J. Microbiol. Biotechnol. (2008), **18**(1), 95–103

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Immunostimulatory Activities of Polysaccharides from Liquid Culture of Pine-Mushroom *Tricholoma matsutake*

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Received: May 5, 2007 / Accepted: July 14, 2007

Mushrooms are regarded as one of the well-known foods and biopharmaceutical materials with a great deal of interest. Polysaccharide β-glucan is the major component of mushrooms that displays various biological activities such as antidiabetic, anticancer, and antihyperlipidemic effects. In this study, we compared the immunostimulatory potency of polysaccharide fractions, prepared from liquid culture of pine-mushroom *Tricholoma matsutake*, with a potent immunogen lipopolysaccharide (LPS), and their molecular mechanisms on the functional activation of macrophages. We found that fraction II (TMF-II) was able to comparably upregulate or highly enhance the phenotypic functions of macrophages such NO production and cytokine (IL-1β, IL-6, IL-12, and TNF-α) expression, to LPS. TMF-II triggered the phosphorylation of IκBα, a critical step for NF-κB activation and translocation. Of the upstream signaling enzymes tested, Src and Akt were thought to be the responsible upstream signaling components in induction of NO production, although TMF-II strongly upregulated the phosphorylation of all MAPK pathways. Therefore, our data suggest that *T. matsutake*-derived β-glucan may exert its immunostimulating activities with similar potency to LPS *via* activation of multiple signaling pathways linked to NF-κB activation.

Keywords: β-Glucan, *Tricholoma matsutake*, macrophage functions, NF-κB, intracellular signaling cascades

Macrophages are a type of differentiated tissue cells that originate as blood monocytes. The cells have several functions such as i)the removal of cell debris, ii)the killing of pathogenic microorganisms, and iii)the processing and presentation of antigens to lymphocytes [8, 17]. 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] [44]. For these cellular events, an intracellular alteration such as signaling cascades is known to be accompanied by a connection from cell surface molecules including pattern-recognition receptors (PRRs) to the intracellular signaling machinery [42]. 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 [11, 20]. 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, 16]. Although large amounts of macrophage-derived inflammatory mediators can cause severe inflammatory diseases such as septic shock and rheumatoid arthritis [22, 41], 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 [5, 30, 31, 45]. For example, some mushrooms such

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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 in Korea, China, and Japan [31]. 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, due 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 [31]. The majority of these components (such as β-glucan) have unique structural features including protein binding and branch structure [β-(1→6) branches] properties.

Pine-mushroom fungus *T. matsutake* is a representative high-class edible mushroom that is used for both medicinal and food purposes in Korea, China, and Japan. Although it has been traditionally known as a biologically excellent mushroom with immunostimulatory, anticancer, and antistress effects [12, 23, 24], difficulty in cultivating the fruit bodies and limited bulk availability have led us to restricted detailed studies. Recently, however, through developing a method of culturing in tanks, enabling the bulk supply of the mycelia, immunologically active polysaccharide fractions (*e.g.*, CM6271 and MPG-1) have been identified and purified. These fractions have been demonstrated to modulate TGF-β activity and NK cell activity *in vitro* and *in vivo*. We also successfully developed an *in vitro* culture method of *T. matsutake* and prepared an industrially useful exopolysaccharide fraction (TMF-II) secreted into the culture medium [21]. In this study, we aimed to prove the activating roles and action mechanisms of the polysaccharide fraction on macrophage-mediated innate immune responses. To do this, functional events mediated by activated macrophages such as the production of cytokines and the release of toxic molecule (NO) were investigated, comparing with LPS. Additionally, mechanism studies in terms of activation signaling pathways were carried out.

