Carbohydrate Polymers 120 (2015) 29–37

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Mechanism of macrophage activation induced by polysaccharide from *Cordyceps militaris* culture broth

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ARTICLE INFO

Article history:
Received 28 November 2013
Received in revised form
22 September 2014
Accepted 28 November 2014
Available online 5 December 2014

Keywords:
Macrophage
MAPKs
NF-κB
Polysaccharide
PRRs

ABSTRACT

Mushroom-derived polysaccharides have been shown to stimulate immune responses. Our previous report showed that the novel polysaccharide PLCM isolated from the culture broth of *Cordyceps militaris* could induce nitric oxide production in the murine macrophage-like cell line RAW264.7. In this study, we show that PLCM enhances immunostimulatory activities such as the release of toxic molecules (nitric oxide and reactive oxygen species), secretion of the cytokine tumor necrosis factor (TNF)-α, and phagocytic uptake in RAW264.7 macrophages. In addition, all the specific inhibitors against the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) (SN50, BAY11-7082, PD98059, SP600125 and SB203580) markedly suppressed the nitric oxide production and phagocytic uptake induced by PLCM. Moreover, antibodies specific to the extracellular domain of Toll-like receptor-2, Toll-like receptor-4 or the macrophage receptor Dectin-1 significantly attenuated PLCM-induced secretion of TNF-α. Our results indicate that the *C. militaris* polysaccharide activates macrophages through the MAPKs and NF-κB signaling pathways *via* Toll-like receptor 2, Toll-like receptor 4, and Dectin-1.

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1. Introduction

Macrophages are cells produced by the differentiation of monocytes in tissues in response to inflammatory stimuli or injury. They are specialized cells that neutralize foreign substances, infectious microbes and cancer cells through phagocytosis (Dentan, Lesnik, Chapman & Ninio, 1996). They function in non-specific defense (innate immunity) as well as help initiate specific defense mechanisms (adaptive immunity). Macrophages defend against external pathogens by releasing cytotoxic and inflammatory molecules such as nitric oxide (NO), reactive oxygen species (ROS) and secreting pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1 (Grimes et al., 2005; Yoon, Kim, Oh, Lee & Hyun, 2009). These defensive mechanisms are activated when various molecules that act as stimuli bind to pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Dectin-1 and complement receptor type 3 (CR3 or CD11b/CD18) on the surface of macrophages and trigger several different signaling pathways including tyrosine kinases, protein kinase C, phosphoinositide-3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPKs), and nuclear factor kappa B (NF-κB) (Biswas & Sodhi, 2002; Kuan, Huang, Li & Chang, 2012; Wu, Chen & Chen, 2009).

Mushrooms have been shown to have several therapeutic effects (Bobek et al., 1991; Joseph, Panicker & Janardhanan, 2012; Selegean, Putz & Rugea, 2009). Most, if not all, mushrooms have biologically active polysaccharides in the fruiting body, culture broth, and cultured mycelia. Many studies have demonstrated that polysaccharides from mushrooms are able to boost the host immune system by stimulating natural killer cells, T-cells, B-cells, and macrophages (Hou et al., 2009; Kim et al., 2008). In a previous study, we identified a novel exo-polysaccharide in the liquid culture broth of *Cordyceps militaris* (PLCM) (Lee et al., 2010b). We also showed that PLCM could induce NO production in the murine macrophage-like cell line RAW264.7.

In the present study, we examined whether PLCM could induce or enhance other immunostimulatory activities in macrophages and attempted to identify the mechanisms underlying macrophage activation by PLCM.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), penicillin G, streptomycin, and RPMI 1640 were purchased from GIBCO (Grand Island, NY,

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http://dx.doi.org/10.1016/j.carbpol.2014.11.059
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USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopropyl alcohol, lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), Polymyxin B(PMB), and fluorescein isothiocyanate (FITC)-labeled dextran were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dichlorodihydrofluorescein diacetate (H_2DCFDA), fluorescein-labeled *E. coli* (FITC-*E. coli*) bioparticles and rhodamine phalloidin were purchased from Molecular Probes (Carlsbad, CA, USA). The cell-permeable inhibitor peptide NF-κB SN50, BAY11-7082, PD98059, SP600125, and SB203580 were purchased from Calbiochem (La Jolla, CA, USA). Anti-β actin, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-NF-κB-p65 antibodies, Dectin-1-specific mAb 2A11, TLR2-specific mAb T2.5, TLR4-specific mAb MTS510 and CD11b mAb were obtained from Cell Signaling (Beverly, MA, USA), Serotec (Kidlington, Oxford, UK), HyCult Biotechnology (Uden, The Netherlands), and BD Bioscience (San Diego, CA, USA), respectively. All other chemicals were analytical grade.

2.2. Preparation of polysaccharide from the culture broth of *C. militaris*

The exo-polysaccharide from *C. militaris* liquid culture was isolated using the methods described previously (Lee et al., 2010a). Briefly, *C. militaris* KCTC 6064 was cultivated for 11 days at 24 °C, 200 rpm, uncontrolled pH, and a 2% (v/v) inoculum size in a modified medium containing 80 g/l glucose, 10 g/l yeast extract, 0.5 g/l $MgSO_4{\cdot}7H_2O$, and 0.5 g/l KH_2PO_4. After 11 days of cultivation, the culture broth was centrifuged at 5000 rpm for 20 min. Polysaccharides were precipitated from the liquid culture broth using 75% ethanol, centrifuged at 5000 rpm for 25 min, resuspended, dialyzed against distilled water for 5 days to remove low-molecular-weight compounds, and then freeze-dried. Polysaccharide fraction, termed "PLCM", derived from the liquid culture broth of *C. militaris*, was a 1,6-branched-glucogalactomannan that had a molecular weight of 36 kDa, the β-linkage configuration, and random coil conformation. Endotoxin levels in the polysaccharide fraction were below the limit of detection (0.0015 EU/ml) as assessed with an E-TOXATE kit (Sigma, St. Louis, MO, USA).

2.3. Cell culture

RAW264.7 cells were purchased from the Korean Cell Line Bank (KCLB, Korea). The cells were maintained in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS at 37 °C in an incubator with a humidified atmosphere and 5% CO_2.

2.4. Cell viability assay

The effect of PLCM on the viability of RAW264.7 cells was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the cleavage of the yellow tetrazolium salt to purple formazan crystals by metabolically active cells. Cells were pre-incubated in 12-well plates for 24 h at 37 °C in a 5% CO_2 incubator and then were incubated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml) for 24 h. After incubation, cells were washed with phosphate-buffered saline (PBS) to remove detached cells and then 50 μl of MTT stock solution (2 mg/ml) was added to each well, and the cells were incubated for another 2 h. After incubation, the medium was removed and isopropyl alcohol was added to solubilize the formazan salt. The amount of formazan salt was determined by measuring absorbance at 595 nm using an ELISA microplate reader (Bio-Rad, model 550).

