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Phagocytic Effects of β-Glucans from the Mushroom *Coriolus versicolor* are Related to Dectin-1, NOS, TNF-α Signaling in Macrophages

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Abstract

The mushroom *Coriolus versicolor* contains biologically active polysaccharides, most of which belong to the β-glucan group. Diverse physicochemical properties, due to different sources and isolated types of β-glucans, can induce distinct biological activities. We investigated the effects of β-glucans from *C. versicolor* on phagocytic activity, nitric oxide (NO), TNF-α production, and signaling of dectin-1, a well-known β-glucan receptor, in macrophages. β-Glucans increased phagocytic activity and TNF-α and NO-iNOS/eNOS production. Laminarin, a specific inhibitor of dectin-1, showed strong inhibitory effects on phagocytosis and subsequent TNF-α, iNOS, and eNOS production increased by β-glucans, indicating that β-glucans reacts with dectin-1 receptors. We examined whether the aforementioned cytokines were involved in the signaling pathway from the dectin-1 receptor to phagocytosis, and found that the inhibition of iNOS, eNOS, and TNF-α receptors significantly decreased β-glucan-induced phagocytosis. In conclusion, our study showed that dectin-1 signaling, triggered by β-glucans, subsequently elicited TNF-α and NO-iNOS/eNOS production, and that these molecules seem to act as secondary molecules that cause eventual phagocytosis by macrophages. These findings suggest that *C. versicolor* could be used as a nutritional medicine that may be useful in the treatment of infectious disease.

Key Words: β-glucans, *Coriolus versicolor*, Dectin-1, Macrophage, NO, TNF-α

INTRODUCTION

Coriolus versicolor (known as "Yun Zhi"), a mushroom fungus of the *Basidiomycetes* family, has been used in Asia for the prevention of infectious diseases and in the United States as a dietary supplement for surgery, chemotherapy, radiation therapy, and rehabilitation (Kidd, 2000; Ho *et al.*, 2005). These mushrooms contain biologically active polysaccharides, most of which belong to the β-glucan group. β-Glucans are naturally occurring (1→3)-β-D-linked polymer glucoses that are found in the cell walls of certain pathogenic bacteria, fungi, mushrooms, algae, and cereal grains (Williams *et al.*, 1992; Muller *et al.*, 1996) Different sources and types of β-glucans result in diverse physicochemical properties, such as solubility, primary structure, molecular weight, and branching. Interestingly, these variables in the β-glucan group can induce distinct biological activities, depending on their origins (Yadomae, 2000) such as antitumor effects (Ross, 2000) and anti-infective properties against bacterial (Kokoshis *et al.*, 1978), viral (Itoh *et al.*, 1990), fungal (Williams *et al.*, 1978), and protozoa (Cook *et al.*, 1982) infections. Generally, biological activities induced by β-glucans have indicated the presence of immunomodulatory properties via stimulating effects on innate immune cells, including macrophages, neutrophils, and natural killer cells, and on the production of cytokines (Di Luzio *et al.*, 1979; Imura *et al.*, 1985).

The macrophage is considered to be an important component of the innate immune response against bacterial infection and cancer (Hahn and Kaufmann, 1981; Verstovsek *et al.*, 1992). During development into the next activated stage, the macrophage undergoes induction of phagocytic activity and an increase in the secretion of various materials, such as cytokines and nitric oxide (NO), which bring about nonspecific immune responses (Adams and hamilton, 1984; Nathan, 1987). Phagocytosis plays a critical role in innate immunity by facilitating the removal and killing of pathogens and by