Materials and Methods

Materials

Polysaccharide fractions (TMF-I: non-exopolysacharide fraction in culture medium; TMF-II: exopolysaccharide fraction in culture medium) were prepared from liquid culture of *T. matsutake* by the ethanol precipitation and lyophilization methods reported previously (Yield: 6.11%) [10, 40]. These fractions contained a level of endotoxin below the detection limits (0.0015 EU/ml) as assessed by an endotoxin assay kit (Sigma, MO, St. Louis, U.S.A.). Of these, TMF-II is composed of large-molecular-weight exopolysaccharides containing more than 300 kDa β-glucan. N^G-monomethyl-L-arginine (N-MMA) and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PP2, LY294002, wortmannin, and Bay 11-7082 were obtained from Calbiochem. (La Jolla, CA, U.S.A.). Cynaropicrin was a gift from Prof. Jee Hyung Jung (Pusan National University, Pusan, Korea) [6]. Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, U.S.A.). RAW264.7 cells were purchased from the American Tissue Culture Center (Rockville, MD, U.S.A.). All other chemicals were of Sigma grade. Phospho-antibodies to Src, Akt, p38, JNK, ERK, and IκBα and the antibody to β-actin were purchased from Cell Signaling (Beverly, MA, U.S.A.).

Cell Culture

RAW264.7, U937, LoVo and C6 cells HEK293 cells were maintained in RPMI1640 supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 10% fetal bovine serum. Cells were grown at 37°C with 5% CO_2.

Determination of NO Production

After the pre-incubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, TMF-I (100 μg/ml), TMF-II (100 μg/ml), or LPS (2.5 μg/ml) were added for 24 h, as reported previously [6]. 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 of samples.

RT-PCR

For the evaluation of LPS-inducible gene mRNA expression levels, the total RNA from TMF-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-dT_{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).

MTT Assay (Colorimetric Assay)

The cytotoxic effects of TMFs and LPS were evaluated by conventional MTT assay. At 3 h prior to culture termination, 10 μl of the MTT solution (10 mg/ml in a phosphate-buffered saline, pH 7.4) was added and the cells were continuously cultured until termination. The incubation was stopped by the addition of 15% sodium dodecyl sulfate into each well for the solubilization of

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-1β	F	5'-CAGGATGAGGACATGAGCACC-3'
	R	5'-CTCTGCAGACTCAAACTCCAC-3'
IL-3	F	5'-GAAGTGGATCCTGAGGACAGATACG-3'
	R	5'-GACCCATGGGCCATGAGGAACATTC-3'
IL-6	F	5'-GTACTCCAGAAGACCAGAGG-3'
	R	5'-TGCTGGTGACAACCACGGCC-3'
IL-12p40	F	5'-CAGAAGCTAACCATCTCCTGGTTTG-3'
	R	5'-TCCGGAGTAATTTGGTGCTTCACAC-3'
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

formazan. Absorbance at 570 nm (OD_{570}) was measured by a Spectramax 250 microplate reader.

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 benzimide, 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 phosphorylation levels of Src, Akt, ERK, p38, JNK, and IκBα were visualized with their phospho-specific antibodies.