2.5. Measurement of NO production

RAW264.7 cells (1×10^6 cells/well) were pre-incubated in 12-well plates for 24 h at 37 °C in 5% CO_2 incubator and then were incubated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml) for 24 h. Next, the culture supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) and were incubated at room temperature for 30 min. Sodium nitrite ($NaNO_2$) was used to generate a standard curve, and nitrite production was determined by measuring absorbance at 540 nm.

2.6. Measurement of ROS generation

Changes in fluorescence resulting from the oxidation of the fluorescent probe H_2DCFDA were used to evaluate the level of intracellular ROS. RAW264.7 cells (2×10^6 cells/well) were pre-incubated in 6-well plates for 24 h in a 37 °C, 5% CO_2 incubator. And then, cells were incubated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml) for 24 h. Next, the cells were incubated with 50 mM of the fluorescent probe H_2DCF-DA 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 intracellular ROS production, RAW 264.7 cells (2×10^6 cells/well) were seeded on coverslip-loaded 6-wells plate and stimulated with PLCM (200 μg/ml) or LPS (2.5 μg/ml) for 24 h. A H_2DCFDA solution was then added to the cells and the plates were incubated for 2 h at 37 °C. Images of the stained cells were captured using a fluorescence microscope (ECCIPSE 50, Nicon, Japan).

2.7. Determination of endotoxin contamination

RAW264.7 cells (1×10^6 cells/well) were pre-incubated in 12-well plates for 24 h in humidified 37 °C, 5% CO_2 incubator and then were incubated with PLCM (200 μg/ml) or LPS (2.5 μg/ml) in the presence or absence of PMB (50 μg/ml) for 24 h. After treatments, the amount of NO in the culture supernatants was measured using the Griess reagent, as described above. Intracellular ROS production was also measured using H_2DCFDA solution, as described above.

2.8. Determination of phagocytic uptake

The phagocytic activity of the macrophages was measured according to a method described previously (Won et al., 2011). Cells (2×10^6 cells/well) were pre-incubated in 6-well plates for 42 h in a humidified 37 °C, 5% CO_2 incubator and then were incubated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml) for 6 h. Finally, 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. The cells were then washed three times with cold PBS containing 1% human serum and 0.02% sodium azide and analyzed by the FACScan flow cytometry (Becton-Dickinson, San Jose, CA, USA). For image analysis of PLCM-mediated phagocytosis, RAW 264.7 cells (2×10^6 cells/well) were seeded on coverslip-loaded 6-well plates and stimulated with PLCM (200 μg/ml) or LPS (2.5 μg/ml) for 6 h. A solution of FITC-labeled *E. coli* was added to the cells, which were then incubated at 37 °C in a humidified incubator containing 5% CO_2 for 30 min. After fixation with a 3.7% paraformaldehyde solution, adherent cells were stained with rhodamine phalloidin (1:250 dilution), mounted under glass coverslips with Vectashield (Brunschwig Chemie, Amsterdam, The Netherlands), and photographed under a fluorescence microscope.

2.9. Determination of TNF-α secretion

RAW264.7 cells (1×10^6 cells/well) were pre-incubated in 12-well plates for 42 h at 37 °C in a humidified incubator at 5% CO_2 and then were incubated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml) for 6 h. Levels of TNF-α in the culture supernatants were then evaluated using an ELISA kit (Biosource International, Camarillo, CA, USA) according to the manufacturer's instructions.

2.10. Nuclear protein extraction

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

2.11. Western blot analysis

After various treatments, cells were washed with PBS and were lysed in a 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, and 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 and the protein content of the supernatant was measured using a Bio-Rad protein assay kit prior to analysis. The cytoplasmic or nuclear protein samples were loaded, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10–15% gels and were 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., Billerica, MA, USA).

2.12. Inhibition of MAPKs and NF-κB using specific inhibitors

To identify the signal transduction pathways that mediate the effects of PLCM on RAW264.7 cell activation, RAW264.7 cells were pre-treated with the p38 MAPK inhibitor SB203580 (25 μM), the ERK inhibitor PD98059 (25 μM), the JNK inhibitor SP600125 (25 μM), and the NF-κB inhibitors BAY11-7082 (10 μM) or SN50 (50 μM) for 2 h in RPMI 1640 medium supplemented with 0.5% FBS. This medium was then replaced with medium with or without LPS (2.5 μg/ml) or PLCM (200 μg/ml) for 6 h at 37 °C. Then, cells were harvested and analyzed for phagocytic uptake by FACScan flow cytometry, as described above. TNF-α was measured in the culture supernatants using an ELISA kit, as described above.

2.13. Inhibition of cytokine production using anti-PRR antibodies

To investigate the role of TLR-2, TLR-4, Dectin-1, and CR3 in cytokine production, RAW264.7 macrophages in 12-well plate were pre-incubated with medium containing the TLR-2-specific mAb T2.5, the TLR-4-specific mAb MTS510, the Dectin-1-specific mAb2A11 or a 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. Next, cells were treated with LPS (2.5 μg/ml) or PLCM (200 μg/ml) for 6 h at 37 °C. Then, levels of TNF-α in the culture supernatants were measured using an ELISA kit, as described above.

2.14. Statistical analysis

Data are expressed as means ± standard error (SE), and the results were taken from at least three independent experiments performed in triplicate. Student's *t*-test was used to determine the statistical significance of the differences between the values determined for the various experimental and control groups. A * $p < 0.05$ was considered statistically significant.

3. Results

3.1. PLCM induces the production of NO and ROS in macrophages

To investigate the effect of PLCM on the viability of RAW.264.7 macrophages, cells were treated with different concentrations of PLCM (10–1000 μg/ml) for 24 h, and then cell viability was determined using the MTT assay. PLCM at concentrations of 10–200 μg/ml did not affect cell viability significantly (Fig. 1A). To investigate whether PLCM was capable of inducing NO production from macrophages, RAW264.7 cells were treated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml, positive control) for 24 h. PLCM induced NO production in RAW264.7 cells in a dose-dependent manner. In particular, cells treated with PLCM (200–1000 μg/ml) showed significantly higher (1.6-fold increase) NO production than that in the control (Fig. 1B). To investigate whether PLCM could induce intracellular ROS generation in macrophages, RAW264.7 cells were incubated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml) for 24 h. Significantly higher ROS generation was observed in cells treated with PLCM or LPS than in untreated cells. The fluorescence intensity in cells treated with PLCM was significantly increased in a dose-dependent manner (Fig. 1C), which was confirmed under fluorescence microscopy (Fig. 1D). These data showed that PLCM-treated macrophages could induce generation of NO and ROS which are known to be fatal for invading pathogens. To ensure that the effects of PLCM were not due to endotoxin contamination, NO production and ROS generation were evaluated in RAW264.7 macrophages treated with PLCM with or without PMB. As shown in Fig. 2, generation of NO and ROS in PLCM-treated macrophages was not affected by the presence of PMB, but LPS-induced NO production and ROS generation were inhibited in the presence of PMB, indicating that macrophage activation by PLCM was not due to endotoxin contamination.

