihydrofluorescein diacetate (H_2DCFDA), FITC-labeled *E. coli* bioparticles and rhodamine phalloidin were purchased from Molecular Probes (USA). Cell-permeable inhibitor peptide (NF-κB SN50), BAY11-7082, PD98059, SP600125 and SB203580 were obtained from Calbiochem (USA). The following antibodies, β-actin monoclonal antibody (mAb), extracellular activated signal-regulated kinase (ERK) MAPK Ab, phospho-ERK MAPK Ab, stress-activated protein kinases/c-Jun N-terminal kinase (SAPK/ JNK) Ab, phospho-SAPK/JNK Ab, p38 MAPK Ab, phospho-p38 MAPK Ab, NF-κB p65 mAb, dectin-1-specific mAb 2A11, Toll-like receptor (TLR) 2-specific mAb T2.5, TLR4-specific mAb MTS510 and complement receptor type 3 (CR3 or CD11b/CD18) mAb were purchased from Cell Signaling (USA), Serotec (UK), HyCult Biotechnology (The Netherlands) and BD Bioscience (USA), respectively. All other chemicals were of Sigma grade.

Animals and cell lines

6 week-old female C57BL/6 mice (weight: 18-20 g) were purchased from the Charles River Orient Experimental Animal Breeding Center (Korea). The mice were maintained under constant conditions, i.e., 20-22°C ambient temperature, relative humidity of 50 ± 5%, 12/12 h light-dark cycle with free access to standard commercial diet and water. RAW264.7 macrophages were purchased from the American Type Culture Collection (USA). RAW264.7 macrophages were maintained in RPMI 1640 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% heat-inactivated fetal bovine serum.

In vitro mouse splenocyte culture

The spleen was aseptically removed from 6 week-old female C57BL/6 mice and transferred to serum-free RPMI 1640 medium. The spleen cell suspensions were prepared by passing spleen cells through a sterile nylon mesh column (BD Biosciences, USA) in RPMI 1640 medium containing 10 mM HEPES. In order to lyse the erythrocytes, the pellet was suspended in 3 ml of tris ammonium chloride (0.26 M NH_4Cl, 17 mM Tris, pH 7.6), which was agitated for 2 min at room temperature, and then washed twice with RPMI 1640 medium. The splenocytes (2×10^6 cells/ml) were cultured in 96-well plates in the presence or absence ConA (5 μg/ml) or various concentrations of PFIO (from 10 to 1,000 μg/ml) in a total volume of 200 μl/well for 48 h under the same conditions.

Culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were obtained by infusing the peritoneal cavity with 5 ml of ice-cold sterile RPMI 1640 medium, and cultured in a humidified incubator containing 5% CO_2 at 37°C in 10% fetal calf serum-RPMI 1640 medium for 3 h. After incubation, non-adherent cells were removed by washing with serum-free medium and adherent cells were seeded in 12-well plates at a density of 5×10^5 cells/well and incubated in 5% fetal calf serum-RPMI 1640 medium in the presence or absence of LPS (2.5 μg/ml) or PFIO (100, 300 and 500 μg/ml) for 24 h.

Determination of NO production

After various treatments, NO in the culture supernatants was measured by the addition of 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl of each sample. The NO concentration was determined at 540 nm using $NaNO_2$ as a standard.

Determination of ROS generation

To evaluate the level of intracellular ROS, the change in fluorescence resulting from the oxidation of the fluorescent probe H_2DCFDA was measured. Briefly, macrophages (1×10^6 cells/well) were pre-incubated for 24 h, followed by incubation with various concentrations (from 50 to 500 μg/ml) of PFIO for 24 h. Cells were then incubated with 50 mM of the fluorescent probe H_2DCFDA for 2 h at 37°C. The degree of fluorescence was detected at 485 nm excitation and at 535 nm emission using a microplate spectrofluorometer. For image analysis of PFIO-mediated or LPS-mediated ROS production, macrophages (1×10^6 cells/well) were seeded on coverslip-loaded 6-well plates and stimulated with PFIO (300 μg/ml) or LPS (2.5 μg/ml) for 24 h. H_2DCFDA solution was then added and incubated with the cells for 2 h at 37°C. Images of the stained cells were obtained using a fluorescence microscope.

In vivo assay of mouse peritoneal macrophage phagocytosis

Female C57BL/6 mice were injected with PFIO (300 and 500 μg/200 μl) or 200 μl of phosphate buffered saline (PBS) intraperitoneally. FITC-labeled *E. coli* (200 μl) was then injected into the peritoneal cavity the following day before the mice were sacrificed. The peritoneal cavity was washed with 1 ml of PBS under aseptic conditions to collect peritoneal macrophages. The macrophages were incubated on a slide in a humidified incubator containing 5% CO_2 at 37°C for 30 min. After fixation with 3.7% paraformaldehyde solution, adherent cells were stained with rhodamine phalloidin (1:250 dilution), and mounted under glass coverslips with a Vectashield (Brunschwig Chemie, The Netherlands), observed and photographed under a fluorescence microscope.

