J. Microbiol. Biotechnol. (2008), **18**(2), 355–364

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Molecular Mechanism of Macrophage Activation by Exopolysaccharides from Liquid Culture of *Lentinus edodes*

Lee, Ji Yeon[1], Joo Young Kim[1], Yong Gyu Lee[1], Man Hee Rhee[2], Eock Ki Hong[3], and Jae Youl Cho[1]*

[1]*School of Bioscience and Biotechnology, and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Korea*
[2]*College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea*
[3]*Department of Bioengineering and Technology, Kangwon National University, Chuncheon 200-701, Korea*

Received: June 14, 2007 / Accepted: August 28, 2007

Mushrooms are regarded as one of the well-known foods and biopharmaceutical materials with a great deal of interest. β-Glucan is the major component of mushrooms that displays various biological activities such as antidiabetic, anticancer, and antihyperlipidemic effects. In this study, we explored the molecular mechanism of its immunostimulatory potency in immune responses of macrophages, using exopolysaccharides prepared from liquid culture of *Lentinus edodes*. We found that fraction II (F-II), with large molecular weight protein polysaccharides, is able to strongly upregulate the phenotypic functions of macrophages such as phagocytic uptake, ROS/NO production, cytokine expression, and morphological changes. F-II triggered the nuclear translocation of NF-κB and activated its upstream signaling cascades such as PI3K/Akt and MAPK pathways, as assessed by their phosphorylation levels. The function-blocking antibodies to dectin-1 and TLR-2, but not CR3, markedly suppressed F-II-mediated NO production. Therefore, our data suggest that mushroom-derived β-glucan may exert its immunostimulating potency *via* activation of multiple signaling pathways.

Keywords: Mushroom-derived β-glucan, *Lentinus edodes*, macrophage functions, pattern-recognition receptors, signaling cascade

Macrophages are a type of differentiated tissue cells that originate as blood monocytes. The cells have several functions such as (1) the removal of cell debris, (2) the killing of pathogenic microorganisms, and (3) the processing and presentation of antigens to lymphocytes [9, 19]. Therefore, the activation of macrophages is a key event for effective innate and adaptive immunity. When the body is stimulated by pathologic stimuli or injury, macrophages release (1) numerous proinflammatory cytokines [*e.g.*, tumor necrosis factor (TNF)-α and interleukin (IL)-1], (2) chemokines and chemoattractants [*e.g.*, IL-8, macrophage inhibitory protein (MIP-1), and monocyte chemoattractant protein (MCP)-1], and (3) cytotoxic and inflammatory molecules [*e.g.*, nitric oxide (NO), reactive oxygen species (ROS), and prostaglandin (PG)E_2] [47]. For these cellular events, an intracellular alteration such as signaling cascades has known to be accompanied by a connection from cell surface molecules including pattern-recognition receptors (PRRs) to the intracellular signaling machinery [44]. So far, receptor-type or nonreceptor-type tyrosine kinases (*e.g.*, Src kinase), protein kinase C, phosphatidylinositol 3-kinase (PI3K)/Akt, and mitogen-activated protein kinases (MAPKs), as well as transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1, have been reported to be the major components of the signaling machinery [13, 23]. In addition, the macrophages upregulate the surface levels of PRRs such as toll-like receptor (TLR)-2 [a receptor for peptidoglycan (PGN) derived from G(+) bacteria], TLR-4 [a receptor for lipopolysaccharide (LPS) derived from G(−) bacteria], and dectin-1 (a receptor for β-glucan derived from fungi and yeast cell walls)] [2, 18]. Although large amounts of macrophage-derived inflammatory mediators can cause severe inflammatory diseases such as septic shock and rheumatoid arthritis [31, 42], proper regulation of these macrophage functions by immunomodulatory molecules could help a host to protect itself from various pathologic and cancerous attacks.

Mushrooms are one of the well-known foods and biopharmaceutical materials generating a great deal of interest [7, 28, 29, 48]. For example, some mushrooms such as *Lentinus edodes* (Shiitake) and *Inonotus obliquus* (Chaga) have also been used for medicinal purposes (to treat ailments such as allergic asthma, atopic dermatitis, autoimmune joint inflammation, tuberculosis, and cancer) for hundreds of years

*Corresponding author
Phone: 82-33-250-6562; Fax: 82-33-253-6560;
E-mail: jaecho@kangwon.ac.kr

in Korea, China, and Japan [29]. The main pharmacological merit of the food materials is to stimulate the host immune defense mechanisms. This is of vast importance, since the population of elderly people whose immune systems have entered a weak state is greatly increasing, owing to the improvement of medicinal benefits. Additionally, the number of cancer patients is growing quickly. In fact, various chemical components from mushrooms such as PG101 (a water-soluble extract that consists of protein-bound polysaccharides), grifolan (an antitumor β-glucan), and PL (an acidic polysaccharide) have been identified as immunostimulating principles upregulating the functions of macrophages and natural killer cells [29]. The majority of these components (such as β-glucan) have unique structural features including protein binding and branch structure [β-(1→6) branches)] properties.

Lentinus edodes is a representative mushroom that is used for both medicinal and food purposes. Owing to aggressive systemic studies, the value of *L. edodes* as a folk medicine has been greatly demonstrated. Indeed, recently, various substances (*e.g.*, β-glucan, eritadenine and lenthionine) that have immunopotentiating, antiatherogenic, anti-HIV, hepatoprotective, and hypercholesterolemic effects have been isolated from *L. edodes* [1, 6, 27, 49]. In spite of numerous studies, the exact biochemical (*e.g.*, molecular weight, pI value, and chemical structures) and immunopharmacological (*e.g.*, immunopotentiating mechanism) properties of the active polysaccharides (β-glucan) have remained largely unelucidated. In this study, therefore, we aimed to prove the activating roles and action mechanisms of protein-bound polysaccharide fractions from *L. edodes* on macrophage-mediated innate immune responses. To do this, functional events mediated by activated macrophages such as phagocytic uptake, the production of cytokines, the release of toxic molecules (NO and ROS), and morphological changes with the dendritic process were investigated. Additionally, mechanism studies in terms of activation signaling pathways were carried out.

Materials and Methods

Materials

Exopolysaccharide fractions (F-1, F-II, and F-III) prepared from liquid culture of *L. edodes* and F-II's were prepared by the ethanol precipitation and lyophylization methods reported previously (Yield: 6.11%) [12, 40, 41]. These fractions contained a level of endotoxin below the detection limits (0.0015 EU/ml) as assessed by an E-TOXATE kit (Sigma, St. Louis, MO, U.S.A.). Of the fractions, F-II is composed of three large molecular mass exopolysaccharides including 308, 580, and 658 kDa with random coiled helical conformation of β-glucan. The composition of monosaccharide was 99.42% glucose and 0.58% mannose. N^G-monomethyl-L-arginine (N-MMA), FITC-dextran, peptidoglycan (PGN), and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PP2, cell-permeable SN50, LY294002, and Bay 11-7082 were obtained from Calbiochem (La Jolla, CA, U.S.A.). Antibodies to TLR-2 [39], CR3 [32], and dectin-1 [5] were from Hycult Biotechnology (Uden, The Netherlands), BD Bioscience (San Diego, CA, U.S.A.), and Serotec (Kidlington, Oxford, U.K.), respectively. Dichlorodihydrofluorescein diacetate (H_2DCFDA), Hoechst 33258, and rhodamine phalloidin were purchased from Molecular Probes (Carlsbad, CA, U.S.A.). Cynaropicrin was a gift from Prof. Jung Jee Hyung (Pusan National University, Pusan, Korea) [8]. Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, U.S.A.). RAW264.7 and HEK293 cells were purchased from the American Tissue Culture Center (Rockville, MD, U.S.A.). NF-κB promoter-containing luciferase construct was a gift from Prof. Chung Hae Young (Pusan National University, Pusan, Korea). All other chemicals were of sigma grade. Phospho-specific antibodies to Src, P85, Akt, IκBα, p38, C-jun terminal N-kinase (JNK), and early activated signal-related kinase (ERK), and antibodies to p65, β-actin, Akt, p85, IκBα, ERK, p38, and JNK were purchased from Cell Signaling (Beverly, MA, U.S.A.), Promega (Madison, WI, U.S.A.), Biovision (Mountainview, CA, U.S.A.), and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture

RAW264.7 cells were maintained in RPMI1640 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum. Cells were grown at 37°C with 5% CO_2.