3.2. PLCM enhances phagocytic uptake capacity and TNF-α secretion in macrophages

To investigate whether PLCM was capable of enhancing the phagocytic uptake capacity of macrophages, RAW264.7 cells were incubated with PLCM (10–1000 μg/ml) and LPS (2.5 μg/ml) for 6 h and uptake of FITC-labeled dextran by PLCM-treated, LPS-treated and untreated cells was compared. As shown in Fig. 3A, the results of flow cytometry demonstrate that the uptake of FITC-labeled dextran was enhanced in cells treated with PLCM or LPS. In addition, fluorescence microscopy images showed greater phagocytic uptake of FITC-labeled *E. coli* in cells treated with PLCM (200 μg/ml) or LPS (2.5 μg/ml) than in untreated RAW264.7 cells (Fig. 3B). TNF-α is a cytokine produced mainly by activated macrophages that is exceedingly important for killing and degrading tumor cells. To investigate whether PLCM could induce TNF-α secretion in macrophages, RAW264.7 cells were incubated with PLCM (10–1000 μg/ml) or LPS (2.5 μg/ml) for 6 h. As shown in Fig. 3C, PLCM-treated cells secreted significantly TNF-α than untreated cells did. These data indicate that PLCM-treated macrophages can

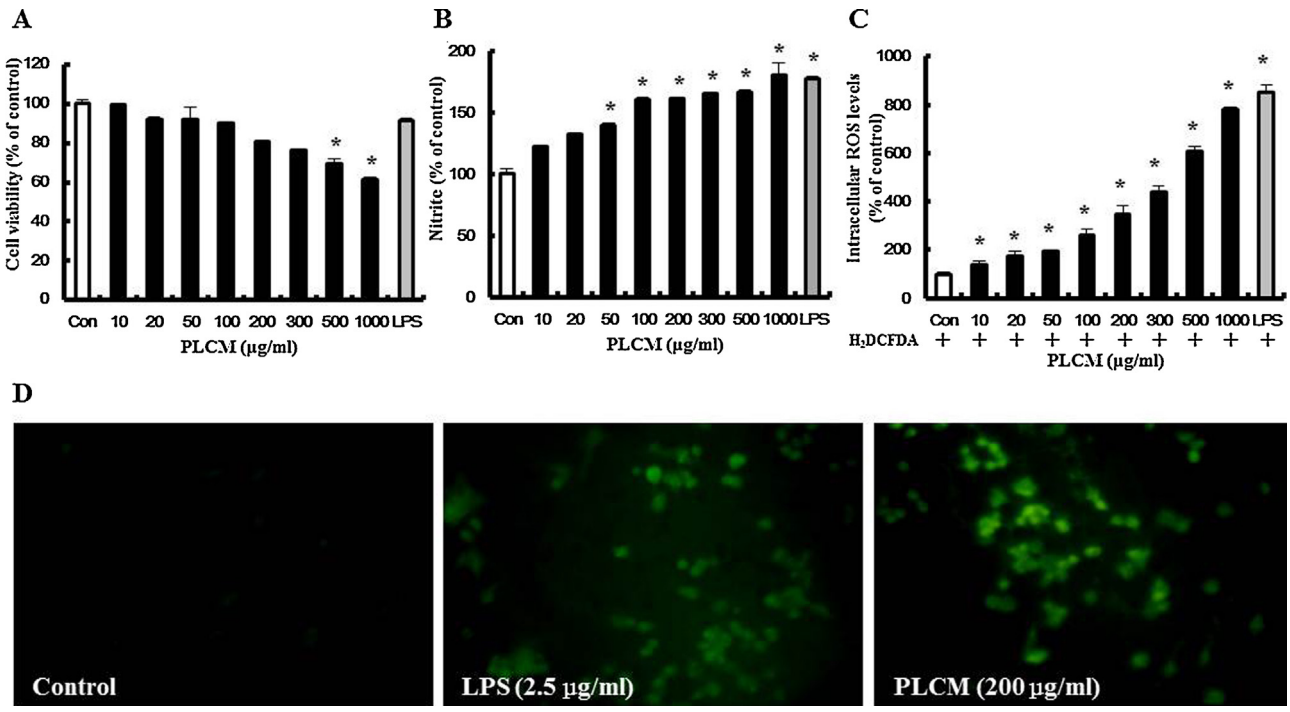

Fig. 1. Effect of PLCM on NO and ROS production in RAW264.7 macrophages. (A) Effect of PLCM on cell viability. (B) Effect of PLCM on NO production. (C) Effect of PLCM on intracellular ROS generation. (D) Fluorescence microscopic images of cells stained for ROS. Data (A, B and C) are expressed as the means ± SE. * Indicate significant difference compared to the control by Student's *t*-test ($p < 0.05$).

enhance phagocytic uptake capacity and TNF-α secretion, suggesting that this might be the mechanism by which PLCM acts to stimulate the killing and degradation of invading pathogens and tumor cells.

3.3. MAPK and NF-κB pathways participate in macrophage activation induced by PLCM

To investigate whether MAPK signaling pathways were involved in macrophage activation by PLCM, RAW264.7 cells were stimulated with PLCM (200 μg/ml) at the indicated times, and then western blot analysis was performed to detect the levels of phosphorylated ERK, SAPK/JNK and p38 MAPK (Fig. 4A). Increased phosphorylation of all three MAPKs was observed in cells treated with PLCM (200 μg/ml). To confirm that MAPK signaling pathways were involved in macrophage activation by PLCM, specific MAPK pathway inhibitors were used. As shown in Fig. 4B, phagocytic uptake induced by PLCM was suppressed by ERK (PD98059), JNK (SP600125) and p38 MAPK (SB203580) inhibitors. In particular, the p38 MAPK (SB 203580) inhibitor suppressed PLCM-induced phagocytic uptake by 40%. These results suggest that all three MAPKs participate in macrophage activation by PLCM.

To investigate whether the NF-κB signaling pathway was involved in macrophage activation by PLCM, RAW264.7 cells were stimulated with PLCM at the indicated times, and then effect of PLCM on the translocation of NF-κB from the cytosol to the nucleus was determined by western blot analysis using an anti-NF-κB subunit p65 antibody. As shown in Fig. 5A, the expression