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priming the adaptive immune response (Janeway and Medzhitov, 2002; Uthaisangsook *et al.*, 2002). The phagocytic process is initiated by the cross-linking of so-called pattern recognition receptors (PRRs), an array of dedicated surface receptors that are capable of innately recognizing non-self structures, such as pathogen-associated molecular patterns (PAMPs). Thus, β-glucans probably act like PAMPs, and subsequent recognition by appropriate cell surface receptors, such as PRRs, initiates immune responses. Until now, identified β-glucan receptor candidates acting as PRRs include dectin-1, complement receptor 3 (CR3; CD11b/CD18), scavenger receptors (SRs), lactosylceramide (LacCer), and Toll-like receptors (TLRs). Among these, dectin-1 has emerged as the major receptor mediating β-glucan activity in leukocytes, especially macrophages (Brown *et al.*, 2002; Willment *et al.*, 2005) and dendritic cells (Gantner *et al.*, 2003, Rogers *et al.*, 2005). Activated macrophages can engulf pathogens through phagocytosis and digest them with lysosomal enzymes. In this process, dectin-1 plays an important role in fungal recognition by macrophages. Genetic studies recently demonstrated that mice deficient in dectin-1 are more susceptible to infection by *Candida albicans* (Taylor *et al.*, 2002) and *Pneumocystis carinii* (Saijo *et al.*, 2007).

Our previous studies showed comparable significant effects on phagocytosis and NO production in RAW264.7 macrophages by β-glucans from yeast, bacteria, and mushrooms, including *C. versicolor*, *in vitro*. Among them, β-glucans from *C. versicolor* showed a highly significant effect on phagocytosis (Jang *et al.*, 2009). Thus, we further studied whether the effect of β-glucans from *C. versicolor* on phagocytosis is mediated via the dectin-1 receptor and whether this phagocytic activity correlates with the production of cytokines in macrophages.

MATERIALS AND METHODS

Preparation of β-glucans from mushrooms

Mushroom β-glucans isolated from *C. versicolor* were used. The process of isolating and purifying a water-soluble glucan from *C. versicolor* was achieved by hot water extraction, filtration, solvent precipitation, dialysis, and freeze-drying. Acidic fractions of the polysaccharide were separated from crude polysaccharides by DEAE-cellulose anion exchange chromatography at 0.7 M NaCl. The molecular weight of the proteoheteroglycan after Sepharose CL-4B gel filtration chromatography was approximately 750 kDa. These products have been shown to possess an 85% purity level.

Chemicals

Unless stated otherwise, all chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). The XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] cell viability assay kit was purchased from WelGENE (Daegu, South Korea). Antibodies against TNF-α, iNOS and eNOS were purchased from BD Transduction Laboratories (Lexington, KY). All tissue culture reagents and α-glucans were assayed for endotoxin contamination using the E-TOXATE Limulus lysate test (Sigma), and the endotoxin levels were found to be <10 pg/ml.

Cell culture

Primary peritoneal macrophages isolated from male C57BL/6 mice were collected 4 days after an intraperitoneal injection of 1 ml of 24 mg/ml solution thioglycollate. The mouse monocyte/macrophage cell line RAW264.7 was maintained in RPMI 1640 medium (Cambrex, Walkersville, MD), supplemented with 10% FBS and 2% penicillin/streptomycin (Cambrex), and incubated at 37°C in 5% CO_2.

Nitrite determination

The cells were treated with various doses of β-glucans for 24 h, and the accumulation of nitrite in culture supernatants was measured using the assay system described by Ding *et al.* (1988). Aliquots (100 μl) of culture supernatants were mixed with an equal volume of Griess reagent (mixture of 1:1 N-(1-naphthyl)ethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4) and incubated at room temperature for 10 min. The nitrite concentration was calculated from an $NaNO_2$ standard curve.

NBT assay for phagocytosis

Phagocytosis was measured using the nitro blue tetrazolium (NBT) reduction assay (Okimura *et al.*, 1986). Peritoneal macrophages were seeded in 96-well plates at a density of 5×10^4 cells per well, treated with various concentrations of β-glucans, and cultured for 24 h. The cultured media was then removed, and 50 μl of 5×10^6 particles/ml zymosan and 0.6 mg/ml NBT was added to each well. After an additional 1-h incubation, wells were washed twice with cold D-PBS, and the optical density of the reduction product of NBT, a purple insoluble formazan, was determined at 540 nm using a microplate reader. Solubilization of the formazan before measurement of absorbance was not required.