Determination of phagocytic uptake

The phagocytic activity of macrophages was measured according to a previously described method with slight modifications (Duperrier et al., 2000). After pre-incubation for 48 h, macrophages (2×10^6 cells/well) were treated with varying concentrations of PFIO (from 50 to 500 μg/ml) at 37°C for 6 h. Cells were resuspended in 1 ml of PBS containing 1% human Ab serum with FITC-labeled dextran (1 mg/ml) and incubated at 37°C for 30 min. Phagocytosis was then stopped by the addition of 2 ml of ice-cold PBS containing 1% human serum and 0.02% so-

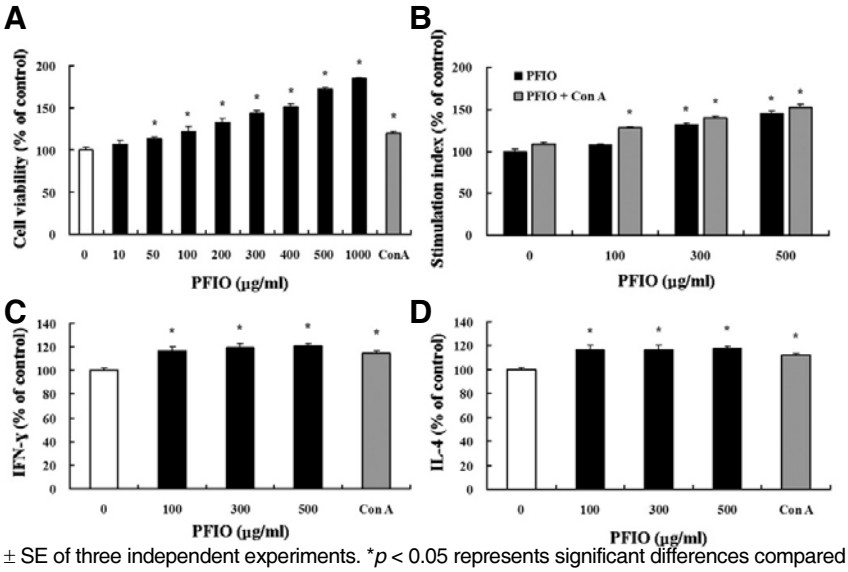

Fig. 1. The effects of PFIO on proliferation, comitogenic effect and cytokine secretion in mouse splenocytes. (A) Proliferation: Splenocytes were incubated with various concentrations of PFIO (from 10 to 1,000 μg/ml) or ConA (5 μg/ml) for 48 h, and the viability of the cells was determined using the MTT assay. (B) Comitogenic effect: Splenocytes were stimulated with PFIO (100, 300 and 500 μg/ml) in the presence of ConA (5 μg/ml) for 48 h, and cell viability was examined using the MTT assay. (C, D) Cytokine secretion: Splenocytes were incubated with PFIO (100, 300 and 500 μg/ml) or ConA (5 μg/ml) for 48 h, after which supernatants were collected for measurement of IFN-γ (C) and IL-4 (D) secretion. IFN-γ and IL-4 concentrations in the supernatants were determined by ELISA. Data (A-D) are represented as the means ± SE of three independent experiments. *$p < 0.05$ represents significant differences compared to the control by Student's *t*-test.

dium azide. The cells were then washed three times with cold PBS-azide and analyzed by the FACScan flow cytometry (Becton-Dickinson, USA).

Determination of cytokine secretion

The levels of IL-4, IFN-γ and TNF-α in the culture supernatants of splenocytes and macrophages were evaluated using an ELISA kit (Biosource International, USA) according to the manufacturer's instructions.

Nuclear protein extraction

Nuclear extracts were prepared by lysing nuclei in high salt buffer supplemented with protease and phosphatase inhibitors using a nuclear extraction kit (Panomics Inc., USA) according to the manufacturer's protocol. Protein concentrations were quantified by the Bio-Rad protein assay (Bio-Rad Laboratories, USA).

Western blot analysis

After treatment, cells were washed in 1× PBS and lysed in lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM NaH_2PO_4/$NaHPO_4$ (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaPPi, 1 mM phenylmethylsulphonyl fluoride, 2 μg/ml pepstatin A] for 30 min on ice. Lysates were then centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was collected, after which protein content was measured using a Bio-Rad protein assay kit before analysis. The cytoplasmic or nuclear protein samples were loaded at 10 μg of protein/lane, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10-15% gel, and transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF membrane, 0.2 μm; Bio-Rad). Membranes were blocked with 5% nonfat powdered milk in 1× tris buffered saline (TBS) containing 0.1% Tween 20 for 1 h, and then incubated with primary antibodies at 4°C overnight. Finally, the membranes were treated with horseradish peroxidase-coupled secondary antibodies for 1 h at 4°C. The membranes were washed with T-TBS after each antibody binding reaction. Detection of each protein was performed using an enhanced chemiluminescence (ECL) kit (Millipore Co., USA).

Inhibition of MAPKs and NF-κB activation

To examine the signal transduction pathways that mediate the effects of PFIO on macrophage activation, macrophages were pre-treated with ERK inhibitor PD98059 (25 μM), JNK inhibitor SP600125 (25 μM), p38 MAPK inhibitor SB203580 (25 μM) and NF-κB inhibitor BAY11-7082 (10 μM) or SN50 (50 μM) for 2 h in RPMI 1640 medium containing 0.5% FBS. This medium was then replaced with medium with or without LPS (2.5 μg/ml) or PFIO (300 μg/ml) for 6 h at 37°C. Then, as described previously, cells were harvested and analyzed through phagocytic uptake by the FACScan flow cytometry.