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

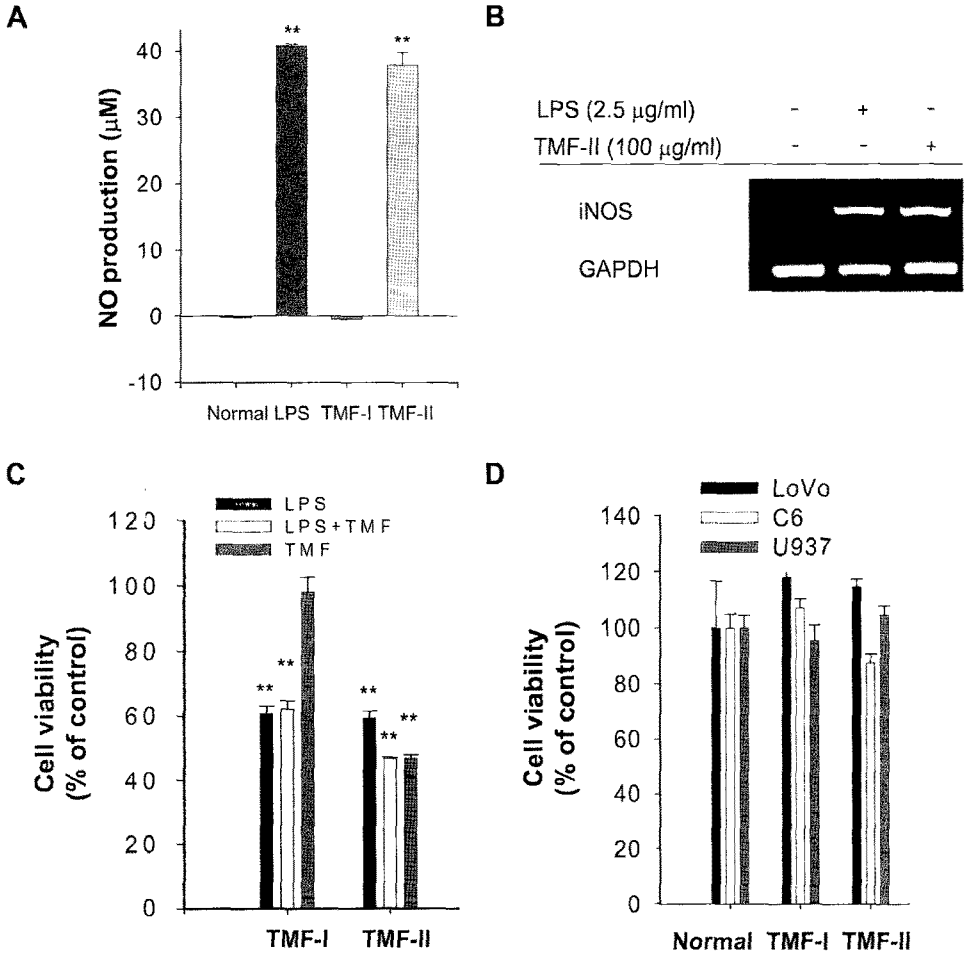

Fig. 1. The effects of TMF-II and LPS on NO production of RAW264.7 cells.

A. RAW264.7 cells (1×10^6 cells/ml) were stimulated with LPS (2.5 μg/ml) or fractions [TMF-I or TMF-II (100 μg/ml each)] for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined with Griess reagent, as described in Materials and Methods. **B.** RAW264.7 cells (1×10^7 cells/ml) were incubated with TMF-II (100 μg/ml) or LPS (2.5 μg/ml) for 6 h. The mRNA level of iNOS was determined by semiquantitative RT-PCR. The results show one experiment out of three. **C.** RAW264.7 cells (1×10^6 cells/ml) were incubated with TMF-I (100 μg/ml) or TMF-II (100 μg/ml), LPS (2.5 μg/ml), or both for 24 h. **D.** LoVo, C6, or U937 cells (1×10^6 cells/ml) were incubated with TMF-I (100 μg/ml) or TMF-II (100 μg/ml) for 24 h. The viability of the cells was determined by MTT assay as described in Materials and Methods. Data (**A**, **C**, and **D**) [% of control (NO levels induced by LPS or TMF-II alone or viability of untreated cells set as 100%)] represent mean±SEM of three independent observations performed in triplicate. **: $p<0.01$ compared with immunogen-untreated normal (RAW264.7) cells.

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 on NO Production

To address whether polysaccharides from *T. matsutake* and LPS from *E. coli* were different in inducing macrophage activation, we first examined the effects of the immunogens on NO production using a macrophage-like cell line, RAW264.7 cells. As Fig. 1 shows, both LPS and TMF-II upregulated effectively and similarily NO release from the macrophages. TMF-II also dose-dependently triggered NO production, maximized at 100 μg/ml (data not shown). In particular, NO upregulation by these immunogens was also accompanied with increased iNOS expression, according to semiquantitative RT-PCR analysis (Fig. 1B). Cytotoxicity analysis indicated that these immunogens were able to affect RAW264.7 cells viability (Fig. 1C). However, these cytotoxic effects of the immunogens were not exhibited in cancer cell lines (LoVo, C6, and U937 cells), NO non-producing cells (Fig. 1D), indicating that the cytotoxic effects of LPS and TMF-II were not caused by their direct toxicity but by producing NO, a strong cytotoxic molecule. Therefore, we continued further experiments to evaluate the various immunological activities of polysaccharides from *T. matsutake* in triggering macrophage activation, comparing with those of LPS under the same conditions.