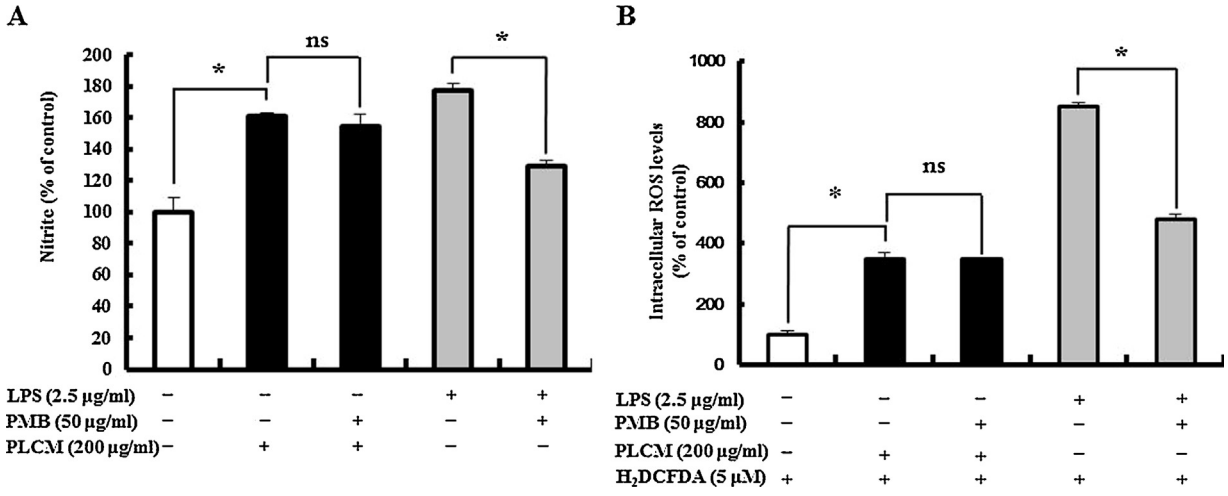

Fig. 2. Endotoxin contamination tests using PMB in PLCM-treated RAW264.7 macrophages. (A) Endotoxin contamination tests through PMB treatment on PLCM- or LPS-induced NO production. (B) Endotoxin contamination tests through PMB treatment on PLCM- or LPS-induced ROS generation. Data are expressed as the means ± SE. * Indicate significant difference compared to the control by Student's *t*-test ($p < 0.05$).

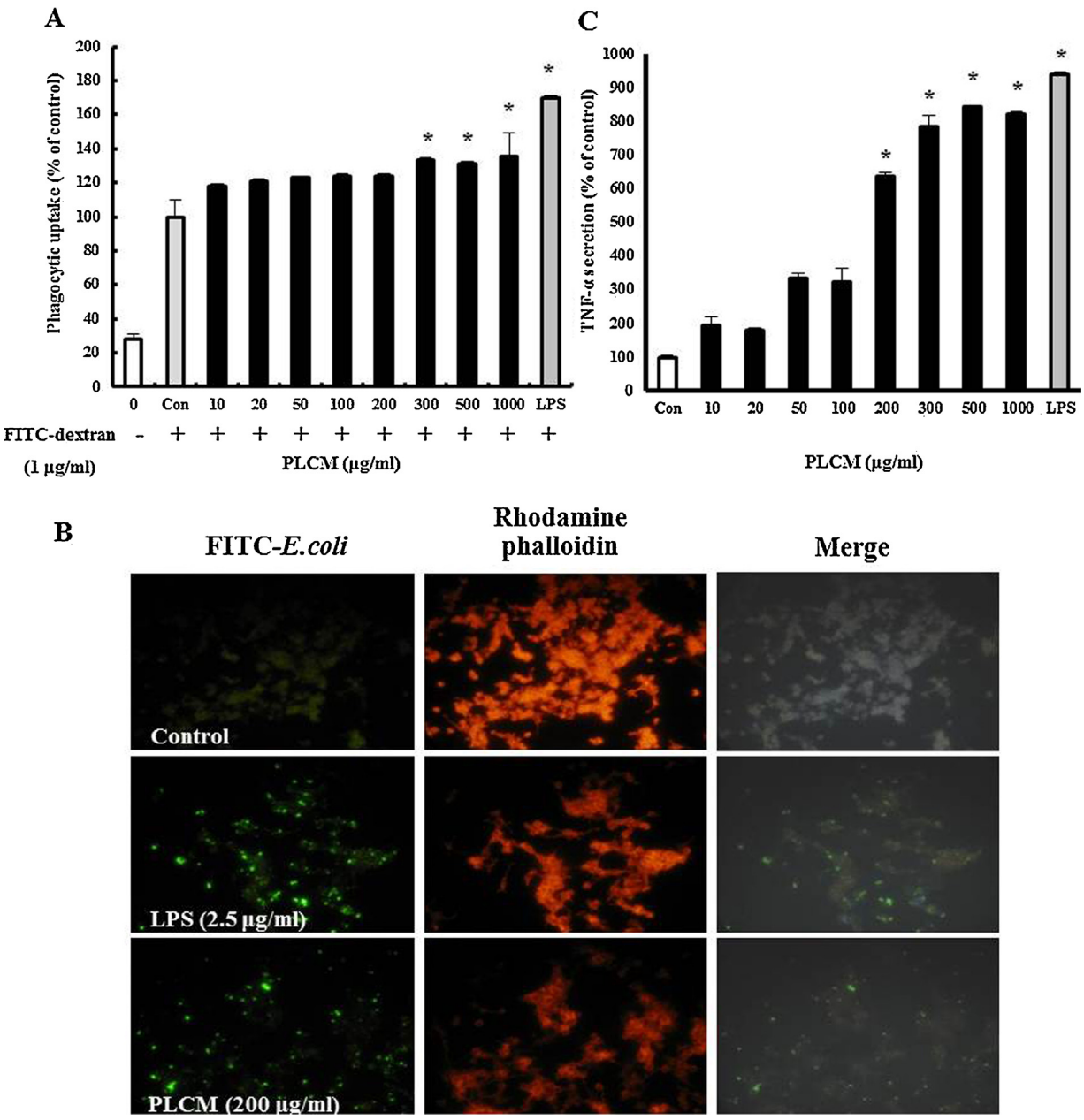

Fig. 3. Effect of PLCM on phagocytic uptake and TNF-α secretion in RAW264.7 macrophages. (A) Effect of PLCM on phagocytic uptake. (B) Fluorescence microscopic images of PLCM-induced phagocytosis. (C) Effect of PLCM on TNF-α secretion. Data are expressed as the means ± SE. * Indicate significant difference compared to the control by Student's *t*-test ($p < 0.05$).

level of nuclear p65, a key subunit of NF-κB, was highly increased after treatment for 15–30 min with PLCM. To confirm that the NF-κB pathway participated in macrophage activation by PLCM, RAW264.7 cells were treated with NF-κB inhibitors and then inhibition of phagocytic uptake was evaluated. The NF-κB inhibitors BAY11-7082 and SN50 attenuated the PLCM-induced enhancement in the phagocytic uptake by 40% and 18%, respectively (Fig. 5B). These results suggest that the translocation of NF-κB from the cytosol to the nucleus is involved in macrophage stimulation by PLCM.

To provide further evidence that the MAPK and NF-κB pathways are involved in macrophage activation by PLCM, several specific NF-κB and MAPKs inhibitors were added to macrophages treated with PLCM and the level of inhibition of TNF-α secretion was determined. As shown in Fig. 6, RAW264.7 cells treated with PLCM secreted high level of TNF-α, however all the inhibitors (BAY11-7082 and SN50 [NF-κB], PD98059 [ERK], SP600125 [JNK], and SB203580 [p38 MAPK]) suppressed the induction of TNF-α by PLCM. In particular, BAY11-7082, SP600125, and SB203580 significantly suppressed PLCM-induced TNF-α secretion.