Determination of Phagocytotic Uptake

To measure the phagocytic activity of RAW264.7 cells, a previously reported method was used with slight modifications [14]. RAW264.7 (2×10^6) cells were resuspended in 1 ml of PBS containing 1% human AB serum and incubated with FITC-dextran (1 mg/ml) in the presence or absence of LPS (2.5 μg/ml) or exopolysaccharide fractions (300 μg/ml) at 37°C for 30 min. The incubations were stopped by adding 2 ml of ice-cold PBS containing 1% human serum and 0.02% sodium azide. The cells were then washed three times with cold PBS-azide and analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.).

Determination of ROS Generation

The level of intracellular ROS was determined by the change in fluorescence resulting from the oxidation of the fluorescent probe H_2DCFDA. Briefly, 2×10^6 cells/well were exposed to F-II (300 μg/ml) for 30 min. After incubation, cells were then incubated with LPS (2.5 μg/ml) as inducer of ROS production at 37°C for 6 h. Cells were incubated with 50 mM of the fluorescent probe H_2DCFDA for 1 h at 37°C. The degree of fluorescence, corresponding to intracellular ROS, was determined using flow cytometry.

TNF-α Production

The induction effect of fractions from *L. edodes*, solubilized with culture medium, on TNF-α production from LPS-treated RAW264.7 cells was determined as described previously [8]. Supernatants were harvested and assayed for murine TNF-α by enzyme-linked immunosorbent assay (ELISA).

Determination of NO Production

After the preincubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, F-I, F-II, or F-III (300 μg/ml) with LPS (2.5 μg/ml) was added for

24 h, as reported previously. The nitrite in the culture supernatants was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100-μl samples.

RT-PCR

For the evaluation of LPS-inducible gene mRNA expression levels, the total RNA from F-II-treated (or untreated) RAW264.7 cells was prepared by adding TRIzol Reagent (Gibco BRL), according to the manufacturer's protocol. The total RNA solution was stored at −70°C for further use. Semiquantitative RT reactions were conducted using MuLV reverse transcriptase. The total RNA (1 μg) was incubated with oligo-$d(T)_{15}$ for 5 min at 70°C, and was mixed with a 5× first-strand buffer, 10 mM dNTPs, and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37°C, and for 60 min after the addition of MuLV reverse transcriptase (2 U). Reactions were terminated for 10 min at 70°C and the total RNA was depleted by adding RNase H. The PCR reaction was conducted with the incubation mixture (2 μl of cDNA, 4 μM 5' and 3' primers, a 10× buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100], 250 μM dNTPs, 25 mM $MgCl_2$, and 1 unit of *Taq* polymerase [Promega, U.S.A.]) under the following incubation conditions: a 45 sec denaturation time at 94°C, an annealing time of 45 sec between 55 and 60°C, an extension time of 60 sec at 72°C, and a final extension of 7 min at 72°C at the end of 30 cycles. The primers (Bioneer, Seoul, Korea) used in this experiment are indicated in Table 1 (F: forward; R: reverse).

Luciferase Reporter Gene Activity Assay

Luciferase assays were performed using the Luciferase Assay System (Promega) [25]. Briefly, the transfected cells were lysed in the culture dishes with reporter lysis buffer, and the lysates were centrifuged at maximum speed for 10 min in an Eppendorf microcentrifuge. Ten μl of the supernatant fraction was incubated with 50 μl of luciferase substrate, and the relative luciferase activity was determined with a Luminoskan Ascent (Thermo Labsystems Oy, Helsinki, Finland). Luciferase activity was normalized to β-galactosidase activity.

Table 1. The primer sequences of the investigated genes in a RT-PCR analysis.

Gene		Primer sequences
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'
	R	5'-CCTGTAGCCCACGTCGTAGC-3'
IL-12p40	F	5'-CAGAAGCTAACCATCTCCTGGTTTG-3'
	R	5'-TCCGGAGTAATTTGGTGCTTCACAC-3'
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
MCP-1	F	5'-ACTGAAGCCAGCTCTCTCTTCCTC-3'
	R	5'-TTCCTTCTTGGGGTCAGCACAGAC-5'
MIP-2	F	5'-GAACAAAGGCAAGGCTAACTGA-3'
	R	5'-AACATAACAACATCTGGGCAAT-3'
IL-23p19	F	5'-AATAATGTGCCCCGTATCCA-3'
	R	5'-CTGGAGGAGTTGGCTGAGTC-3'
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

F, forward; R, reverse.

Confocal Microscopy

Confocal analysis of RAW264.7 cells (1×10^5 cells/ml) treated with F-II (300 μg/ml) was performed on glass-bottom culture dishes (MatTek Corp., Ashland, MA, U.S.A.) using directly labeled antibodies [to TLR-2 (T2.5, 1:20 dilution), dectin-1 (2A11, 1:20 dilution), and p65 (NF-κB, 1:15 dilution)], rhodamine phalloidin (1:250 dilution), or Hoechst solution (0.2 mg/ml). Intensity changes in TLR-2, dectin-1, p65, the actin cytoskeleton, and nuclei were imaged by LSCM on a Zeiss LSM 510 META confocal microscope equipped with a Zeiss 37°C incubation system. Images were analyzed using the Zeiss LSM Image Examiner.

Immunoblotting

Cells (5×10^6 cells/ml) were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM ben, and 2 mM hydrogen peroxide) for 30 min under rotation in a cold-room. The lysates were clarified by centrifugation at 16,000 ×*g* for 10 min at 4°C. Soluble cell lysates were immunoblotted, and phosphotyrosine, ERK, or phospho-ERK were visualized as reported previously.

Statistical Analysis

A Student's *t*-test and a one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as means±standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. *P* values of 0.05 or less were considered to be statistically significant.

RESULTS

Effect of Macrophage Activation

To address whether exopolysaccharides from *L. edodes* were able to stimulate the functional activation of macrophages, various cellular activities (innate immune responses) of macrophages (such as phagocytosis, ROS generation, cytokine expression, and NO production) and their morphological changes were evaluated using a macrophage-like cell line, RAW264.7 cells. As Fig. 1 shows, exopolysaccharide fraction II (F-II) upregulated very effectively the macrophage functions. Thus, F-II increased the phagocytotic uptake of RAW264.7 cells, ROS generation, TNF-α production, and NO release, as LPS did (Fig. 1). Furthermore, it induced morphological changes, showing numerous dendritic processes, one of macrophage activation markers [10], as shown in the case of LPS, indicating that it was able to induce the functional and phenotypical activation of macrophages. Therefore, we continued further experiments to understand the molecular mechanism by which F-II stimulates the cellular activities of macrophages.