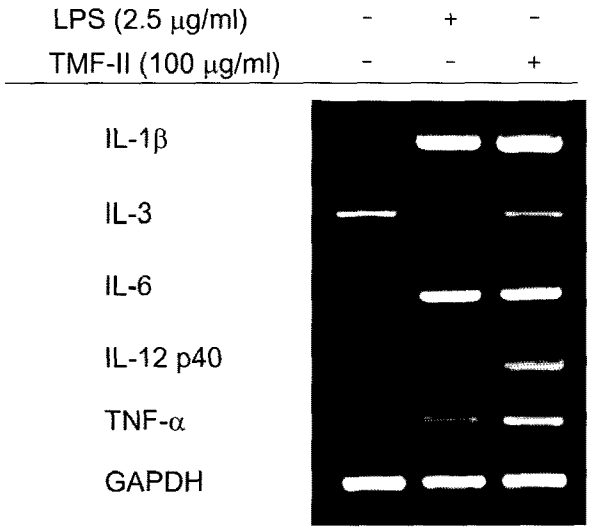

Fig. 2. The effect of TMF-II and LPS on the expression of cytokines in RAW264.7 cells.
RAW264.7 cells (1×10^7 cells/ml) were incubated with TMF-II (100 μg/ml) or LPS (2.5 μg/ml) for 6 h. The mRNA levels of cytokines were determined by semiquantitative RT-PCR. The results show one experiment out of three.

Induction of Transcriptional Expression of Proinflammatory Cytokines

To elicit innate immune responses by macrophages, newly synthesized proteins such as proinflammatory cytokines and inflammatory enzymes are required. Therefore, we next compared whether there is a difference between TMF-II and LPS in inducing transcriptional upregulation of these genes by RT-PCR. Indeed, Fig. 2 depicts that TMF-II more strongly stimulated the expression of IL-1β, IL-12, and TNF-α than LPS.

Distinct Activation of NF-κB Activation by LPS and TMF-II

Based on previous papers that LPS and mushroom-derived immunostimulants such as β-glucan activate a series of signaling pathways for NF-κB activation [22, 23], we first