3.4. Anti-PRR antibodies attenuated PLCM-induced TNF-α secretion

Although Dectin-1 is known to be a β-glucan specific receptor involved in the binding of polysaccharides to macrophages (Herre, Gordon & Brown, 2004), several PRRs such as TLR2/6 and CR3 are also reported to participate in the regulation of β-glucan-induced signaling, which leads to macrophage activation (Le Cabec, Carreno, Moisand, Bordier & Maridonneau-Parini, 2002). Therefore, we determined whether these molecules also were capable of acting as receptors for PLCM to modulate macrophage activation. For these experiments, specific antibodies against the extracellular

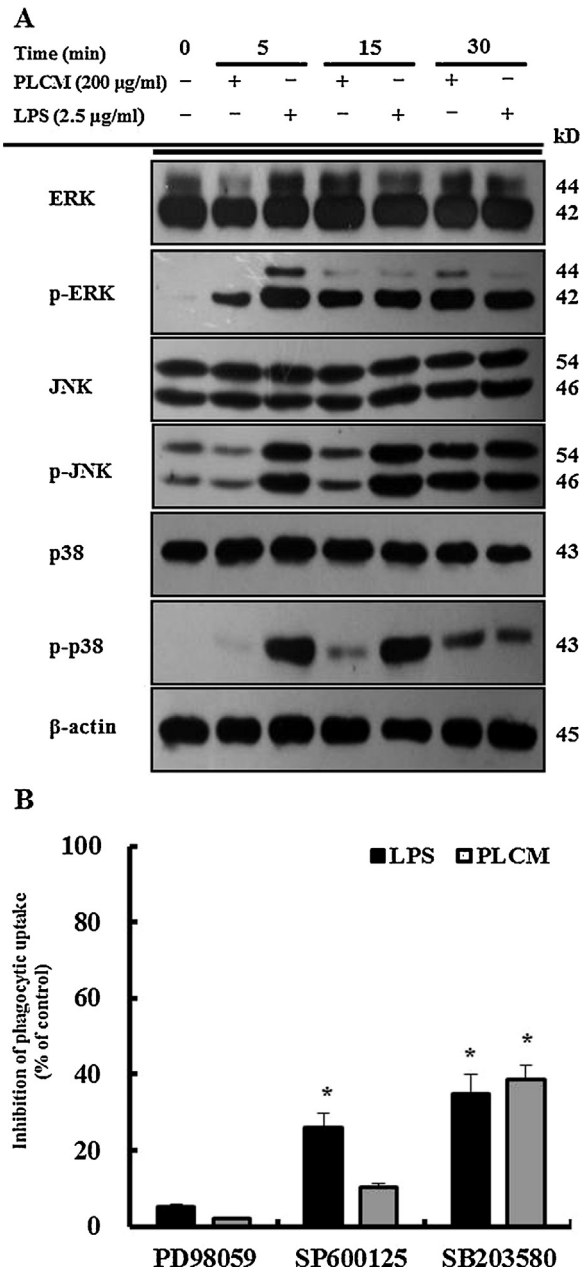

Fig. 4. Effect of PLCM on MAPK pathways in RAW264.7 macrophages. (A) Effect of PLCM on phosphorylation of MAPKs. (B) Effect of specific MAPK inhibitors on PLCM-induced or LPS-induced phagocytic uptake. Data are expressed as the means ± SE. * Indicate significant difference compared to the control by Student's *t*-test ($p < 0.05$).

domains of TLR-2, TLR-4, Dectin-1, or CR3 were used to block their activities. We found that these antibodies reduced PLCM-induced TNF-α production (Fig. 7). These data suggest that TLR-2, TLR-4, and Dectin-1 might be involved in PLCM-induced TNF-α secretion in macrophages.

4. Discussion

Immunostimulation is regarded as an important strategy for improving the body's defense mechanism in elderly people as well as in cancer patients (Rihova, Kovar, Kovar & Hovorka, 2009). Biological response modifiers isolated from mushrooms are being used increasingly to treat a wide variety of clinical conditions, albeit with relatively little knowledge of their modes of action (Yoshida et al., 2012). In particular, some of the major active substances in mushrooms are polysaccharides. Polysaccharides isolated from mushrooms can exert considerable effects on the innate and adaptive immune response and can therefore be used as immunostimulators, with wide clinical and medicinal applications (Hsiao et al., 2004). Many mushroom-derived polysaccharides are used as therapeutic and preventive agents for cancer, immune disorders, etc. (Fullerton et al., 2000). For example, polysaccharides isolated from *Lentinus edodes*, *Tricholoma matsutake*, *Hericium erinaceus* and *C. militaris* are well known to play important roles in immunostimulation. Of these, *C. militaris* is commonly used both as a food and as a treatment for various diseases. Studies have shown that polysaccharides isolated from *C. militaris* have potent immunostimulating activity, anti-cancer and anti-inflammation effects both *in vivo* and *in vitro* (Lee & Hong, 2011; Qian, Pan & Guo, 2012; Smiderle, Sassaki, Van Griensven & Iacomini, 2013). In our previous study, PLCM, which was a 1,6-branched-glucogalactomannan that had a molecular weight of 36 kDa, could induce NO production in the murine macrophage-like cell line RAW264.7 (Lee et al., 2010b). However, the signaling pathway through polysaccharides isolated from *C. militaris* activated immunocytes such as macrophages remain to be elucidated. In this study, therefore, the immunostimulatory effect of PLCM on macrophages and the mechanisms involved in macrophage activation by PLCM were investigated. Macrophage activation is accepted as one of the most important events in the immune response (Hino et al., 2005). During activation, macrophages initiate phagocytosis, the first step in the macrophage response against invading pathogens and microbes, and macrophage activation signifies the up-regulation of the innate immune response (Henneke & Golenbock, 2004). During this response, activated macrophages produce cytotoxic molecules and inflammatory cytokines such as NO, ROS, and TNF-α (Hess et al., 2009). In the present study, PLCM was shown to increase NO production, ROS generation and TNF-α secretion in RAW264.7 macrophages. PLCM also enhanced the phagocytic uptake capacity of RAW264.7 macrophages. Notably, PLCM-induced NO production was not affected by treatment with PMB and therefore was not due to the presence of endotoxins. LPS, a major macrophage activation factor, induces NF-κB activation and the phosphorylation of MAPKs such as ERK1/2, JNK, and p38 in macrophages. MAPKs are a family of serine/threonine-specific protein kinases that participate in the initiation of NF-κB activation following various extracellular stimuli such as LPS, pro-inflammatory cytokines, and polysaccharides (Ma et al., 2011; Ruimi et al., 2010). Phosphorylation of MAPKs can activate the transcription of several targets such as NF-κB and AP-1 or other transcription factors. Recent studies have reported that polysaccharides derived from *L. edodes*, *Ganoderma lucidum*, and *C. militaris* induce immunocyte activation through NF-kB activation and MAPK phosphorylation (Park, Kunieda & Kubo, 2003). Therefore, we attempted to demonstrate the PLCM-mediated activation of NF-κB and all three MAPK pathways during macrophage activation. All three MAPKs were found to be phosphorylated in RAW264.7 macrophages in response to PLCM treatment. Moreover, the blockade of their activities by specific inhibitors (PD98059, ERK inhibitor; SP600125, JNK inhibitor; and SB20358, p38 inhibitor) also suppressed both LPS- and PLCM-induced phagocytic uptake. The transcription factor NF-κB belongs to 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, and apoptosis. In resting cells, NF-κB exists in a latent form in the cytosol, in inactivated complexes formed through non-covalent interactions with IκB. In response to various stimuli, the IκB in the cytosolic complexes is phosphorylated, which causes the release of NF-κB and its translocation into the nucleus, where it activates gene expression. In this study, the levels of cytosolic NF-κB were decreased, whereas the levels of nuclear NF-κB increased