Induction of Transcriptional Expression of Proinflammatory Cytokines

To elicit innate immune responses by macrophages, newly synthesized proteins such as proinflammatory cytokines

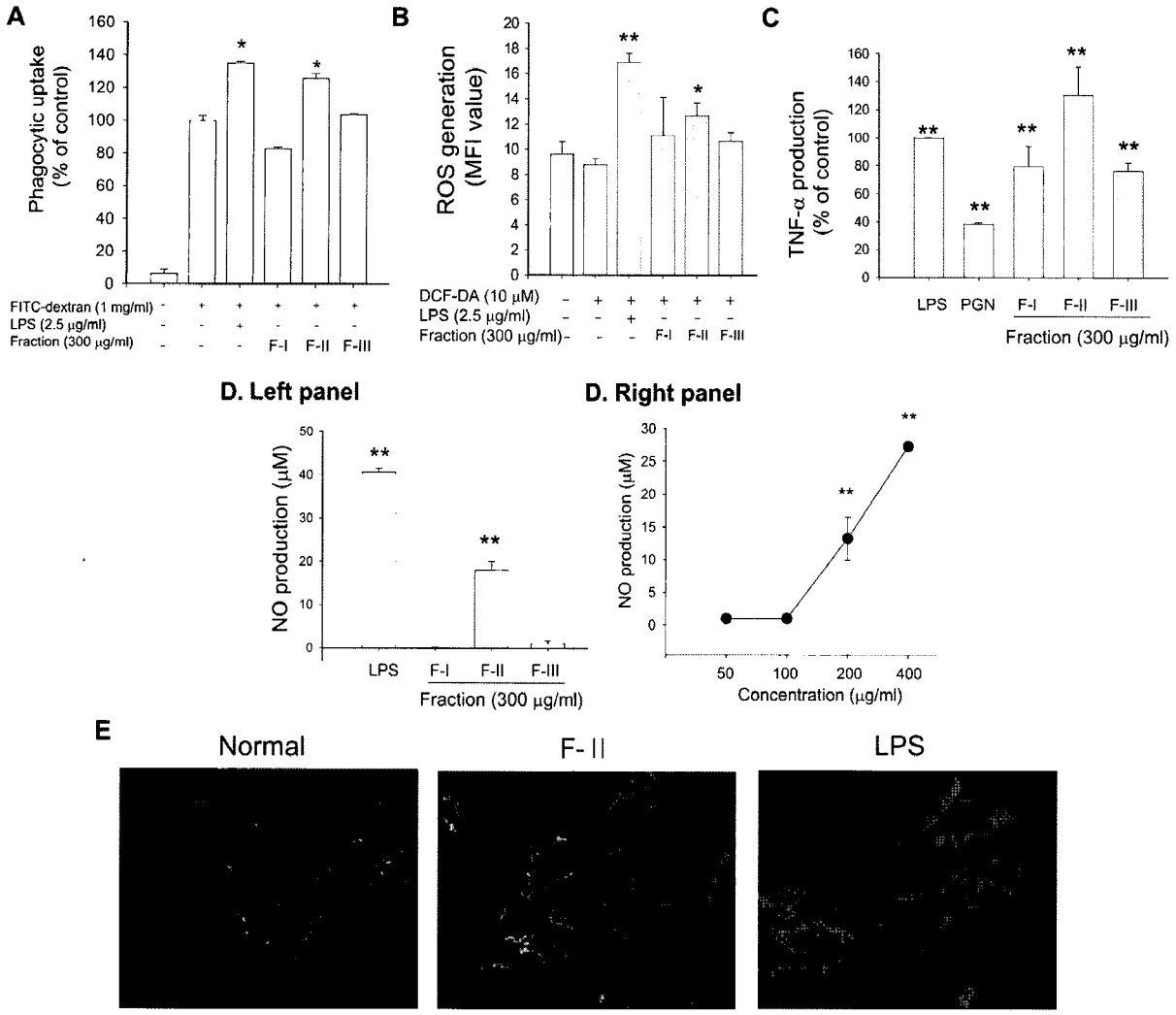

Fig. 1. The effect of protein-polysaccharide-containing fractions on cellular activation of RAW264.7 cells.
A. Phagocytosis: RAW264.7 cells (2×10^6 cells/ml) were stimulated by FITC-dextran (1 mg/ml), in the presence or absence of fractions [F-I, F-II, or F-III (300 μg/ml)] and LPS (2.5 μg/ml) for 6 h. The extent of the phagocytotic uptake was determined by flow cytometric analysis, as described in Materials and Methods. *: $p<0.05$ compared with FITC-dextran alone. **B**. ROS generation: RAW264.7 cells (1×10^6 cells/ml) were stimulated by LPS (2.5 μg/ml) or fractions [F-I, F-II, or F-III (300 μg/ml each)] for 3 h. ROS was determined by flow cytometic analysis as described in Materials and Methods. *: $p<0.05$ and **: $p<0.01$ compared with DCF-DA alone. **C**. TNF-α production: RAW264.7 cells (1×10^6 cells/ml) were stimulated by LPS (2.5 μg/ml) or fractions [F-I, F-II, or F-III (300 μg/ml)] for 6 h. Supernatants were collected and TNF-α concentrations from the supernatants were determined by ELISA, as described in Materials and Methods. **: $p<0.01$ compared with normal. **D**. NO production: Left panel, RAW264.7 cells (1×10^6 cells/ml) were stimulated by LPS (2.5 μg/ml) or fractions [F-I, F-II, or F-III (300 μg/ml)] for 24 h. Right panel, Dose response curve of F-II-induced NO production was obtained under the same conditions. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. Data (**A** to **D**) represent mean±SEM of three independent observations performed in triplicate. **: $p<0.01$ compared with normal. **E**. Morphological changes of actin cytoskeleton: RAW264.7 cells (1×10^6 cells/ml) were stimulated by LPS (2.5 μg/ml) or F-II (300 μg/ml)] for 24 h. Staining of actin cytoskeleton was performed by confocal microscopic analysis, as described in Materials and Methods. The result (**E**) shows one experiment out of three.

and enzymes are required. Therefore, we next examined whether F-II induced transcriptional upregulation of these genes by RT-PCR. Fig. 2 depicts that F-II was strongly able to trigger the proinflammatory cytokines and enzymes such as iNOS, TNF-α, IL-12, IL-23, MCP-1, and MIP-2, in a similar manner to LPS.