Inhibition of cytokine production using pattern recognition receptor antibodies

To study the role of TLR2, TLR4, dectin-1 and CR3 in cytokine production, macrophages cultivated in 12-well plates were pre-incubated with medium containing TLR2-specific mAb T2.5, TLR4-specific mAb MTS510, dectin-1-specific mAb2A11 or CD11b mAb (10 μg/ml) for 1 h. Isotype control antibodies were used at 10 μg/ml for rat IgG_{2B} and 35 μg/ml for mouse IgG_1. Cells were then treated with PFIO (300 μg/ml) for 6 h at 37°C.

***In vivo* experiments using mice**

The inhibitory effect of PFIO against a melanoma tumor was investigated. Six week-old female C57BL/6 mice were randomly divided into four groups consisting of eight mice per group. Control: Group 1 (Normal control, n = 8), saline implantation; Group 2 (Tumor control, n = 8), B16-F10 melanoma cells implantation (200 μl of 2×10^5 cells/mouse). Oral group: Group 3 (n = 8), pre-oral PFIO diluted in PBS; Group 4 (n = 8), post-oral PFIO diluted in PBS. The pre-treated PFIO was administered orally at a dose of 200 mg/kg/day to the mice three days before implantation of B16-F10 melanoma cells and then administered daily an oral dose of 200 mg/kg/day to the mice after B16-F10 melanoma cells implantation. The post-treated PFIO was administered orally at a dose of 200 mg/kg/day to the mice simultaneously with the implantation of B16-F10 melanoma cells. After the mice were intraperitoneally implanted with 200 μl of 2×10^5 B16-F10 melanoma cells per mouse on day 0, all of the groups were administered daily an oral dose of PFIO (200 μl) for 12 days after tumor implantation. The body weights of the mice were measured every three days until they were sacrificed by cervical dislocation on the 13th day after tumor implantation.

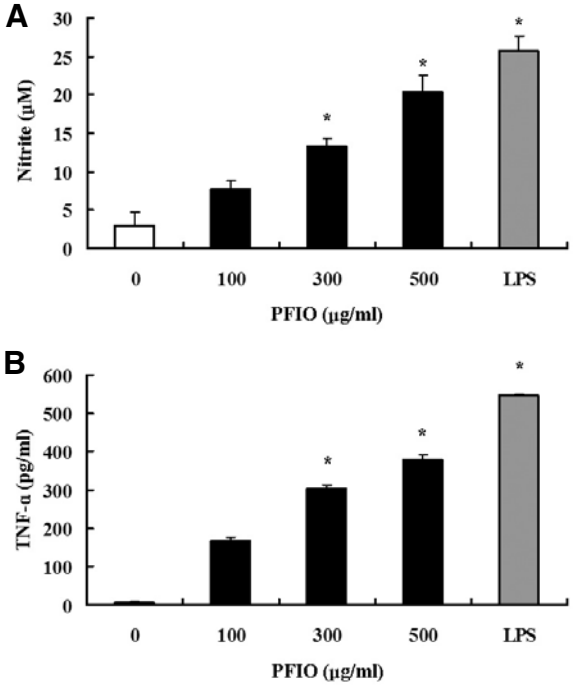

Fig. 2. The effects of PFIO on immunostimulating activity in mouse peritoneal macrophages. (A) NO production: Peritoneal macrophages were incubated with PFIO (100, 300 and 500 μg/ml) or LPS (2.5 μg/ml) for 24 h, and NO concentrations in the culture supernatants were assessed using the Griess assay. (B) TNF-α secretion: Peritoneal macrophages were incubated with PFIO (100, 300 and 500 μg/ml) or LPS (2.5 μg/ml) for 6 h. After incubation, supernatants were collected, and the TNF-α concentrations in the supernatants were determined by ELISA. Data (A, B) are represented as the means ± SE of three independent experiments. *$p < 0.05$ represents significant differences compared to the control by Student's *t*-test.

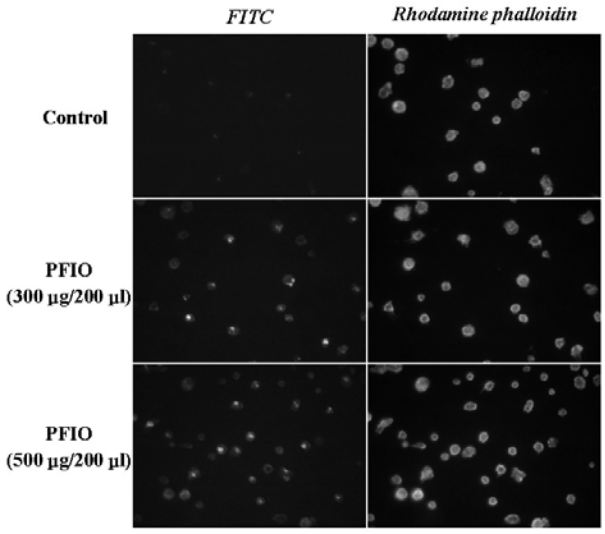

Fig. 3. The effects of PFIO on *in vivo* phagocytosis in mouse peritoneal macrophages. Phagocytosis of FITC-labeled *E. coli* by peritoneal macrophages was examined after treatment with saline or PFIO (300 and 500 μg/200 μl) *in vivo*. After treatment, peritoneal macro- phages were injected with FITC-labeled *E. coli* for 30 min, followed by staining with rhodamine-phalloidin. Lastly, *in vivo* phagocytosis in peritoneal macrophages was detected using a fluorescence microscope.

The peritoneal all viscera masses where then collected and weighed together.