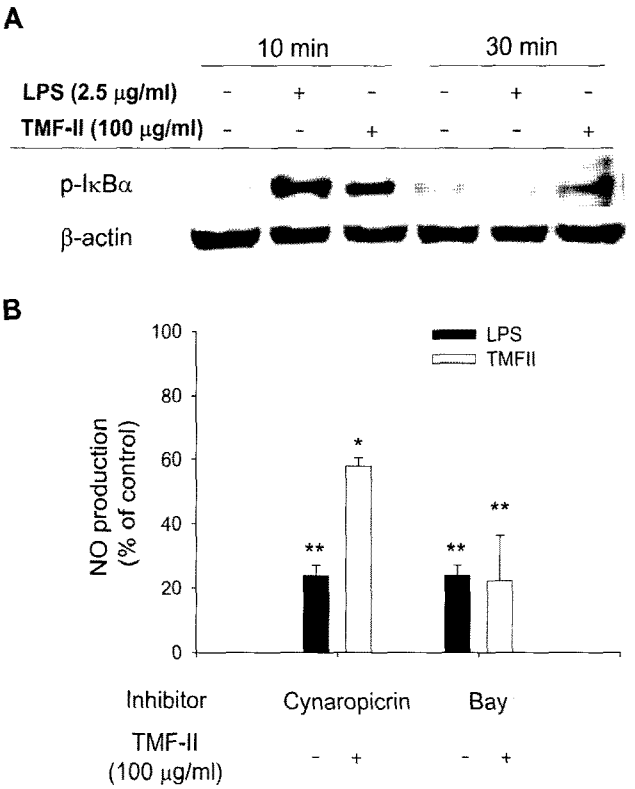

Fig. 3. The effect of TMF-II and LPS on the upregulation of NF-κB activation.
A. RAW264.7 cells (5×10^6 cells/ml) were stimulated with LPS (2.5 μg/ml) or TMF-II (100 μg/ml) for 10 and 30 min. After immunoblotting, the level of phospho-IκBα and β-actin was identified with its phopho-specific or specific antibody. The results show one experiment out of three. **B.** RAW264.7 cells (1×10^6 cells/ml) were pretreated with NF-κB inhibitors [cynaropicrin (20 μM) and Bay 11-7082 (10 μM)] in the presence or absence of TMF-II (100 μg/ml) or LPS (2.5 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined with Griess reagent, as described in Materials and Methods. Data [% of control (NO levels induced by LPS or TMF-II alone set as 100%)] represent mean±SEM of three independent observations performed in triplicate. **: $p<0.01$ compared with control (LPS or TMF-II treated group).

investigated the involvement of NF-κB related molecular events. Several lines of evidence obtained by biochemical and pharmacological analyses also supported this possibility. Thus, LPS strongly increased the phosphorylation of IκBα at 10 min, whereas TMF-II triggered the phosphorylation at both 10 min and 30 min, according to immunoblotting analysis (Fig. 3A). Furthermore, strong NF-κB inhibitors (cynaropicrin and Bay11-7082) also diminished both LPS- and TMF-II-mediated NO production (Fig. 3B), suggesting that NF-κB activation is tightly linked to upregulation of nitric oxide production by these immunogens.

TMF-II Induces the Activation of Src/PI3K/Akt and MAPK

Whether NF-κB activation by TMF-II and LPS is triggered by upstream signaling cascades was carefully explored using immunoblotting analysis and pharmacological assay. Fig. 4A shows that TMF-II as well as LPS were able to strongly induce the phosphorylation of Src and Akt. In particular, the phosphorylation level of Src was more increased in the TMF-II treatment group. LPS upregulated the phosphorylation of Akt at 10 min, whereas TMF-II was shown to highly trigger Akt phosphorylation at 30 min. In agreement, selective pharmacological inhibitors [PP2 (a Src inhibitor); LY294002 and wortmannin (PI3K inhibitors); and cynaropicrin and Bay11-7082 (IκB inhibitors)] of these enzymes were also exhibited to inhibit TMF-II (as well as LPS)-mediated NO production up to 70 to 95% (Fig. 4B).

In addition, the activation pattern of MAPKs (ERK, p38, and JNK) under LPS or TMF-II treatment was also distinct. Thus, TMF-II highly triggered the phosphorylation of p38 and ERK at 10 min, compared with LPS, whereas LPS