NF-κB Activation is Required for F-II-mediated Macrophage Activation

Based on previous papers that LPS and mushroom-derived immunostimulants such as β-glucan activate a series of signaling pathways for NF-κB activation [15], we first investigated the involvement of NF-κB-related molecular events. A luciferase assay with a plasmid construct composed of the NF-κB binding promoter site and the luciferase gene was initially chosen. As expected, LPS or F-II fraction both enhanced the luciferase activity from 6 h or 12 h (Fig. 3A), indicating NF-κB activation. Continuously, several lines of evidence obtained by cell biological, biochemical, and pharmacological analyses also supported this possibility. Thus, F-II induced the translocation of NF-κB into the nuclei as LPS did (Fig. 3A right panel). Furthermore, it

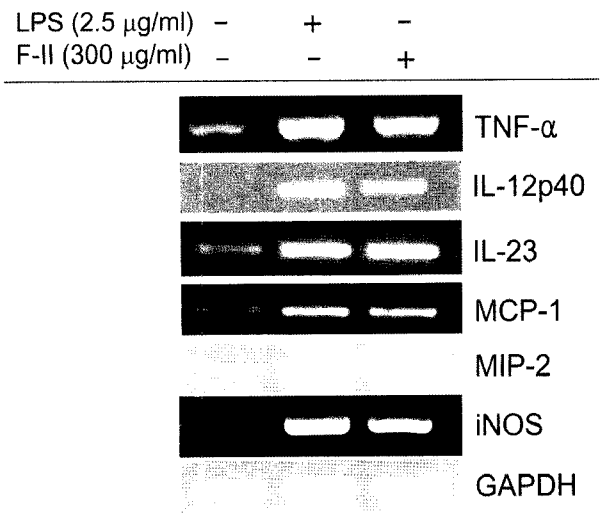

Fig. 2. The effect of F-II on the expression of cytokines, chemokines, and iNOS in RAW264.7 cells.
RAW264.7 cells (1×10^7 cells/ml) were incubated with F-II (300 μg/ml) or LPS (2.5 μg/ml) for 6 h. The mRNA levels of cytokines, chemokines, and iNOS were determined by semiquantitative RT-PCR. The results show one experiment out of three.

also increased the phosphorylation of IκBα at 15 and 30 min, whereas LPS did so at 5 and 30 min. Additionally, F-II remarkably activated the upstream signaling involved in NF-κB activation. Fig. 3B (left panel) shows that Akt, p85 (a regulatory subunit of PI3K), and Src were strongly phosphorylated by F-II as well as LPS, suggesting that these activation signals for NF-κB translocation may be effectively upregulated. In agreement, selective pharmacological inhibitors of these enzymes for Src (PP2), PI3K (LY29004), and IκB (SN50, cynaropicrin, and Bay 11-7082) were shown to inhibit F-II (as well as LPS)-mediated NO production up to 70 to 95% (Fig. 3B, right panel). In addition, like LPS, F-II also activated all MAPKs (ERK, p38, and JNK), according to immunoblotting analysis. Thus, it clearly triggered the phosphorylation of ERK, JNK, and p38 at 30 min, whereas LPS-induced phosphorylation of the proteins was remarkably shown from 5 min (Fig. 3C, left panel). Of these, however, JNK seemed to clearly participate in F-II-mediated NO production signaling, unlike LPS, indicating a different signaling pattern. Thus, the JNK inhibitor SP600125 significantly suppressed F-II-mediated NO production (Fig. 3C, right panel).

Role of Dectin-1 and TLR-2 in F-II-mediated NO Production

It has been known that dectin-1 is a major receptor for β-glucan [4]. Therefore, the role of dectin-1 in F-II-mediated macrophage responses was elucidated using flow cytometry and function blocking assays with a specific monoclonal antibody to dectin-1. Firstly, to know whether F-II binds to dectin-1, the binding effects of anti-dectin-1 antibody to the cells pretreated or post-treated with F-II were compared. As Fig. 4A (left panel) shows, F-II pretreatment almost completely blocked the antibody binding, suggesting that dectin-1 is a receptor for F-II. Secondly, the function blocking effect of anti-dectin-1 antibody on F-II-induced NO production was examined. Fig. 4A (right panel) displays that there was a significant alteration of the NO production in blocking antibody-treated cells compared with isotype control-treated cells. In contrast, little or no change in NO production in LPS- and PGN-treated groups was observed, suggesting that dectin-1 may be limited in β-glucan-mediated responses. Owing to the fact that there was no dramatic alteration, another potential receptor of β-glucan, TLR-2 (a major receptor of PGN), was also selected to get additional information. The function-blocking antibody to TLR-2 also significantly diminished F-II-mediated NO production (Fig. 4B), suggesting the involvement of this receptor. PGN-induced NO production was also affected by this antibody, indicating that the blocking antibody was working very well. Confocal data also supported the importance of these proteins. Although the membrane intensity of dectin-1 was relatively lower than that of TLR-2, TLR-2 and dectin-1 seem to be in part colocalized at the membrane, as reported previously (Fig. 4C). In spite of this, there was no major alteration in surface dectin-1 and TLR-2 by F-II when it was treated for 12 h, unlike LPS by which TLR-2 was significantly downregulated (data not shown). The NO-producing activity of F-II was not affected by a blocking antibody to CR3 unlike β(1→4) D-glucan (Fig. 4D), indicating a structural difference [32].

Discussion

Immunostimulation itself is regarded as one of the important strategies to improve the body's defense mechanism in elderly people as well as in cancer patients. One of the most important immunostimulating materials is known to be polysaccharides (such as β-glucan) derived from mushrooms including *L. edodes*, *Phellinus linteus*, and *Agaricus blazei* [29]. Of them, *L. edodes* is the most well-known one used as food and for medicinal purposes. Owing to numerous studies, it has been demonstrated that polysaccharides (*e.g.*, lentinan) from *L. edodes* display potent *in vivo* immunostimulating activities such as anticancer and antibacterial effects [1, 16, 27]. However, the molecular mechanism by which the β-glucan upregulates innate and adaptive immunity of macrophages and T cells remains largely unelucidated. In this study, therefore, functional activation of macrophages by β-glucan from *L. edodes*, and their molecular mechanism and structural features in upregulating macrophage-mediated innate immune responses were carefully explored.