RNA preparation and mRNA analysis by RT-PCR

Total RAW264.7 cells were plated at 1×10^6 cells/ml and treated with β-glucans (1, 10, and 100 μg/ml) for 16 h. Total RNA from the treated cells was prepared with the Trizol reagent (Invitrogen) according to the manufacturer's protocol and stored at −80°C until use. Total RNA was extracted after stimulation and treatment. The sequences of the primers used in this study were iNOS forward: 5'-CAG CTG GGC TGT ACA AAC CTT3'; iNOS reverse: 5'-TGA ATG TGA TGT TTG CTT CGG-3'; GAPDH forward: 5'-GTG GCA AAG TGG AGA TTG TTG CC-3'; and GAPDH reverse: 5'-GAT GAT GAC CCG TTT GGC TCC-3'. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of GAPDH, a housekeeping gene.

Western blot analysis

The amount of iNOS, eNOS and TNF-α were measured by Western blot analysis. Cells in six-well plates were washed by D-PBS and lysed with homogenization buffer (50 mM Tris-HCl, pH6.8, 10% glycerol, 2% SDS, 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Each sample (20 μg) was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with 5% skim milk in Tris-buffered saline/non-fat Tween for 1 h. The membranes were incubated with primary antibody

against iNOS for 24 h. They were then washed with Tris-buffered saline/non-fat Tween once for 15 min and three times for 5 min each, and incubated with secondary ALPconjugated anti-rabbit antibody for 1 h. The membranes were washed again as described above. Autoradiography was performed using an enhanced chemiluminescence kit (Amersham Bioscience).

Statistical analysis

Each experiment was performed in triplicate, and the results of one representative experiment are shown. The results were expressed as means ± SEM and analyzed with Student's *t*-test. Statistically significant values are indicated by an asterisk (*p<0.05, #p<0.05, **p<0.01, ##p<0.01).

RESULTS

The effects of β-glucans on phagocytosis and the production of TNF-α and NO

Macrophages are responsible for a diverse range of antimicrobial and cytotoxic activities by means of respiratory bursts via phagocytosis and diverse secretion of inflammatory mediators including TNF-α and NO. In this study, we examined the effects of β-glucans from *C. versicolor* on phagocytosis and production of TNF-α and NO by peritoneal macrophages. The concentrations used had no effect on cell viability or proliferation (data not shown). As shown in Fig. 1, β-glucan-treated macrophages showed dose-dependent increases in phagocytic activity (Fig. 1A), production of TNF-α (Fig. 1B), and release of NO (Fig. 1C).

The effects of β-glucans on iNOS and eNOS expression

NO is related to the cytotoxic activity of macrophages against a variety of tumors and microorganisms, and it is mainly produced by constitutive NO synthase (cNOS) and inducible NO synthase (iNOS) (Kroncke *et al*., 1997). cNOS consists of neural NOS (nNOS) and endothelial NOS (eNOS). In particular, iNOS is known to produce high amounts of NO in macrophages activated by external stimuli (Keller and Keist, 1989; Bredt and Snyder, 1994). To determine the protein levels of NOS in peritoneal macrophages, eNOS and iNOS expressions were assessed by Western blots. Expression of both iNOS and eNOS in peritoneal macrophages was increased by β-glucans in a time-dependent manner (Fig. 2A). We also assessed eNOS protein expression by Western blots in RAW264.7 cells, and the mRNA level of iNOS by RT-PCR. Both eNOS protein expression and the iNOS mRNA level were increased (Fig. 2B).

Dectin-1 mediated phagocytic activity of macrophages by β-glucans

Addition of laminarin, a well-established dectin-1 inhibitor, can significantly block dectin-1 receptors and inhibit surface expression of dectin-1 (Brown *et al*., 2002; Gantner *et al*., 2003). Thus, we used laminarin to examine whether dectin-1 mediates the increased phagocytic activity by β-glucans. β-Glucan-induced phagocytic activity was inhibited by cotreatment with laminarin in peritoneal macrophages (Fig. 3A) and RAW264.7 cells (Fig. 3B), but laminarin itself did not induce phagocytic activity.