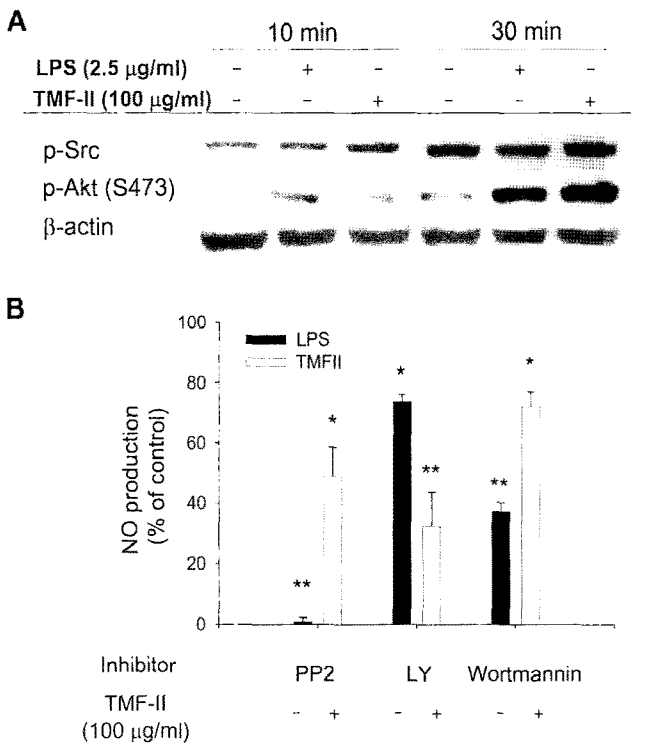

Fig. 4. The effect of TMF-II and LPS on the upregulation of upstream signaling pathways for NF-κB activation.

A. RAW264.7 cells (5×10^6 cells/ml) were stimulated with LPS (2.5 μg/ml) or TMF-II (100 μg/ml) for 10 and 30 min. After immunoblotting, the levels of phospho (p)-Src, p-Akt, and β-actin were identified with their phospho-specific or specific antibodies. The results show one experiment out of three. **B.** RAW264.7 cells (1×10^6 cells/ml) were pretreated with various enzyme inhibitors [PP2 (25 μM), a Src kinase inhibitor; LY29 (LY29004, 25 μM) and wortmannin (25 μM), a PI3K inhibitor] in the presence or absence of TMF-II (100 μmg/ml) or LPS (2.5 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined with Griess reagent, as described in Materials and Methods. Data [% of control (NO levels induced by LPS or TMF-II alone set as 100%)] represent mean±SEM of three independent observations performed in triplicate. **: $p<0.01$ compared with control (LPS or TMF-II treated group).

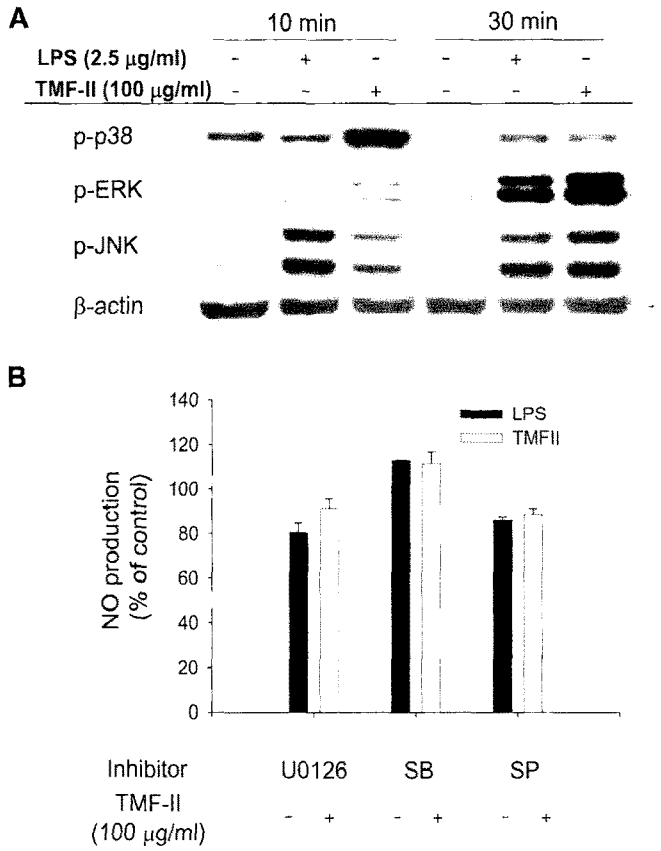

Fig. 5. The effect of TMF-II and LPS on the upregulation of MAPK.