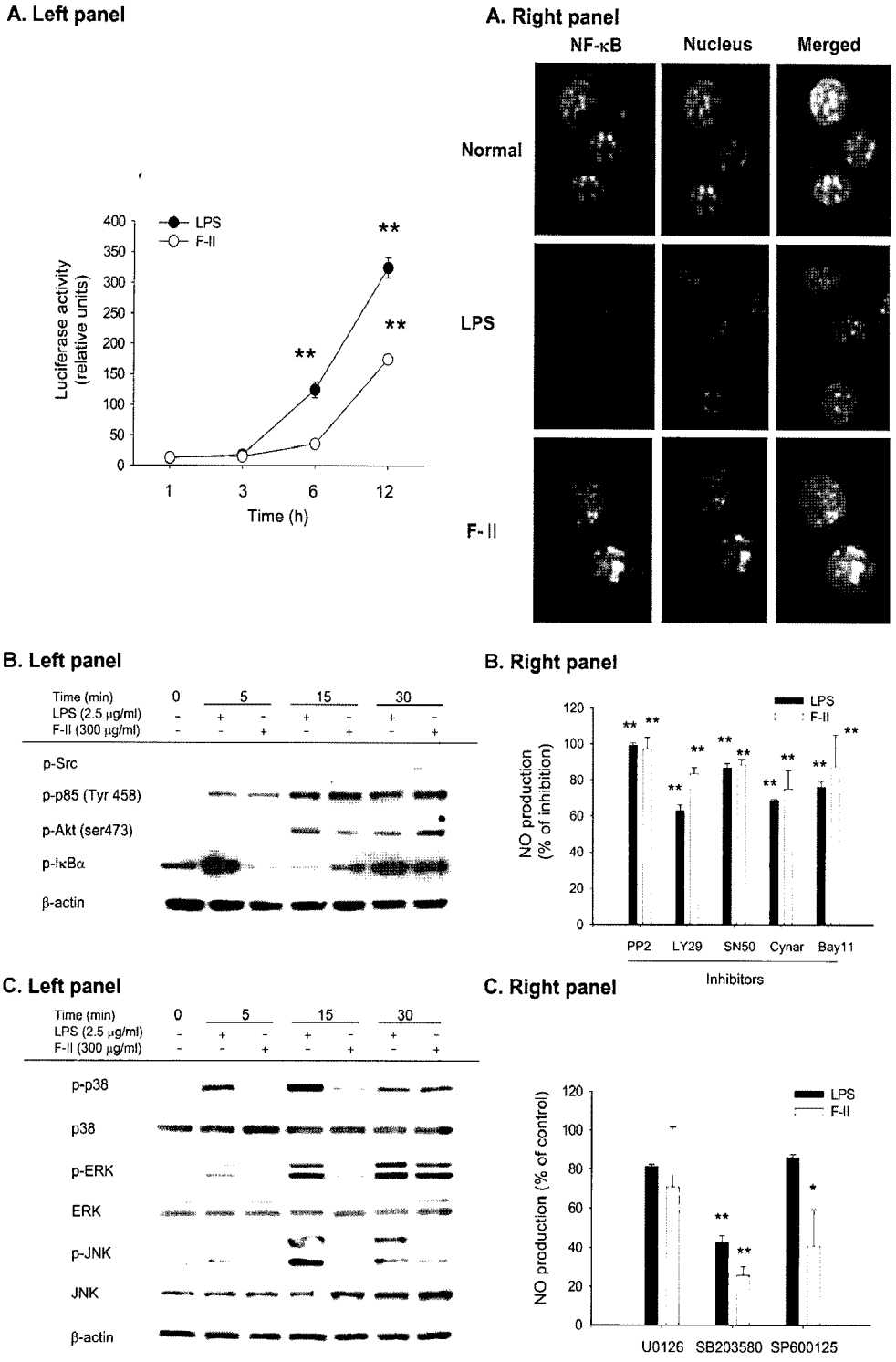

Fig. 3. The effect of F-II on the upregulation of signaling pathways for NF-κB activation.

A. Luciferase assay (left panel) and NF-κB translocation (right panel): RAW264.7 cells (1×10^7 cells/ml) were incubated with F-II (300 μg/ml) or LPS (2.5 μg/ml) for the various times indicated. The NF-κB promoter binding activity was determined by measuring luciferase activity from transfected cells as described in Materials and Methods. The nuclear translocation of NF-κB was confirmed by determining the nuclear translocation of p65 (NF-κB) as described in Materials and Methods. (Left panels of **B** and **C**) Immunoblotting analysis: RAW264.7 cells (5×10^6 cells/ml) were stimulated by LPS (2.5 μg/ml) or F-II (300 μg/ml) for indicated times. After immunoblotting, the levels of phospho (p)-Src, p-p85, p-Akt, p-IκBα, p-ERK, p-p38, p-JNK, and β-actin were identified by their phopho- or nonphospho-specific antibodies. The results show one experiment out of three. (Right panels of **B** and **C**) NO production: RAW264.7 cells (1×10^6 cells/ml) were pretreated by various enzyme inhibitors [PP2 (25 μM), a Src kinase inhibitor; LY29 (LY29004, 25 μM), a PI3K inhibitor; SN50 (50 μM), a cell-permeable NF-κB inhibitor; cynaropicrin (20 μM); Bay 11-7082 (10 μM), a IκB kinase inhibitor; U0126 (50 μM); SB203580 (25 μM); and SP600125 (25 μM)] in the presence or absence of F-II (300 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. Data represent mean±SEM of three independent observations performed in triplicate. *: $p<0.05$ and **: $p<0.01$ compared with control.

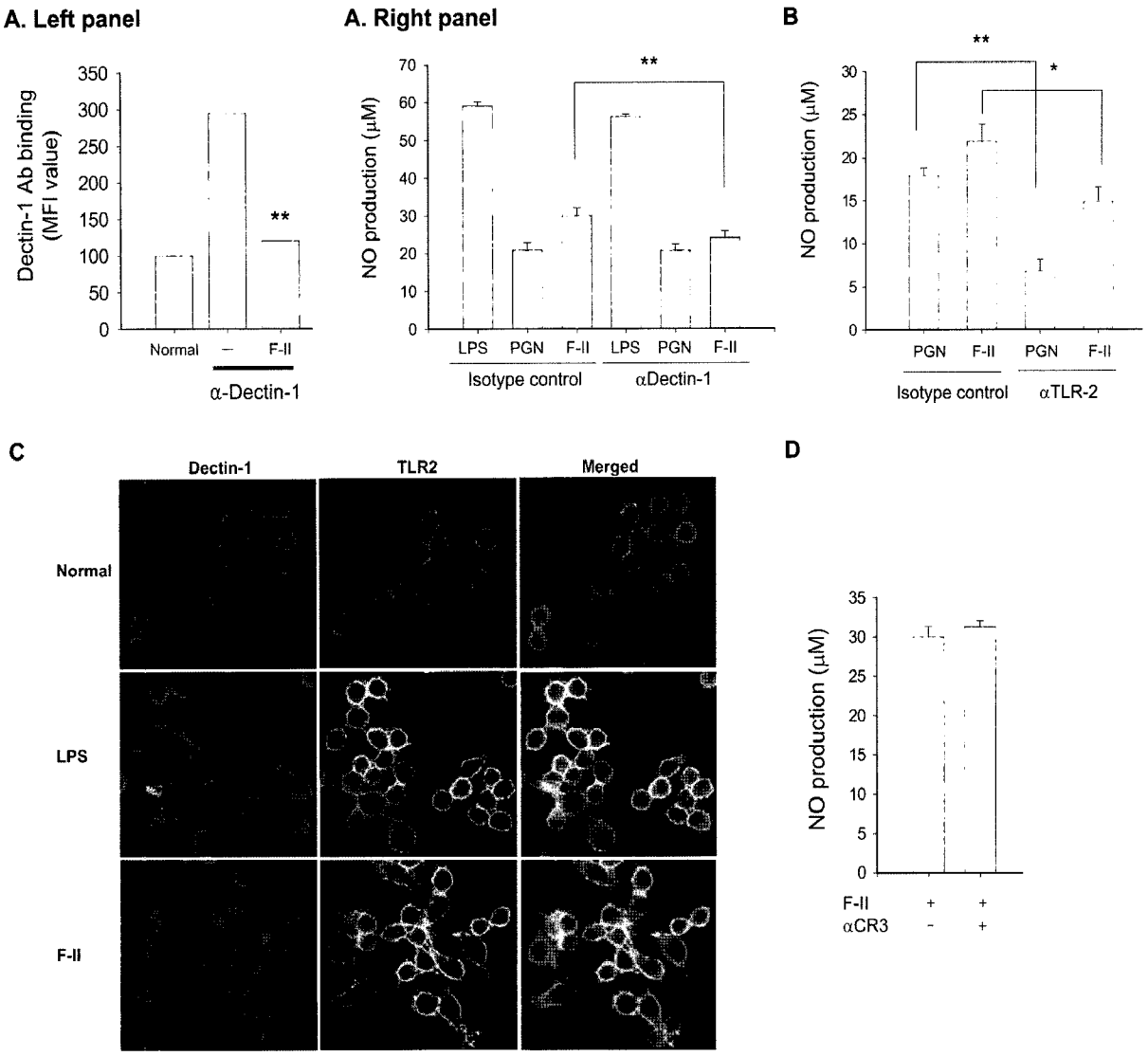

Fig. 4. The involvement of surface glycoproteins, dectin-1, TLR-2, and CR3 in F-II-mediated NO production.
(**A** left panel) Flow cytometry: RAW264.7 cells (2×10^6 cells/ml), pretreated with F-II (300 μg/ml), were stained with antibody to dectin-1 (1 μg/ml each) for 1 h. The extent of the antibody binding was determined by flow cytometric analysis, as described in Materials and Methods. [**A** (right panel), **B**, and **D**] NO production: RAW264.7 cells (1×10^6 cells/ml) were pre-treated by function-blocking antibodies (10 μg/ml each) to dectin-1, TLR-2, CR3, or isotype control (IgG) in the presence or absence of F-II (300 μg/ml), PGN (50 μg/ml), or LPS (2.5 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. Data represent mean±SEM of three independent observations performed in triplicate. **C**. Confocal microscopy: Colocalization of dectin-1 and TLR-2 was determined by confocal microscopic analysis as described in Materials and Methods. The results show one experiment out of three. *: $p<0.05$ and **: $p<0.01$ compared with control.