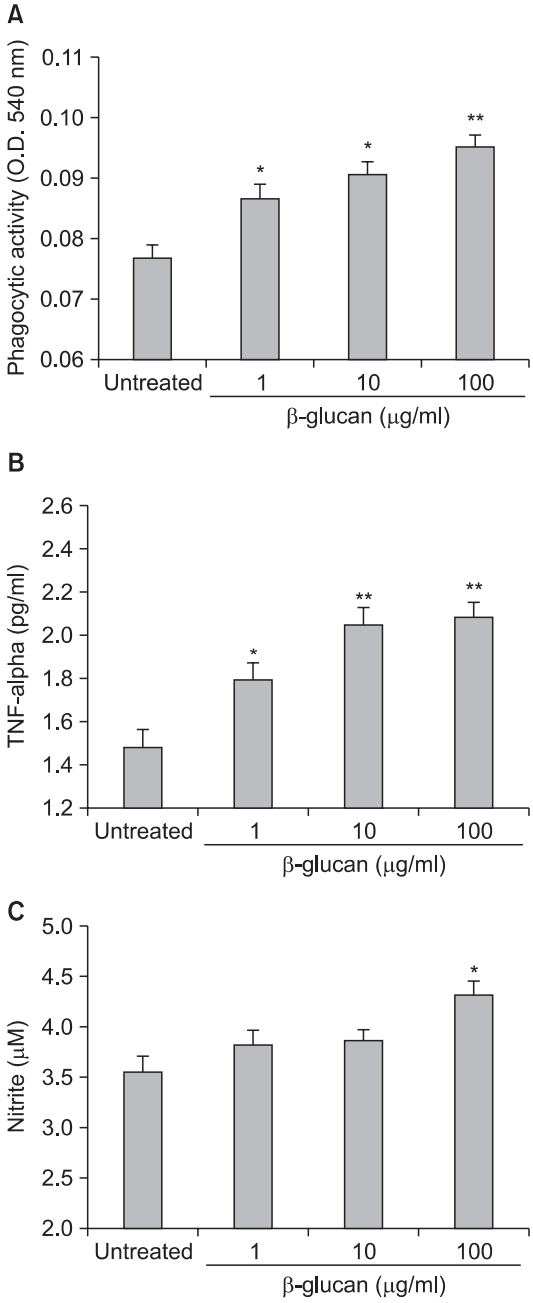

Fig. 1. Effects of β-glucans from *C. versicolor* on (A) phagocytosis, (B) TNF-α and (C) nitric oxide production in peritoneal macrophages. Peritoneal macrophages were treated with β-glucans for 24 h. (a) Cells were incubated with media containing zymosan (1×10^6 particles/ml) and NBT (0.6 mg/ml) for 1 h. Formazan formation was measured at 540 nm as described in materials and method. (B) Culture supernatants were collected and the levels of TNF-α were measured as described in materials and method. (C) Culture supernatants were collected and the levels of nitrite were measured as described in materials and method. The data represents the mean ± S.E.M. of quadruplicate experiments. *p<0.05, **p<0.01: significantly different from the untreated group (no treatment).