Statistical analysis

Data are expressed as means ± standard error (SE), and the results were taken from at least three independent experiments performed in triplicate. The data were analyzed by Student's *t*-test to evaluate significant differences. A level of *$p < 0.05$ was regarded as statistically significant.

RESULTS

Effect of PFIO on splenocyte proliferation and cytokine secretion

We investigated the effects of PFIO on splenocyte proliferation, comitogenic effect, and cytokine secretion of IFN-γ and IL-4. As shown in Fig. 1A, splenocyte proliferation by PFIO was significantly increased in a dose-dependent manner. In addition, PFIO showed dose-dependent comitogenic activity in ConA-stimulated splenocytes (Fig. 1B). We also elucidated the immunostimulating effect of PFIO more clearly, and the secretion levels of the Th1 cell-related cytokine IFN-γ and the Th2 cell-related cytokine IL-4 were measured by ELISA. As shown in Figs. 1C and 1D, secretion levels of IFN-γ and IL-4 were exhibited balanced increase according to PFIO stimulation. As a result, we suggested that PFIO activated T cells according to enhanced proliferation, comitogenic effect and cytokine secretion.

Effect of PFIO on *in vitro* and *in vivo* immunostimulating activity in mouse peritoneal macrophages

Macrophages can play a critical role in the innate immune response against invading pathogens (Kumar et al., 2004; Volman et al., 2008). Therefore, we investigated whether PFIO is capable of stimulating functional activation in mouse peritoneal macrophages, such as NO production, TNF-α secretion and *in vivo* phagocytosis. As shown in Figs. 2A and 2B, PFIO increased NO production and TNF-α secretion in mouse peritoneal macro- phages in a dose-dependent manner. To investigate the direct effect of PFIO on macrophage phagocytosis, the phagocytic activity of macrophages toward FITC-labeled *E. coli* was assessed *in vivo*. As shown in Fig. 3, the phagocytic uptake of FITC-labeled *E. coli* was significantly promoted by PFIO treatment in mouse peritoneal macrophages. These data indicated that PFIO played critical roles in the macrophage-mediated immune responses.

Effect of PFIO on immunostimulating activity in RAW264.7 cells

Another macrophage cell line, RAW264.7 cells were used for measurement of immunostimulating activity. Our results showed that PFIO was capable of enhancing NO production (Fig. 4A) and ROS generation (Figs. 4B and 4C) in a dose-dependent manner. PFIO-induced NO production was not affected by the presence of PMB, whereas LPS-induced NO production was inhibited by the presence of PMB, indicating that macrophage activation by PFIO was not due to endotoxin contamination (Fig. 4D). Moreover, PFIO enhanced TNF-α secretion (Fig. 4E) and phagocytic uptake (Fig. 4F) in a dose-dependent manner.