The exopolysaccharide fraction (F-II) from *L. edodes* remarkably upregulated the innate immune functions of macrophages. Thus. parameters such as phagocytotic uptake (Fig. 1A), ROS generation (Fig. 1B), cytokine production (Figs. 1C and 2), NO production (Fig. 1D), and morphological changes (Fig. 1E), indicating macrophage activation, proposed that F-II is able to upregulate macrophage functions, as in the case of LPS and PGN (data not shown). Considering the immunostimulatory activities of F-II, the expression of cytokines and chemokines such as TNF-α, IL-23, and MIP-2 was found to be comparable in F-II treatment to LPS (Figs. 1 and 2). The upregulation of macrophage functions by mushroom-derived principles has been reported in numerous papers. Indeed, D-fraction, PG101, grifolan, MD fraction, fucogalactan, and lectins (TML-1 and TML-2) from *G. frondosa*, *L. lepidesus*, *S. aspratus*, and *T. monglicum* strongly increased the production of cytokines such as TNF-α, IL-1β, IL-6, IL-12, and GM-CSF [11, 3, 30]. Fractions I and II from Maitake (*Grifola frondosa*) composed mainly of polysaccharides with molecular masses of 43–140 and 13–38 kDa, respectively, showed marked activity in enhancing phagocytosis of human polymorphonuclear neutrophils (PMN) [45]. Acidic polysaccharide (PL) isolated from *Phellinus linteus* has been found to enhance the phagocytotic uptake by peritoneal macrophages, although there was no induction of ROI

production [35]. Taken together, F-II may be regarded as one of the macrophage function upregulating agents in a similar manner to other mushroom-derived products.

The activation of macrophages by microbial products accompanies transcriptional upregulation of large numbers of genes involved in innate immunity. The transcriptional control is known to be managed by redox-sensitive transcription factors such as NF-κB and AP-1 [21]. Other researchers and we have found that polysaccharides from mushrooms indeed triggered transcriptional activation of proinflammatory genes such as cytokines and inflammatory enzymes (Fig. 2). Since β-glucan has been reported to induce nuclear translocation of NF-κB [15, 26], we tried to address whether F-II was capable of controlling the functional role of NF-κB. As Fig. 3 depicts, several lines of evidence suggest that F-II-mediated activation of macrophages including the expression of proinflammatory genes could be mediated by the activation of NF-κB, as well. Thus, reporter gene assay using NF-κB promoter-containing luciferase construct, confocal microscopy data on nuclear translocation of NF-κB, biochemical analysis by Western blotting of p-IκBα, and pharmacological dissection using specific NF-κB inhibitors (SN50: a cell-permeable NF-κB inhibitor; cynaropicrin, a sesquiterpene lactone compound with a selective NF-κB inhibition; and Bay11-7082, an IκB inhibitor) apparently indicated the involvement of the transcription factor NF-κB. Similarly, most microbial products such as zymosan (a mixture prepared from yeast cell wall), (1-->3)-beta-D-glucan, biglycan, a small leucine-rich PGN, and LPS are known to be strong inducers of NF-κB [37, 38], suggesting that NF-κB may play a central role in macrophage-mediated cellular activation by pathogenic or nonpathogenic components.

Since not many papers have been reported on the signaling cascade for NF-κB activation by mushroom-derived immunostimulants, we next carefully explored how F-II was able to induce NF-κB activation. Several major pathways are currently considered to be relevant to the activation of the transcription factor. A signaling cascade composed of Src, PI3K, and Akt and an activation pathway of ERK, p38, and JNK are the examples of the pathways [17, 24]. Although a yeast cell wall product, zymosan, with a similar characteristic to mushroom-originated β-glucan was reported to activate these pathways including Syk kinase [34, 36], it has not yet been proven regarding the regulatory roles of protein polysaccharides complexes (F-II) from fungal mushroom on the activation of PI3K and MAPKs. Interestingly, it has been found that F-II strongly triggered both pathways, and indeed, their blockade by specific inhibitors of these pathways also reduced F-II-mediated NO production. Thus, stimulation of RAW264.7 cells with F-II time-dependently upregulated the phoshorylation (a hallmark of activation) of IκBα, Akt, p85 (a regulatory subunit of PI3K), and Src (Fig. 3B), which are intracellularly required for the activation of NF-κB induced by extracellular stimuli. Moreover, all MAPKs were also found to be markedly phosphorylated (Fig. 3C), according to immunoblotting analysis. Strikingly, the upregulation pattern of these signaling enzymes seems to be different from that induced by LPS. Namely, LPS exposure to the macrophages showed remarkable phosphorylation levels at the early phase (5 min) as reported previously, whereas F-II-mediated activation of these enzymes was shown from 15 min and clearly at 30 min. This signaling pattern seems to suggest that F-II-induced activation signals may indirectly share the cellular signaling enzyme machinery *via* some adaptor proteins, unlike LPS signaling, which is able to trigger directly. Meanwhile, the fact that PP2, a Src inhibitor, LY29004, a PI3K inhibitor, and NF-κB inhibitors (SN50, cynaropicrin, and Bay 11-7082) blocked both LPS- and F-II-induced NO production indicates that a series of the upstream NF-κB activation signals connected from Src to IκBα may commonly play a positive role in cellular events for F-II- and LPS-induced NO production. However, it is not yet clear whether these signaling pathways are distinct from a pathway composed of Src, phospholipase C (PLC), Akt, and Btk, involved in releasing arachidonate [33]. In contrast, MAPK-mediated cellular activation for NO production seems to be distinguished between LPS and F-II groups. Thus, the p38 inhibitor only showed significant NO inhibitory effects upon LPS stimulation, whereas inhibitors of JNK and p38 very strongly diminished F-II-mediated NO production as in dendritic cells [11], suggesting that both JNK and p38 may participate in F-II (but not LPS)-mediated NO production as positive signals. Although we cannot exactly address the difference between the two signals, it is assumed that the activation mode of the cellular proinflammatory signaling machinery linked to each surface receptor of these microbial products may be different, and the difference may be decided by molecular interactions between the receptor and signaling molecules.