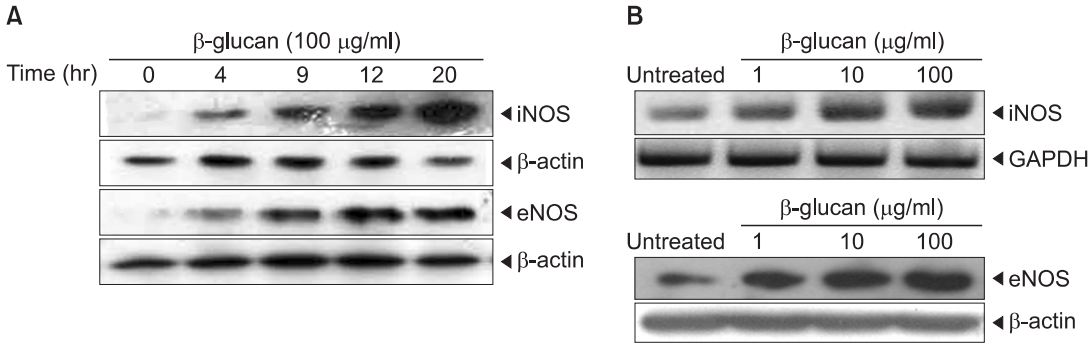

Fig. 2. (A) The effects of β-glucans from *C. versicolor* on production of iNOS and eNOS protein expression in peritoneal macrophages. (B) The effects of β-glucans from *C. versicolor* on production of iNOS gene expression and eNOS protein expression in RAW264.7 macrophages. After cells were treated with β-glucans for 24 hr, total protein was extracted and subjected to Western blot analysis for iNOS, eNOS and β-actin protein. And the cells were treated with β-glucans for 16 h, total mRNA was extracted and subjected to RT-PCR analysis for iNOS and GAPDH. The data represents the mean ± S.E.M. of triplicate experiments.

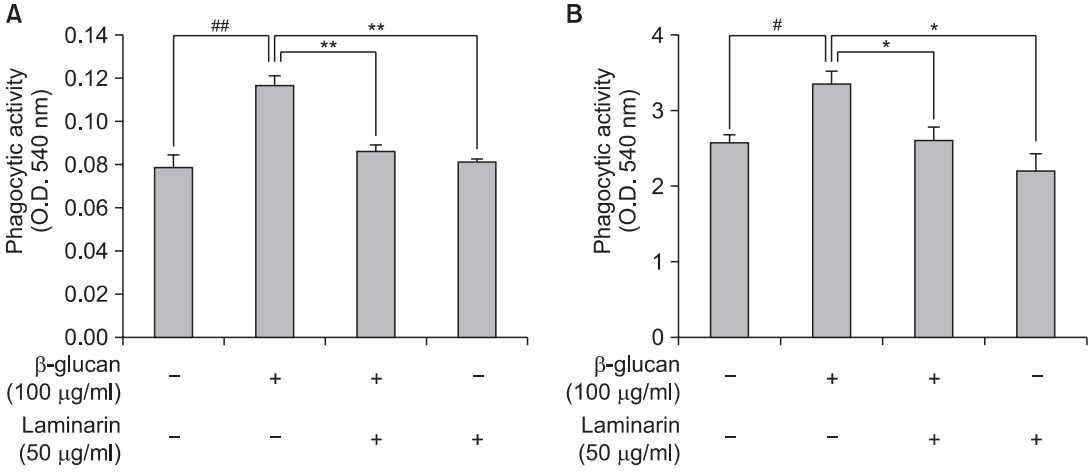

Fig. 3. Inhibitory effects of laminarin, dectin-1 specific inhibitor, on phagocytosis by β-glucans from *C. versicolor* in (A) peritoneal macrophages and (B) RAW264.7 macrophages. Cells were co-treated with laminarin and β-glucans for 24 h. After treatment with β-glucans, cells were incubated with media containing zymosan (1×10^6 particles/ml) and NBT (0.6 mg/ml) for 1 h. Formazan formation was measured at 540 nm as described in materials and method. The data represents the mean ± S.E.M. of quadruplicate experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$: significantly different from no treatment, $^{*}p<0.05$ $^{**}p<0.01$: significantly different from β-glucans-treated group.