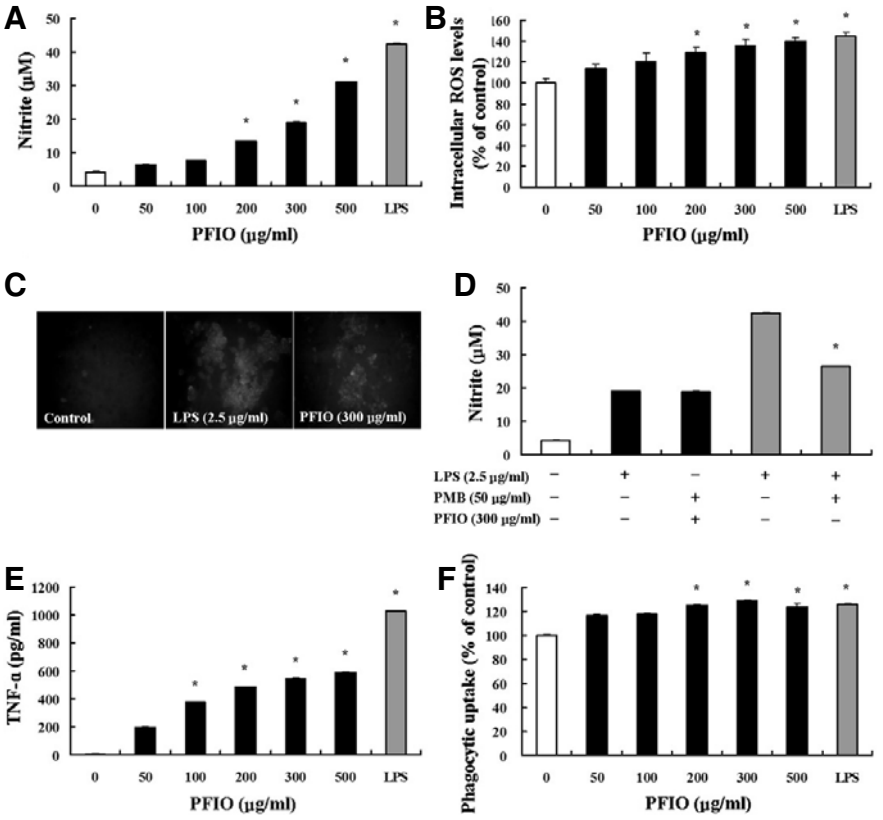

Fig. 4. The effects of PFIO on cellular activation in RAW264.7 cells. (A) NO production: RAW264.7 cells were incubated with PFIO (from 50 to 500 μg/ml) or LPS (2.5 μg/ml) for 24 h, and NO concentrations in the culture supernatants were assessed using the Griess assay. (B) ROS generation: RAW264.7 cells were incubated with PFIO (from 50 to 500 μg/ml) or LPS (2.5 μg/ml) for 24 h, and the level of ROS in the cell lysates was detected at 485 nm excitation and at 535 nm emission using a microplate spectrofluorometer. (C) Fluorescence microscopic image: RAW264.7 cells were incubated with PFIO (300 μg/ml) or LPS (2.5 μg/ml) for 24 h, and the level of ROS in the cells was detected using a fluorescence microscope. (D) Endotoxin test: RAW264.7 cells were incubated with PFIO (300 μg/ml) or LPS (2.5 μg/ml) in the presence or absence of PMB (50 μg/ml) for 24 h. NO concentrations in the culture supernatants were assessed using the Griess assay. (E) TNF-α secretion: RAW264.7 cells were incubated with PFIO (from 50 to 500 μg/ml) or LPS (2.5 μg/ml) for 6 h. After incubation, supernatants were collected and the TNF-α concentration in the supernatants were determined by ELISA. (F) Phagocytic activity: RAW264.7 cells were incubated with PFIO (from 50 to 500 μg/ml) or LPS (2.5 μg/ml) for 6 h. After incubation, FITC-labeled dextran (1 mg/ml) was added for 30 min. Supernatants were removed with unphagocytosed dextran, and the fluorescence intensity was measured using the flow cytometry. Data are represented as the means ± SE of three independent experiments. *$p < 0.05$ represents significant differences compared to the control by Student's *t*-test.

These results indicated that PFIO activated not only mouse peritoneal macrophages but also another macrophage cell line, RAW264.7 cells.

Molecular mechanism of macrophage activation by PFIO

MAPK (ERK, JNK and p38) signaling pathways are known to participate in macrophage activation (Kim et al., 2004b; Naik, 2003). Therefore, to investigate whether the MAPKs signaling pathway participates in macrophage activation by PFIO, RAW264.7 cells were stimulated with PFIO, and the phosphorylation levels of all three MAPKs were estimated by Western blot analysis using respective anti-phospho MAPK mAbs. As shown in Fig. 5A, PFIO induced the phosphorylation of all MAPKs (ERK, JNK and p38) compared to control. Specifically, ERK, JNK and p38 MAPKs were phosphorylated at 5 min, 30 min and 15 min, respectively. And then, we reconfirmed whether the MAPK signaling pathways were involved in macrophage phagocytic uptake by PFIO, all three MAPKs inhibitors were used. PFIO-induced phagocytic uptake by ERK (PD98059), JNK (SP600125) and p38 MAPK (SB203580) inhibitors was suppressed, suggesting that phosphorylation of all three MAPKs were involved in macrophage stimulation by PFIO. In particular, p38 (SB 203580) inhibitor was suppressed 75% compared to PFIO-induced phagocytic uptake (Fig. 5B).

Macrophage activation is induced by NF-κB translocation from the cytosol to the nucleus (Kumar et al., 2004). Therefore, we also investigated whether the NF-κB signaling pathway participates in macrophage activation by PFIO. RAW264.7 cells were stimulated with PFIO at the indicated time periods, and NF-κB translocation was assessed by Western blot analysis using a NF-κB subunit p65 antibody. As shown in Fig. 6A, the total cytosolic NF-κB level in PFIO-treated macrophages gradually decreased thereafter 30 min similar to LPS-treated macrophages. On the other hand, the expression levels of NK-κB in the nucleus increased after treatment with PFIO at 15 min and gradually increased thereafter. In particular, nuclear NF-κB level in PFIO-treated macrophages increased at 60 min and 120 min. LPS also increased the expression levels of nuclear NF-κB at 15 min. To reconfirm whether NF-κB translocation was involved in macrophage phagocytic uptake by PFIO, NF-κB inhibitors were used. PFIO-induced phagocytic uptake by NF-κB inhibitors (SN50 and BAY11-7082) were suppressed 25% and 26%, respectively, compared to PFIO-induced phagocytic uptake (Fig. 6B), suggesting that translocation of NF-κB from the cytosol to the nucleus was involved in macrophage stimulation by PFIO. These data indicated that NF-κB translocation was required for PFIO-mediated macrophage activation.

It has been reported that pattern recognition receptors (PRRs) such as dectin-1, TLR2, TLR4 and CR3 are a major receptor type for β-glucan. Therefore, we investigated whether these receptors are involved in PFIO-mediated macrophage responses. Our results showed that the treatment with anti-TLR2 mAb significantly blocked PFIO-induced TNF-α secretion (45%), whereas treatment with anti-dectin-1 mAb, anti-TLR4 mAb and anti-CR3 mAb failed to inhibit PFIO-induced TNF-α secretion (Fig. 7). These data indicated that TLR2 might be involved in