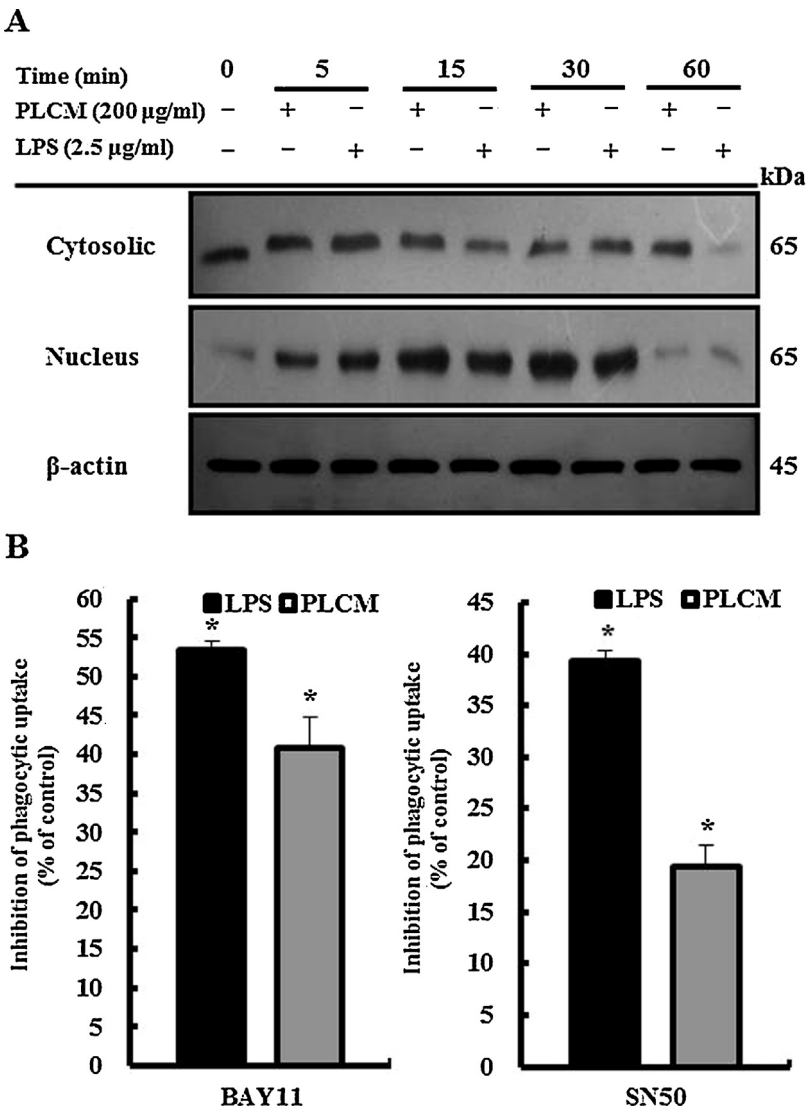

Fig. 5. The role of NF-κB in mediating PLCM activity in RAW264.7 macrophages. (A) Effect of PLCM on the translocation of NF-κB. (B) Effect of specific IκB and NF-κB inhibitors on PLCM-induced or LPS-induced phagocytic functions. Data are expressed as the means ± SE. * Indicate significant difference compared to the control by Student's *t*-test ($p < 0.05$).

in LPS- or PLCM-treated RAW264.7 macrophages. Moreover, treatment with the NF-κB inhibitors SN50 and BAY11-7082 effectively suppressed both LPS- and PLCM-induced phagocytic uptake and TNF-α secretion. Although the major receptor for polysaccharides in fungal immunity is known to be Dectin-1, several PRRs such as TLR-2, TLR-4, and CR3 (CD11b) are also known to participate in the response to plant-derived polysaccharides. Bacterial LPS, the major structural component of the outer wall of gram-negative bacteria, is a potent activator of macrophages (Inagawa et al., 1992). The role of TLR4 in LPS-mediated signaling has also been studied (Hsu et al., 2011; Ikebe et al., 2009). In this study, it was shown that TLR-2, TLR-4, and Dectin-1 are all involved in PLCM-induced TNF-α secretion, which is analogous to the results of our previous study on PLCM. Even though many studies illustrate the distinct biological properties associated with polysaccharide immunomodulators, the lack of defined structural and mechanistic information has limited efforts to study their potential for clinical use (Tzianabos, 2000). Polysaccharides, polymers of monosaccharide residues joined to each other by glycosidic linkages, belong to a structurally diverse class of macromolecules. Because they have the greatest potential for structural variability relative to other biopolymers, polysaccharides have the highest capacity for carrying biological information. As a result of this phenomenon, it is highly important to determine the accurate structures of polysaccharides. Polysaccharides differ greatly in their chemical composition, molecular weight, conformation, glycosidic linkage, degree of branching, etc. (Methacanon, Madla, Kirtikara, & Prasitsil, 2005; Yadomae & Ohno, 1996). Recent investigations have led to a more detailed understanding of structural aspects of polysaccharides that govern biological functions and regulation of cytokine networks that influence host immune responses (Nergard et al., 2005). β-glucans, the most abundant forms of polysaccharides, are one of the active ingredients responsible for the immunomodulation. It was reported that β-glucans of different sizes and branching patterns may have significantly variable immune potency (Chan, Chan, & Sze, 2009). In this study, PLCM, with immunostimulant properties, was a 1,6-branched-glucogalactomannan that had a molecular weight of 36 kDa, the β-linkage configuration, and random coil conformation (Lee et al., 2010b). There are some data suggesting that polysaccharides with low-molecular weight and β-type polymers with no triple-helical conformation were found to exhibit biological activity (Yu et al., 2009, 2007; Kraus, Blaschek,

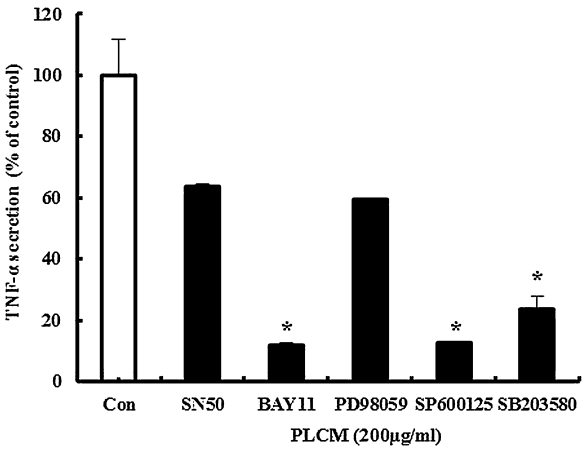

Fig. 6. Effect of specific signaling pathway inhibitors on PLCM- or LPS-induced TNF-α secretion in RAW264.7 macrophages. Data are expressed as the means ± SE. * Indicate significant difference compared to the control by Student's *t*-test ($p < 0.05$).