β-glucan-induced dectin-1 signaling regulates NO, iNOS, eNOS, and TNF-α production

When a pathogen crosses an epithelial barrier, it is affected by phagocytosis and lysosomal enzymes and subsequently cytokines (IL-1, IL-6, TNF-α) and inflammatory mediators (NO and H_2O_2) are secreted (Akramiene *et al.*, 2007). We used laminarin as a dectin-1 antagonist to investigate whether iNOS, eNOS, and TNF-α protein expression was affected by major β-glucan receptor dectin-1 signaling. NO release and expressions of iNOS, eNOS, and TNF-α by β-glucan (100 μg/ml) were all decreased by laminarin in RAW264.7 cells (Fig. 4).

β-glucan-increased productions of iNOS, eNOS, and TNF-α regulate phagocytic activity

We also investigated whether protein expression of iNOS, eNOS, and TNF-α affected the phagocytic activity of macrophages. These effects were used for the iNOS specific inhibitor smethylthiourea (SMT), eNOS inhibitor diphenyleneiodonium (DPI), and TNF-α receptor blocking antibody (Rickard *et al.*, 1999; Akerman *et al.*, 2002; Tipoe *et al.*, 2006). Increased phagocytic activity of macrophages by β-glucan (100 μg/ml) was decreased by SMT, an iNOS inhibitor (Fig. 5A), DPI, a eNOS inhibitor (Fig. 5B), and a TNF-α receptor blocking antibody (Fig. 5C), respectively.

DISCUSSION

In this study, we demonstrated that β-glucans from *C. versicolor* activated the phagocytic function of macrophages and increased gene expression and protein secretion of pro-inflammatory cytokines, such as TNF-α and NO. Additionally, treatment of the cells with laminarin, a dectin-1 receptor blocking agent, reversed the increased expression of iNOS, eNOS, and TNF-α induced by β-glucans from *C. versicolor*. We further determined that the phagocytic activity of macrophages by β-glucans was significantly decreased by the blocking of NO release and TNF-α receptors. Together, these data dem-

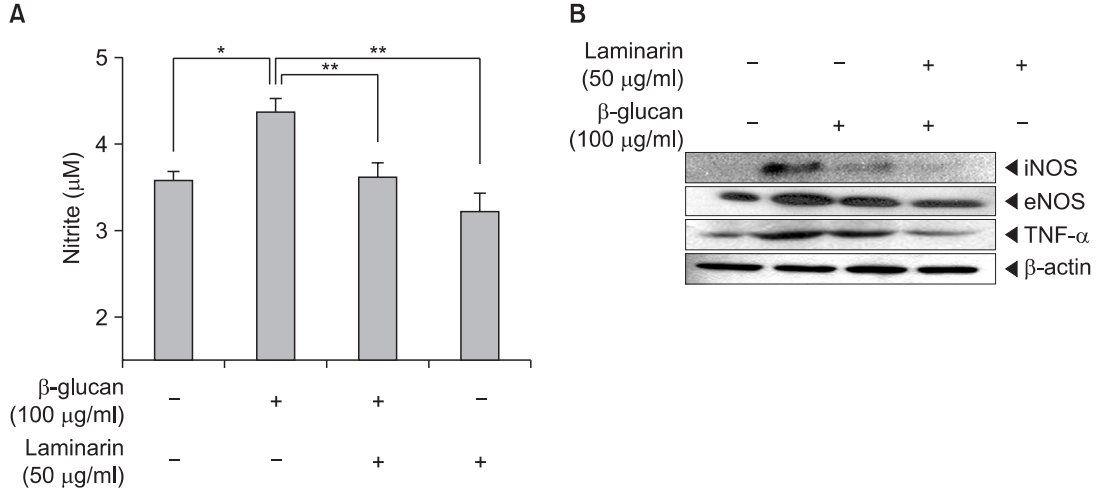

Fig. 4. Inhibitory effects of laminarin, dectin-1 specific inhibitor, on the production of (A) nitric oxide, (B) iNOS, eNOS and TNF-α by β-glucans from *C. versicol* in RAW264.7 macrophages. Cells were co-treated with laminarin and β-glucans for 24 h. Total protein was extracted and subjected to Western blot analysis for iNOS, eNOS, TNF-α and β-actin. The data represents the mean ± S.E.M. of triplicate experiments. *p<0.05: significantly different from no treatment, **p<0.05: significantly different from β-glucans-treated group.

onstrate that dectin-1 is a key receptor for β-glucan-activated macrophages to perform phagocytosis in which TNF-α and iNOS/eNOS are involved.