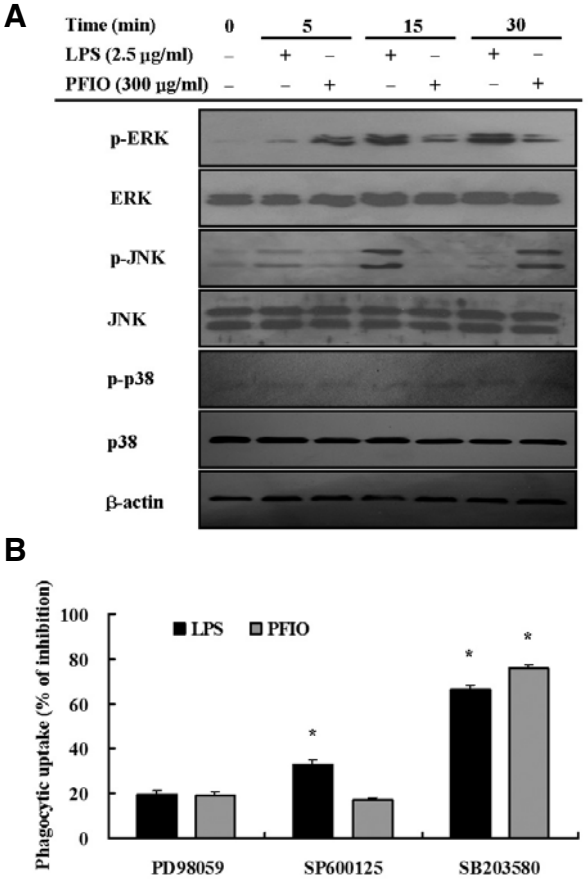

Fig. 5. The effects of MAPKs signaling in PFIO-induced macrophage activation. (A) Western blot: RAW264.7 cells were stimulated by LPS (2.5 μg/ml) or PFIO (300 μg/ml) for the indicated time periods and were collected. The levels of phospho-ERK, phospho-JNK, phospho-p38 and β-actin were assessed by Western blot analysis with primary antibody against each protein. β-actin protein was used as an internal control. (B) Phagocytic analysis: RAW264.7 cells were incubated with various inhibitors [PD98059 (25 μM, ERK1/2 inhibitors), SP600125 (25 μM, JNK inhibitors), SB203580 (25 μM, p38 inhibitors)] in the presence of LPS (2.5 μg/ml) or PFIO (300 μg/ml) for 6 h. After incubation, FITC-labeled dextran (1 mg/ml) was added for 30 min and the fluorescence intensity was mea-sured using the flow cytometry. Data are represented as the means ± SE of three independent experiments. *$p < 0.05$ represents significant differences compared to the control by Student's *t*-test.

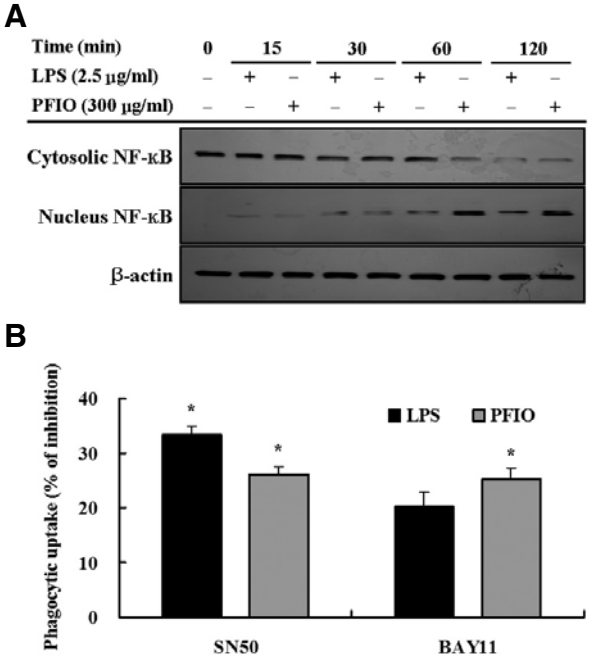

Fig. 6. The effects of NF-κB signaling in PFIO-induced macrophage activation. (A) Western blot: RAW264.7 cells were stimulated by LPS (2.5 μg/ml) or PFIO (300 μg/ml) for the indicated time periods and were collected. The levels of cytosolic NF-κB, nuclear NF-κB and β-actin were assessed by Western blot analysis with primary antibody against each protein. β-actin protein was used as an internal control. (B) Phagocytic analysis: RAW264.7 cells were incubated with BAY 11-7082 (10 μM, IκB inhibitors) or SN50 (50 μM, NF-κB inhibitors) in the presence of LPS (2.5 μg/ml) or PFIO (300 μg/ml) for 6 h. After incubation, FITC-labeled dextran (1 mg/ml) was added for 30 min and the fluorescence intensity was measured using the flow cytometry. Data are represented as the means ± SE of three independent experiments. *$p < 0.05$ represents significant differences compared to the control by Student's *t*-test.

PFIO-mediated TNF-α secretion.

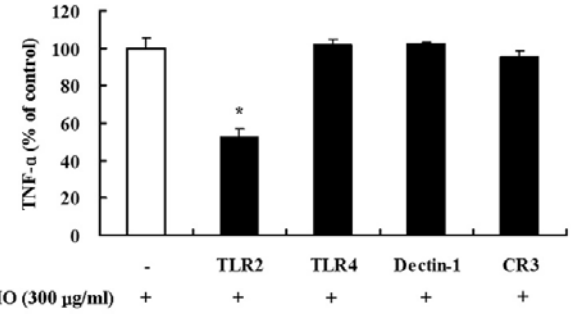

Fig. 7. The effects of TLR2, TLR4, dectin-1 and CR3 pattern recognition receptors in PFIO-mediated TNF-α secretion. RAW264.7 cells were incubated with various function-blocking antibodies specific to TLR2 (10 μg/ml), TLR4 (10 μg/ml), dectin-1 (10 μg/ml) and CR3 (20 μg/ml) in the presence of PFIO (300 μg/ml) for 6 h. After incubation, the TNF-α concentrations in the supernatants were determined by ELISA. Data are represented as the means ± SE of three independent experiments. *$p < 0.05$ represents significant differences compared to the control by Student's *t*-test.