Although the major receptor for β-glucan is known to be dectin-1 in fungal immunity [43], several pattern recognition receptors (PPRs) such as TLR-2, CR3 (CD11b), and CDw17 (lactosylceramide) are also reported to participate in regulating β-glucan-induced signaling [4, 20, 22, 46]. Therefore, whether these molecules were capable of acting as a receptor for F-II in modulating macrophage activation was also investigated using flow cytometry and confocal microscopy. Fig. 4 shows that both dectin-1 and TLR-2 but not CR3 may be involved in F-II-mediated NO production, although blockade of these molecules with function-blocking antibodies did not completely suppress NO production of activated RAW264.7 cells, similar to previous reports [48]. Since F-II clearly inhibited the binding of anti-dectin-1 antibody, this incomplete inhibition seems to be due to the fact that either these blocking antibodies do not perfectly work or that other molecules are also required for F-II (as

well as β-glucan)-mediated NO production. Furthermore, confocal analysis also indicated that these two molecules may be colocalized at the same membrane site upon stimulation with β-glucan F-II, although a similar effect was previously demonstrated with dectin-1 and TLR-2-overexpressing cells [6]. Thus, F-II exposure enhanced the intensity of these two molecules at the membrane part, compared with the normal group, despite the fact that extended treatment with β-glucan (as well as LPS) decreased the surface level of TLR-2 (data not shown). Nonetheless, our data seem to suggest a possibility that there may be other molecules responsive to β-glucan, according to functional assay with blocking antibodies. Potential β-glucan-responsive PRRs will be further explored in the future.

In summary, we found that F-II with large molecular weight protein polysaccharides is able to upregulate functional activation-indicating parameters of macrophages such as phagocytotic uptake, ROS/NO production, cytokine expression, and morphological changes. The activation seems to be managed by NF-κB activation and its upstream signaling cascades such as the PI3K/Akt and MAPK pathways. More interestingly, F-II-mediated activation of macrophages may be required for the functional activation of PRRs such as dectin-1 and TLR-2, but not CR3. Therefore, our data suggest that mushroom-derived β-glucan may have immunostimulating potency *via* activation of multiple PRRs and signaling pathways.

Acknowledgments

We acknowledge the Central Laboratory of Kangwon National University for allowing us to use a FACScan, and the Korea Basic Science Institute Chuncheon Center for help with the confocal experiments. This study was also supported by grants (2006) from ARPC and KRF of the Korean government.

REFERENCES

1. Akamatsu, S., A. Watanabe, M. Tamesada, R. Nakamura, S. Hayashi, D. Kodama, M. Kawase, and K. Yagi. 2004. Hepatoprotective effect of extracts from *Lentinus edodes* mycelia on dimethylnitrosamine-induced liver injury. *Biol. Pharm. Bull.* **27:** 1957–1960.
2. Allavena, P., M. Chieppa, P. Monti, and L. Piemonti. 2004. From pattern recognition receptor to regulator of homeostasis: The double-faced macrophage mannose receptor. *Crit. Rev. Immunol.* **24:** 179–192.
3. Borchers, A. T., J. S. Stern, R. M. Hackman, C. L. Keen, and M. E. Gershwin. 1999. Mushrooms, tumors, and immunity. *Proc. Soc. Exp. Biol. Med.* **221:** 281–293.
4. Brown, G. D. 2006. Dectin-1: A signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* **6:** 33–43.
5. Brown, G. D., P. R. Taylor, D. M. Reid, J. A. Willment, D. L. Williams, L. Martinez-Pomares, S. Y. Wong, and S. Gordon. 2002. Dectin-1 is a major beta-glucan receptor on macrophages. *J. Exp. Med.* **196:** 407–412.
6. Brown, G. D., J. Herre, D. L. Williams, J. A. Willment, A. S. Marshall, and S. Gordon. 2003. Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* **197:** 1119–1124.
7. Cho, H. J., J. Y. Cho, M. H. Rhee, H. S. Kim, H. S. Lee, and H. J. Park. 2007. Inhibitory effects of cordycepin (3'-deoxyadenosine), a component of *Cordyceps militaris*, on human platelet aggregation induced by thapsigargin. *J. Microbiol. Biotechnol.* **17:** 1134–1138.
8. Cho, J. Y., K. U. Baik, J. H. Jung, and M. H. Park. 2000. *In vitro* anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from *Saussurea lappa*. *Eur. J. Pharmacol.* **398:** 399–407.
9. Cutolo, M. 1999. Macrophages as effectors of the immunoendocrinologic interactions in autoimmune rheumatic diseases. *Ann. NY Acad Sci.* **876:** 32–41; discussion 41–42.
10. Dai, Q. and S. B. Pruett. 2006. Ethanol suppresses LPS-induced Toll-like receptor 4 clustering, reorganization of the actin cytoskeleton, and associated TNF-alpha production. *Alcohol. Clin. Exp. Res.* **30:** 1436–1444.
11. Dillon, S., S. Agrawal, K. Banerjee, J. Letterio, T. L. Denning, K. Oswald-Richter, D. J. Kasprowicz, K. Kellar, J. Pare, T. van Dyke, S. Ziegler, D. Unutmaz, and B. Pulendran. 2006. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J. Clin. Invest.* **116:** 916–928.
12. Dong, Q., L. M. Jia, and J. N. Fang. 2006. A beta-D-glucan isolated from the fruiting bodies of *Hericium erinaceus* and its aqueous conformation. *Carbohydr. Res.* **341:** 791–795.
13. Doyle, S. L. and L. A. O'Neill. 2006. Toll-like receptors: From the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochem. Pharmacol.* **72:** 1102–1113.
14. Duperrier, K., A. Eljaafari, C. Dezutter-Dambuyant, C. Bardin, C. Jacquet, K. Yoneda, D. Schmitt, L. Gebuhrer, and D. Rigal. 2000. Distinct subsets of dendritic cells resembling dermal DCs can be generated *in vitro* from monocytes, in the presence of different serum supplements. *J. Immunol. Methods* **238:** 119–131.
15. Evans, S. E., P. Y. Hahn, F. McCann, T. J. Kottom, Z. V. Pavlovic, and A. H. Limper. 2005. Pneumocystis cell wall beta-glucans stimulate alveolar epithelial cell chemokine generation through nuclear factor-kappaB-dependent mechanisms. *Am. J. Respir. Cell Mol. Biol.* **32:** 490–497.
16. Fang, N., Q. Li, S. Yu, J. Zhang, L. He, M. J. Ronis, and T. M. Badger. 2006. Inhibition of growth and induction of apoptosis in human cancer cell lines by an ethyl acetate fraction from shiitake mushrooms. *J. Altern. Complement. Med.* **12:** 125–132.
17. Fishman, P., S. Bar-Yehuda, L. Madi, L. Rath-Wolfson, A. Ochaion, S. Cohen, and E. Baharav. 2006. The PI3K-NF-kappaB signal transduction pathway is involved in mediating the anti-inflammatory effect of IB-MECA in adjuvant-induced arthritis. *Arthritis Res. Ther.* **8:** R33.
18. Gordon, S. 2002. Pattern recognition receptors: Doubling up for the innate immune response. *Cell* **111:** 927–930.
19. Gordon, S. B. and R. C. Read. 2002. Macrophage defences against respiratory tract infections. *Br. Med. Bull.* **61:** 45–61.
20. Hahn, P. Y., S. E. Evans, T. J. Kottom, J. E. Standing, R. E. Pagano, and A. H. Limper. 2003. *Pneumocystis carinii* cell