The discovery of dectin-1 and the study of its functions have helped to assess many of the previously reported roles for β-glucan receptors in immunity, including the innate recognition of and response to fungal pathogens and β-glucan-mediated immunomodulation. Although there are other β-glucan receptors on leukocytes, such as CR3, SRs, LacCer, and TLRs, the use of specific antagonists and blocking monoclonal antibodies has clearly shown that dectin-1 is the main receptor mediating immune-modulating activity in leukocytes, particularly macrophages and dendritic cells (Brown *et al.*, 2002; Willment *et al.*, 2005). The importance of dectin-1 in the activation of innate immune responses of macrophages is also supported by a report showing that knockout of the dectin-1 gene resulted in the abolition of all macrophage-mediated responses (Taylor *et al.*, 2007).

In our study, the inhibitory effects of laminarin, a specific inhibitor of dectin-1, on phagocytosis (Fig. 3) and TNF-α, iNOS, and eNOS production (Fig. 4) induced by β-glucans indicate the involvement of the dectin-1 receptor in the binding and internalization of β-glucans from *C. versicolor*. Several studies have reported the possibility that dectin-1 could trigger the secretion of cytokines and chemokines, including TNF-α, CXC-chemokine ligand 2 (CXCL2, also known as MIP2), IL-2, IL-10, and IL-12, as well as the production of phospholipase A2, through the recognition of soluble and particulate β-(1,3) and/or β-(1,6) glucans (Brown *et al.*, 2003; Gantner *et al.*, 2003; Steele *et al.*, 2003; Rogers *et al.*, 2005). We determined that increased TNF-α and NO-iNOS/eNOS by β-glucans from *C. versicolor* occur via the dectin-1 signal. We further confirmed that these molecules (TNF-α and NO-iNOS/eNOS) are correlated with phagocytosis induced by β-glucans.

In this study, we showed that the inhibition of iNOS, eNOS, and TNF-α signals significantly blocked β-glucaninduced phagocytosis, partially or completely (Fig. 5). The results of some other reports are consistent with our results, demonstrating that inflammatory cytokines, such as TNF-α, induced by pathogens, in turn, promote phagocytosis (Murray *et al.*, 2005; Van der Graaf *et al.*, 2005). It seems likely that inflammatory cytokines induced by β-glucans assist macrophages in activating phagocytosis as a secondary messenger. Taken as a whole, dectin-1 signaling triggered by β-glucans subsequently elicits cytokine production, including TNF-α and NO-iNOS/eNOS, and eventually induces phagocytosis by macrophages.

Dectin-1-mediated signaling is sufficient for most of these responses, but the induction of certain pro-inflammatory cytokines (such as TNF-α, IL-12, and possibly IL-2) also requires signals from TLR2 and TLR6 through the TLR pathway (Gantner *et al.*, 2003). This concept is supported by a report that ROS production by β-glucans was partially inhibited by laminarin, which could indicate the involvement of other receptors (Gersuk *et al.*, 2006). However, recent evidence also showed that SCG and NaClO-oxidized zymosan (which contained only β-glucan)-induced production of TNF-α, IL-12, and IFN-γ, via dectin-1 signaling, was not affected by a deficiency in MyD88, indicating its effect was independent of the TLR pathway (Saijo *et al.*, 2007). Thus, we cannot rule out the possibility that β-glucans from *C. versicolor* can stimulate not only dectin-1, but also TLR2. It seems likely that dectin-1 is able to trigger pro-inflammatory cytokine production independent of TLRs in certain cells or to form a complex with TLRs after being phosphorylated to induce a synergistic effect. Further studies are necessary to determine whether the various responses of macrophages to β-glucans of diverse structures depending on origin are mediated via dectin-1 only or in collaboration with TLRs. That is, determining the specific correlations between dectin-1 receptors and distinct β-glucans would be an important way to understand the versatile biological activities and the use of β-glucans.