Effect of PFIO on tumor growth *in vivo*

To investigate whether PFIO inhibits the *in vivo* growth of melanoma solid tumor in C57BL/6 mice, the growth-inhibitory effects by pre-oral or post-oral administration of PFIO on the melanoma solid tumor mass in B16-F10 melanoma cells-implanted mice were examined. As a first step, the body weights of the mice were recorded every three days during the experiments to evaluate the inhibition of tumor growth in each group. The changes in body weight of the experimental groups were shown in Fig. 8A. In the tumor control group, the body weights of mice increased abnormally from the 3rd day after implantation of B16-F10 melanoma cells, most likely due to the rapid growth of melanoma cells in the peritoneal cavity. However, in the pre-oral or post-oral administration group, the body weights of mice were not significantly increased compared to the tumor

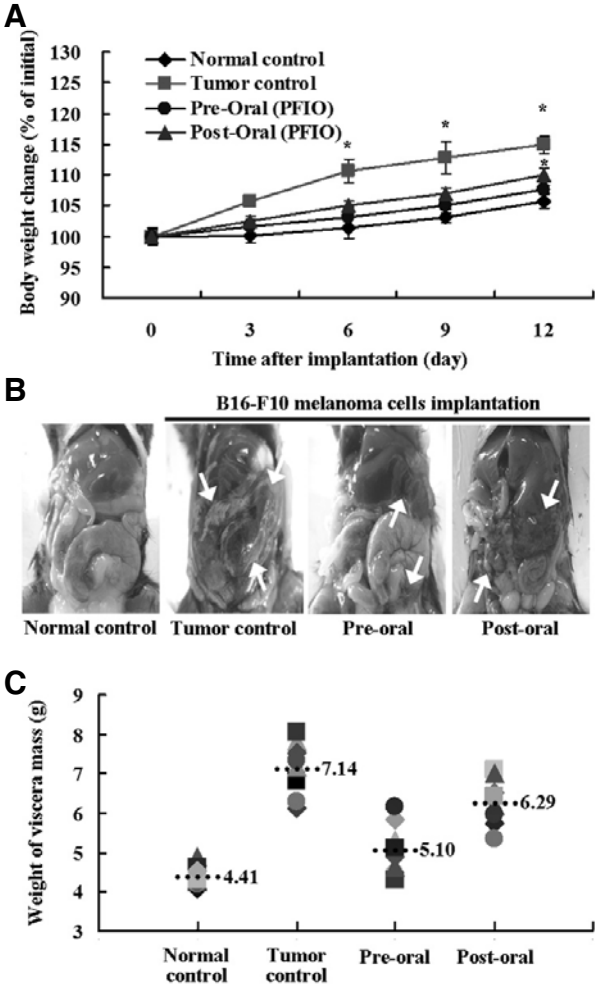

Fig. 8. Inhibitory effects of PFIO on *in vivo* tumor growth in tumor-bearing mice. C57BL/6 mice were implanted intraperitoneally with B16-F10 melanoma cells, after which the mice were treated with PFIO according to an administration schedule. (A) Body weight: Changes in body weight of melanoma tumor-bearing mice were measured. The body weights of mice were measured every 3 days during the experimental period. Changes in body weight of mice were expressed as a percentage of the initial body weight. Data are represented as the means ± SE (n = 8). *$p < 0.05$ represents significant differences compared to the control by Student's *t*-test. (B, C) Growth inhibition effects of PFIO on the peritoneal melanoma tumor mass were measured. The animals in all groups were sacrificed. The peritoneal all viscera masses were collected and weighed together. Arrows are indicated melanoma solid tumor mass in mouse peritoneum.

control group. In particular, in the post-oral administration group, the mice were slightly increased in body weight compared to the pre-oral administration group (Fig. 8A). As shown in Figs. 8B and 8C, pre-oral administration of PFIO significantly inhibited the growth of the peritoneal tumor mass compared to the tumor control group. These data indicated that PFIO had anti-tumor effects against melanoma solid tumor.

DISCUSSION

Various mushroom-derived polysaccharides are used as remedies and prevention agents for cancer and immune disorders, and so on (Goodridge et al., 2009; Kim et al., 2004a). For instance, polysaccharides extracted from mushrooms such as *L. edodes* (Lee et al., 2008), *T. matsutake* (Byeon et al., 2009), *H. erinaceus* (Son et al., 2006) and *I. obliquus* (Kim et al., 2006) are well-known to possess important immunostimulating properties. However, the signaling pathway toward the activation of immunocytes such as macrophages and T cells by polysaccharides isolated from *I. obliquus* is still unclear. Therefore, in this study, immunostimulating activity by PFIO on macrophages and T cells was evaluated, and the signaling pathway involved in macrophage-mediated immune response by PFIO was demonstrated.