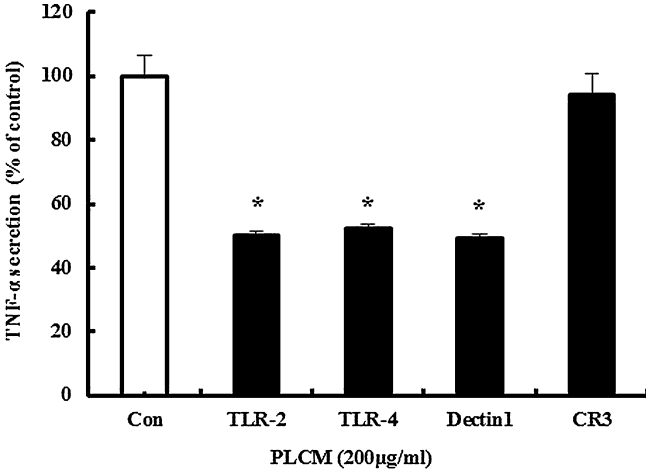

Fig. 7. Effect of anti-PRR antibodies on PLCM-mediated TNF-α secretion in RAW264.7 macrophages. Data are expressed as the means ± SE. * Indicate significant difference compared to the control by Student's *t*-test ($p < 0.05$).

Schutz, & Franz, 1992). However, to understand of structural aspects of PLCM that influence immunostimulating activity, particular structure-immunomodulating activity relationships of PLCM needed to be further investigated. In summary, our results present evidence that PLCM has potent immunostimulatory activity *in vitro* and suggest that PLCM induces up-regulation of NO, ROS, TNF-α production and enhanced phagocytic uptake of macrophages through MAPK and NF-κB signaling pathways *via* several PRRs such as TLR-2, TLR-4, and Detin-1.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2011-0011522 and NRF-2013R1A6A3A01064375).

References

Biswas, S. K., & Sodhi, A. (2002). In vitro activation of murine peritoneal macrophages by monocyte chemoattractant protein-1: Upregulation of CD11b, production of proinflammatory cytokines, and the signal transduction pathway. *Journal of Interferon & Cytokine Research*, *22*(5), 527–538 (the official journal of the International Society for Interferon and Cytokine Research).

Bobek, P., Ginter, E., Kuniak, L., Babala, J., Jurcovicova, M., Ozdin, L., et al. (1991). Effect of mushroom *Pleurotus ostreatus* and isolated fungal polysaccharide on serum and liver lipids in Syrian hamsters with hyperlipoproteinemia. *Nutrition*, *7*(2), 105–108.

Chan, G. C., Chan, W. K., & Sze, D. M. (2009). The effects of beta-glucan on human immune and cancer cells. *Journal of Hematology & Oncology*, *2*, 25–35.

Dentan, C., Lesnik, P., Chapman, M. J., & Ninio, E. (1996). Phagocytic activation induces formation of platelet-activating factor in human monocyte-derived macrophages and in macrophage-derived foam cells. Relevance to the inflammatory reaction in atherogenesis. *European Journal of Biochemistry/FEBS*, *236*(1), 48–55.

Fullerton, S. A., Samadi, A. A., Tortorelis, D. G., Choudhury, M. S., Mallouh, C., Tazaki, H., et al. (2000). Induction of apoptosis in human prostatic cancer cells with beta-glucan (Maitake mushroom polysaccharide). *Molecular Urology*, *4*(1), 7–13.

Grimes, G. R., Moodie, S., Beattie, J. S., Craigon, M., Dickinson, P., Forster, T., et al. (2005). GPX-Macrophage Expression Atlas: A database for expression profiles of macrophages challenged with a variety of pro-inflammatory, anti-inflammatory, benign and pathogen insults. *BMC Genomics*, *6*, 178.

Henneke, P., & Golenbock, D. T. (2004). Phagocytosis, innate immunity, and host-pathogen specificity. *The Journal of Experimental Medicine*, *199*(1), 1–4.

Herre, J., Gordon, S., & Brown, G. D. (2004). Dectin-1 and its role in the recognition of beta-glucans by macrophages. *Molecular Immunology*, *40*(12), 869–876.

Hess, D. J., Henry-Stanley, M. J., Bendel, C. M., Zhang, B., Johnson, M. A., & Wells, C. L. (2009). *Escherichia coli* and TNF-alpha modulate macrophage phagocytosis of *Candida glabrata*. *The Journal of Surgical Research*, *155*(2), 217–224.

Hino, M., Kohchi, C., Nishizawa, T., Yoshida, A., Nakata, K., Inagawa, H., et al. (2005). Innate-immune therapy for lung carcinoma based on tissue-macrophage activation with lipopolysaccharide. *Anticancer Research*, *25*(6A), 3747L 3754.

Hou, J. M., Zhao, X., Tian, L., Li, G., Zhang, R., Yao, B., et al. (2009). Immunotherapy of tumors with recombinant adenovirus encoding macrophage inflammatory protein 3beta induces tumor-specific immune response in immunocompetent tumor-bearing mice. *Acta Pharmacologica Sinica*, *30*(3), 355–363.

Hsiao, W. L., Li, Y. Q., Lee, T. L., Li, N., You, M. M., & Chang, S. T. (2004). Medicinal mushroom extracts inhibit ras-induced cell transformation and the inhibitory effect requires the presence of normal cells. *Carcinogenesis*, *25*(7), 1177–1183.

Hsu, R. Y., Chan, C. H., Spicer, J. D., Rousseau, M. C., Giannias, B., Rousseau, S., et al. (2011). LPS-induced TLR4 signaling in human colorectal cancer cells increases beta1 integrin-mediated cell adhesion and liver metastasis. *Cancer Research*, *71*(5), 1989–1998.

Ikebe, M., Kitaura, Y., Nakamura, M., Tanaka, H., Yamasaki, A., Nagai, S., et al. (2009). Lipopolysaccharide (LPS) increases the invasive ability of pancreatic cancer cells through the TLR4/MyD88 signaling pathway. *Journal of Surgical Oncology*, *100*(8), 725–731.

Inagawa, H., Nishizawa, T., Tsukioka, D., Suda, T., Chiba, Y., Okutomi, T., et al. (1992). Homeostasis as regulated by activated macrophage. II. LPS of plant origin other than wheat flour and their concomitant bacteria. *Chemical & Pharmaceutical Bulletin*, *40*(4), 994–997.