Although we still do not understand what structural features of β-glucans are best for inducing ideal activities (such as macrophage phagocytosis), clarification of the roles of TNF-α and NO-iNOS/eNOS as secondary messengers via dectin-1

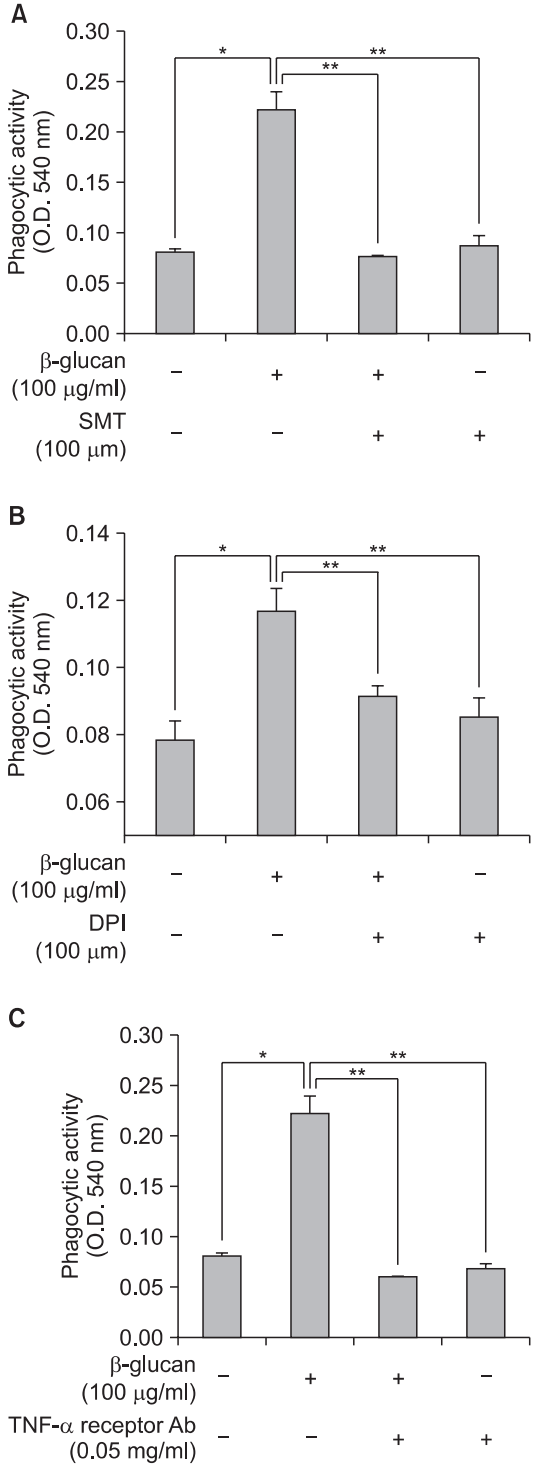

Fig. 5. Inhibitory effects on β-glucans-induced phagocytosis by (A) s-methylsulfothiourea (SMT, iNOS inhibitor), (B) diphenyleneiodonium (DPI, eNOS inhibitor) and (C) TNF-α receptor blocking antibody in RAW264.7 macrophages. Cells were pre-treated with inhibitors for 1 h and subsequently treated with β-glucans for 24 h. After treatment with β-glucans, cells were incubated with media containing zymosan (1×10^6 particles/ml) and NBT (0.6 mg/ml) for 1 h. Formazan formation was measured at 540 nm as described in materials and method. The data represents the mean ± S.E.M. of quadruplicate experiments. *p<0.01: significantly different form no treatment, **p<0.01: significantly different from β-glucans-treated group.

signaling (through β-glucans) may provide meaningful opportunities for the development of nutritional medicines that could be applied to the treatment of infectious disease.

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