The activation of macrophages and T cells is considered to be one of the most important events happened after infection. Briefly, after infection, macrophages mediate phagocytosis through the innate immune response. During the phagocytic process, activated macrophages produce several inflammatory and cytotoxic molecules such as NO, ROS and TNF-α (Balkwill, 2009; Kuo et al., 2005). Moreover, macrophages act as accessory cells in the presenting procession of an antigen to T cells as well as effectors in cell-mediated immune response against tumor cells and intracellular microorganisms. On the other hand, T cells are able to proliferate and subsequently to secrete the cytokines of two different types (Neves et al., 2009; Tsan and Baochong, 2007), including Th1 and Th2 type cellular response. In the present study, PFIO remarkably increased the proliferation of mouse splenocytes in a dose-dependent manner and showed a significant comitogenic effect on ConA-activated splenocytes. Furthermore, PFIO enhanced IFN-γ and IL-4 secretion, that is, PFIO could stimulate both the Th1 and Th2 type responses while maintaining balance (Fujihara et al., 2003; Kidd, 2003). In addition, PFIO was capable of enhancing NO production, ROS generation and TNF-α secretion in mouse peritoneal macrophages and RAW264.7 cells. PFIO also enhanced the phagocytic abilities of RAW264.7 cells and mouse peritoneal macrophages *in vitro* and *in vivo*. In particular, PFIO-induced NO production was not affected by treatment with PMB (a decapeptide cyclic antibiotic with strong binding affinity for endotoxin lipid A) (Schorey and Cooper, 2003), and therefore enhancement of NO production seemed not to be due to endotoxin contamination.

It was previously reported that LPS, a potent macrophage activation factor, induced NF-κB activation and phosphorylation of MAPKs, such as ERK1/2, JNK and p38, in macrophages (Kim et al., 2004b; Lee et al., 2009a). MAPKs are a family of serime/threonine protein kinases that participate in the initiation of NF-κB activation following various extracellular stimuli such as LPS, pro-inflammatory cytokines, polysaccharides, and so on (Schorey and Cooper, 2003). Phosphorylation of MAPKs can accordingly activate the transcription of several targets such as NF-κB (Chang et al., 2010) and AP-1 (Zaidman et al., 2005). Therefore, this study attempted to demonstrate the activation of NF-κB and all three MAPK pathways by PFIO during RAW264.7 macrophage activation. Our results showed that all three MAPKs were phosphorylated in RAW264.7 macrophages. Moreover, blockade of their phosphorylation by specific inhibitors (PD98059, ERK inhibitor; SP600125, JNK inhibitor; and SB20358, p38 inhibitor) also suppressed both LPS- and PFIO-induced phagocytic uptake. NF-κB transcription factors are a family of structurally related eukaryotic transcription factors that play an essential role in controlling a large number of normal cellular and whole-organism responses, such as the immune and inflammatory responses, cellular growth, apoptosis, and so on. In resting cells, inactive NF-κB complexes exist as a latent

form in the cytosol via non-covalent interaction with IκBα. In response to various stimuli, cytosolic IκBα is phosphorylated, which causes translocation of cytosolic NF-κB into the nucleus (Kumar et al., 2004) and activation of gene expression (Zaidman et al., 2005). Our results showed that expression levels of cytosolic NF-κB were decreased, whereas expression levels of nuclear NF-κB were increased on LPS- or PFIO-induced RAW264.7 macrophage activation. More- over, treatment with SN50 and BAY11-7082, inhibitors of NF-κB nuclear translocation, effectively inhibited the activation of NF-κB complexes and the phosphorylation of IκB, and consequently suppressed both LPS- and PFIO-induced phagocytic uptake. Although the major receptor for polysaccharides in fungal immunity is known to be dectin-1 (Herre et al., 2004), several PRRs such as TLR2, TLR4 and CR3 (Schepetkin and Quinn, 2006; Yoon et al., 2000) have also been shown to participate in the response to plant-derived polysaccharides. Therefore, this study investigated whether these molecules are capable of acting as a receptor for PFIO in the modulation of macrophage activation through PFIO-induced TNF-α secretion. Our results showed that TLR2 but not TLR4, dectin-1 and CR3 were involved in PFIO-induced TNF-α secretion, similar to previous reports (Herre et al., 2004; Lee et al., 2008).

It has been reported that oral and intraperitoneal administration of endo-polysaccharides extracted from *I. obliquus* inhibits the *in vivo* growth of melanoma cells in mice via humoral immunity of the host defense system (Kim et al., 2005; 2006). Our results showed that oral administration was effective for inhibition of melanoma solid tumor. In particular, pre-oral administration of PFIO was more rapid and effective for the inhibition of melanoma solid tumor than post-oral administration. These results suggested that inhibition of melanoma solid tumor occurred through upregulation and activation of the intestinal immune system. β-glucans, a major component of polysaccharides, is taken up by intestinal macrophages, after which it is transported to lymph nodes, spleen and bone marrow; accordingly, it can be concluded that oral administration of polysaccharides in as effective as parenteral administration for protecting against pathogen infections (Rop et al., 2009; Volman et al., 2008). Therefore, our results elucidated that the anti-tumor effects of PFIO may have been exerted through activation of several immune response systems (Youn et al., 2009).

Taken together, our results provided evidence that PFIO had potent immunostimulating activity *in vitro* and *in vivo*. PFIO enhanced the mitogenic and comitogenic activities of mouse splenocytes and induced IL-4 and IFN-γ secretion. Furthermore, PFIO might be induced upregulation of NO, ROS, TNF-α and phagocytosis by MAPKs and NF-κB signaling pathways via the TLR2 receptor in macrophages. Our study also exhibited that PFIO suppressed the *in vivo* growth of melanoma solid tumor in an experimental mouse model after both pre-oral and post-oral administration.

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