Joseph, J., Panicker, S. N., & Janardhanan, K. K. (2012). Protective effect of polysaccharide–protein complex from a polypore mushroom, *Phellinus rimosus* against radiation-induced oxidative stress. *Redox Report: Communications in Free Radical Research*, *17*(1), 22–27.

Kim, J. Y., Byeon, S. E., Lee, Y. G., Lee, J. Y., Park, J., Hong, E. K., et al. (2008). Immunostimulatory activities of polysaccharides from liquid culture of pine-mushroom *Tricholoma matsutake*. *Journal of Microbiology and Biotechnology*, *18*(1), 95–103.

Kraus, J., Blaschek, W., Schutz, M., & Franz, G. (1992). Antitumor activity of cell wall β-1,3/1,6-glucans from *Phytophthora* spp. *Planta Medica*, *58*, 39–42.

Kuan, Y. H., Huang, F. M., Li, Y. C., & Chang, Y. C. (2012). Proinflammatory activation of macrophages by bisphenol A-glycidyl-methacrylate involved NFkappaB activation via PI3K/Akt pathway. *Food and Chemical Toxicology*, *50*(11), 4003–4009 (an International Journal Published for the British Industrial Biological Research Association).

Le Cabec, V., Carreno, S., Moisand, A., Bordier, C., & Maridonneau-Parini, I. (2002). Complement receptor 3 (CD11b/CD18) mediates type I and type II phagocytosis during nonopsonic and opsonic phagocytosis, respectively. *Journal of Immunology*, *169*(4), 2003–2009.

Lee, J. S., & Hong, E. K. (2011). Immunostimulating activity of the polysaccharides isolated from *Cordyceps militaris*. *International Immunopharmacology*, *11*(9), 1226–1233.

Lee, J. S., Kwon, J. S., Won, D. P., Lee, J. H., Lee, K. E., Lee, S. Y., et al. (2010). Study of macrophage activation and structural characteristics of purified polysaccharide from the fruiting body of *Cordyceps militaris*. *Journal of Microbiology and Biotechnology*, *20*(7), 1053–1060.

Lee, J. S., Kwon, J. S., Won, D. P., Lee, K. E., Shin, W. C., & Hong, E. K. (2010). Study on macrophage activation and structural characteristics of purified polysaccharide from the liquid culture broth of *Cordyceps militaris*. *Carbohydrate Polymers*, *82*(3), 982–988.

Ma, P., Liu, H. T., Wei, P., Xu, Q. S., Bai, X. F., Du, Y. G., et al. (2011). Chitosan oligosaccharides inhibit LPS-induced over-expression of IL-6 and TNF-alpha in RAW264.7 macrophage cells through blockade of mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways. *Carbohydrate Polymers*, *84*(4), 1391–1398.

Methacanon, P., Madla, S., Kirtikara, K., & Prasitsil, M. (2005). Structural elucidation of bioactive fungi-derived polymers. *Carbohydrate Polymers*, *60*, 199–203.

Nergard, C. S., Kiyohara, H., Reynolds, J. C., Thomas-Oates, J. E., Matsumoto, T., Yamada, H., et al. (2005). Structure-immunomodulating activity relationships of a pectic arabinogalactan from *Vernonia kotschyana* Sch. Bip. ex Walp. *Carbohydrate Research*, *340*, 1789–1801.

Park, J. M., Kunieda, T., & Kubo, T. (2003). The activity of Mblk-1, a mushroom body-selective transcription factor from the honeybee, is modulated by the ras/MAPK pathway. *The Journal of Biological Chemistry*, *278*(20), 18689–18694.

Qian, G. M., Pan, G. F., & Guo, J. Y. (2012). Anti-inflammatory and antinociceptive effects of cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis*. *Natural Product Research*, *26*(24), 2358–2362.

Rihova, B., Kovar, L., Kovar, M., & Hovorka, O. (2009). Cytotoxicity and immunostimulation: Double attack on cancer cells with polymeric therapeutics. *Trends in Biotechnology*, *27*(1), 11–17.

Ruimi, N., Rwashdeh, H., Wasser, S., Konkimalla, B., Efferth, T., Borgatti, M., et al. (2010). *Daedalea gibbosa* substances inhibit LPS-induced expression of NOS by suppression of NF-kappa B and MAPK activities in RAW 264.7 macrophage cells. *International Journal of Molecular Medicine*, *25*(3), 421–432.

Selegean, M., Putz, M. V., & Rugea, T. (2009). Effect of the polysaccharide extract from the edible mushroom *Pleurotus ostreatus* against infectious bursal disease virus. *International Journal of Molecular Sciences*, *10*(8), 3616–3634.

Smiderle, F. R., Sassaki, G. L., Van Griensven, L. J., & Iacomini, M. (2013). Isolation and chemical characterization of a glucogalactomannan of the medicinal mushroom *Cordyceps militaris*. *Carbohydrate Polymers*, *97*(1), 74–80.

Tzianabos, A. O. (2000). Polysaccharide immunomodulators as therapeutic agents: Structural aspects and biologic function. *Clinical Microbiology Reviews*, *13*(4), 523–533.

Won, D. P., Lee, J. S., Kwon, D. S., Lee, K. E., Shin, W. C., & Hong, E. K. (2011). Immunostimulating activity by polysaccharides isolated from fruiting body of *Inonotus obliquus*. *Molecules and Cells*, *31*(2), 165–173.

Wu, T. T., Chen, T. L., & Chen, R. M. (2009). Lipopolysaccharide triggers macrophage activation of inflammatory cytokine expression, chemotaxis, phagocytosis, and oxidative ability via a toll-like receptor 4-dependent pathway: Validated by RNA interference. *Toxicology Letters*, *191*(2–3), 195–202.

Yadomae, T., & Ohno, N. (1996). Structure-activity relationship of immunomodulating (1-3)-β-D-glucans. *Recent Research Developments in Chemical & Pharmaceutical Sciences*, *1*, 23–33.

Yoon, W. J., Kim, S. S., Oh, T. H., Lee, N. H., & Hyun, C. G. (2009). *Abies koreana* essential oil inhibits drug-resistant skin pathogen growth and LPS-induced inflammatory effects of murine macrophage. *Lipids*, *44*(5), 471–476.

Yoshida, M., Hida, T. H., Takeshita, K., Tsuboi, M., Kanamori, M., Akachi, N., et al. (2012). Effect of *Sasa veitchii* extract on immunostimulating activity of beta-glucan (SCG) from culinary-medicinal mushroom *Sparassis crispa* Wulf.: Fr. (higher Basidiomycetes). *International Journal of Medicinal Mushrooms*, *14*(6), 537–547.

Yu, R. M., Yang, W., Song, L., Yan, C., Zhang, Z., & Zhao, Y. (2007). Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps militaris*. *Carbohydrate Polymers*, *70*, 430–436.

Yu, R. M., Yin, Y., Yang, W., Ma, W., Yang, L., Chen, X., et al. (2009). Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*. *Carbohydrate Polymers*, *75*, 166–171.