wall beta-glucan induces release of macrophage inflammatory protein-2 from alveolar epithelial cells *via* a lactosylceramide-mediated mechanism. *J. Biol. Chem.* **278:** 2043–2050.
21. Hardy, K. and N. H. Hunt. 2004. Effects of a redox-active agent on lymphocyte activation and early gene expression patterns. *Free Radic. Biol. Med.* **37:** 1550–1563.
22. Herre, J., S. Gordon, and G. D. Brown. 2004. Dectin-1 and its role in the recognition of beta-glucans by macrophages. *Mol. Immunol.* **40:** 869–876.
23. Hoebe, K., Z. Jiang, P. Georgel, K. Tabeta, E. Janssen, X. Du, and B. Beutler. 2006. TLR signaling pathways: Opportunities for activation and blockade in pursuit of therapy. *Curr. Pharm. Des.* **12:** 4123–4134.
24. Hsu, T. C., M. R. Young, J. Cmarik, and N. H. Colburn. 2000. Activator protein 1 (AP-1)- and nuclear factor kappaB (NF-kappaB)-dependent transcriptional events in carcinogenesis. *Free Radic. Biol. Med.* **28:** 1338–1348.
25. Jung, K. K., H. S. Lee, J. Y. Cho, W. C. Shin, M. H. Rhee, T. G. Kim, J. H. Kang, S. H. Kim, S. Hong, and S. Y. Kang. 2006. Inhibitory effect of curcumin on nitric oxide production from lipopolysaccharide-activated primary microglia. *Life Sci.* **79:** 2022–2031.
26. Kataoka, K., T. Muta, S. Yamazaki, and K. Takeshige. 2002. Activation of macrophages by linear (1→3)-beta-D-glucans. Implications for the recognition of fungi by innate immunity. *J. Biol. Chem.* **277:** 36825–36831.
27. Kupfahl, C., G. Geginat, and H. Hof. 2006. Lentinan has a stimulatory effect on innate and adaptive immunity against murine *Listeria monocytogenes* infection. *Int. Immunopharmacol.* **6:** 686–696.
28. Kweon, M. H., W. J. Lim, H. C. Yang, and H. C. Sung. 2000. Characterization of two glucans activating an alternative complement pathway from the fruiting bodies of mushroom *Pleurotus ostreatus*. *J. Microbiol. Biotechnol.* **10:** 267–271.
29. Lull, C., H. J. Wichers, and H. F. Savelkoul. 2005. Antiinflammatory and immunomodulating properties of fungal metabolites. *Mediators Inflamm.* **2005:** 63–80.
30. Mayell, M. 2001. Maitake extracts and their therapeutic potential. *Altern. Med. Rev.* **6:** 48–60.
31. Morand, E. F. and M. Leech. 2005. Macrophage migration inhibitory factor in rheumatoid arthritis. *Front. Biosci.* **10:** 12–22.
32. Nair, P. K., S. J. Melnick, R. Ramachandran, E. Escalon, and C. Ramachandran. 2006. Mechanism of macrophage activation by (1,4)-alpha-D-glucan isolated from *Tinospora cordifolia*. *Int. Immunopharmacol.* **6:** 1815–1824.
33. Olsson, S. and R. Sundler. 2007. The macrophage beta-glucan receptor mediates arachidonate release induced by zymosan: Essential role for Src family kinases. *Mol. Immunol.* **44:** 1509–1515.
34. Olynych, T. J., D. L. Jakeman, and J. S. Marshall. 2006. Fungal zymosan induces leukotriene production by human mast cells through a dectin-1-dependent mechanism. *J. Allergy Clin. Immunol.* **118:** 837–843.
35. Park, S. K., G. Y. Kim, J. Y. Lim, J. Y. Kwak, Y. S. Bae, J. D. Lee, Y. H. Oh, S. C. Ahn, and Y. M. Park. 2003. Acidic polysaccharides isolated from *Phellinus linteus* induce phenotypic and functional maturation of murine dendritic cells. *Biochem. Biophys. Res. Commun.* **312:** 449–458.
36. Rogers, N. C., E. C. Slack, A. D. Edwards, M. A. Nolte, O. Schulz, E. Schweighoffer, D. L. Williams, S. Gordon, V. L. Tybulewicz, G. D. Brown, and C. Reis e Sousa. 2005. Syk-dependent cytokine induction by dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* **22:** 507–517.
37. Sato, M., H. Sano, D. Iwaki, K. Kudo, M. Konishi, H. Takahashi, T. Takahashi, H. Imaizumi, Y. Asai, and Y. Kuroki. 2003. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-kappa B activation and TNF-alpha secretion are down-regulated by lung collectin surfactant protein A. *J. Immunol.* **171:** 417–425.
38. Schaefer, L., A. Babelova, E. Kiss, H. J. Hausser, M. Baliova, M. Krzyzankova, G. Marsche, M. F. Young, D. Mihalik, M. Gotte, E. Malle, R. M. Schaefer, and H. J. Grone. 2005. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *J. Clin. Invest.* **115:** 2223–2233.
39. Schroder, N. W., B. Opitz, N. Lamping, K. S. Michelsen, U. Zahringer, U. B. Gobel, and R. R. Schumann. 2000. Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by *Treponema glycolipids*. *J. Immunol.* **165:** 2683–2693.
40. Son, C. G., J. W. Shin, J. H. Cho, C. K. Cho, C. H. Yun, W. Chung, and S. H. Han. 2006. Macrophage activation and nitric oxide production by water soluble components of *Hericium erinaceum*. *Int. Immunopharmacol.* **6:** 1363–1369.
41. Sorensen, H. R., A. S. Meyer, and S. Pedersen. 2003. Enzymatic hydrolysis of water-soluble wheat arabinoxylan. 1. Synergy between alpha-L-arabinofuranosidases, endo-1,4-beta-xylanases, and beta-xylosidase activities. *Biotechnol. Bioeng.* **81:** 726–731.
42. Stuhlmuller, B., U. Ungethum, S. Scholze, L. Martinez, M. Backhaus, H. G. Kraetsch, R. W. Kinne, and G. R. Burmester. 2000. Identification of known and novel genes in activated monocytes from patients with rheumatoid arthritis. *Arthritis Rheum.* **43:** 775–790.
43. Taylor, P. R., S. V. Tsoni, J. A. Willment, K. M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G. D. Brown. 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* **8:** 31–38.
44. Trinchieri, G. and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* **7:** 179–190.
45. Wu, M. J., T. L. Cheng, S. Y. Cheng, T. W. Lian, L. Wang, and S. Y. Chiou. 2006. Immunomodulatory properties of *Grifola frondosa* in submerged culture. *J. Agric. Food Chem.* **54:** 2906–2914.
46. Yadav, M. and J. S. Schorey. 2006. The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood* **108:** 3168–3175.
47. Yrlid, U., M. Svensson, C. Johansson, and M. J. Wick. 2000. *Salmonella* infection of bone marrow-derived macrophages and dendritic cells: Influence on antigen presentation and initiating an immune response. *FEMS Immunol. Med. Microbiol.* **27:** 313–320.
48. Zhao, M. W., W. Q. Liang, D. B. Zhang, N. Wang, C. G. Wang, and Y. J. Pan. 2007. Cloning and characterization of squalene synthase (SQS) gene from *Ganoderma lucidum*. *J. Microbiol. Biotechnol.* **17:** 1106–1112.
49. Zheng, R., S. Jie, D. Hanchuan, and W. Moucheng. 2005. Characterization and immunomodulating activities of polysaccharide from *Lentinus edodes*. *Int. Immunopharmacol.* **5:** 811–820.