A. RAW264.7 cells (5×10^6 cells/ml) were stimulated with LPS (2.5 μg/ml) or TMF-II (100 μg/ml) for 10 and 30 min. After immunoblotting, the levels of phospho (p)-p38, p-ERK, p-JNK, and β-actin were identified with their phospho-specific or specific antibodies. The results show one experiment out of three. **B.** RAW264.7 cells (1×10^6 cells/ml) were pretreated with MAPK inhibitors [U0126 (20 μM), SB203580 (10 μM), and SP600125 (10 μM)] in the presence or absence of TMF-II (100 μg/ml) or LPS (2.5 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined with Griess reagent, as described in Materials and Methods. Data [% of control (NO levels induced by LPS or TMF-II alone set as 100%)] represent mean±SEM of three independent observations performed in triplicate.

remarkably induced phosphorylation of JNK at 5 min (Fig. 5A). Interestingly, pharmacological dissection of these enzymes with specific inhibitors [SB203580 (p38), SP600125 (JNK), and U0126 ERK)] did not block the production of TMF-II-induced NO production (Fig. 5B), suggesting that MAPK (but not Src/PI3K/Akt)-mediated signaling may be not involved in both TMF-II- and LPS-induced NO production pathways consistent with previous papers [7, 27]. However, we found that the MAPK inhibitors at these concentrations strongly blocked prostaglandin (PG)E_2 production up to 80 to 90%, indicating that the inhibitors are working (data not shown).

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* [31]. *T. matsutake* is the most famous one, used as food and for medicinal purposes. Owing to numerous studies, it has been demonstrated that polysaccharides (*e.g.*, lentinan) from *T. matsutake* display potent *in vivo* immunostimulating activities such as anticancer and antibacterial effects [1, 14, 29]. However, the molecular mechanism by which the β-glucan upregulates the innate and adaptive immunity of macrophages and T cells remains largely unelucidated. In this study, therefore, functional activation of macrophages by the β-glucan fraction from *T. matsutake* and their molecular mechanism in upregulating macrophage-mediated innate immune responses were carefully explored, comparing with LPS-induced macrophage activation events.

The polysaccharide fraction (TMF-II) from *T. matsutake* remarkably upregulated the innate immune functions of macrophages. Thus, parameters, such as NO production (Fig. 1A) and cytokine expression (Fig. 2), indicating macrophage activation proposed that TMF-II is able to upregulate macrophage functions, as in the case of LPS and PGN (data not shown). Considering the immunostimulatory activities of TMF-II, the expression of cytokines such as TNF-α, IL-1β, IL-6, and IL-12 was found to be comparable in TMF-II treatment to that of the LPS group (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 [31, 32, 40]. Fractions I and II from Maitake (*Grifola frondosa*) mainly composed of polysaccharides with large molecular masses (>300 kDa), respectively, showed marked activity in enhancing phagocytosis of human polymorphonuclear neutrophils (PMN) [43]. Acidic polysaccharide (PL) isolated from *Phellinus linteus* has been found to enhance the phagocytic uptake by peritoneal macrophages, although there was no induction of ROI production [36]. Taken together, TMF-II may be regarded as one of the macrophage function upregulating agents, in a similar manner to other mushroom-derived products and LPS as well.

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 nuclear factor (NF)-κB and activator protein (AP)-1 [19]. Other researchers and we have found that polysaccharides from mushrooms indeed triggered transcriptional activation of proinflammatory genes such as for cytokines and inflammatory enzymes (Fig. 2). Since β-glucan has been reported to induce nuclear translocation of NF-κB [13, 25], we tried to address whether TMF-II was capable of controlling the functional role of NF-κB. As Fig. 3 depicts, several lines of evidence suggest that TMF-II-mediated activation of macrophages including the expression of proinflammatory genes could be mediated by the activation of NF-κB as well. Thus, biochemical analysis by Western blotting of p-IκBα, and pharmacological dissection using specific NF-κB inhibitors (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, as shown in the case of LPS. 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 [38, 39], 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 TMF-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 [15, 22]. 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 [35, 37], it has not yet been proven regarding the regulatory roles of polysaccharides (TMF-II) from fungal mushroom *T. matsutake* on the activation of PI3K and MAPKs. Interestingly, TMF-II was strongly found to trigger both pathways, and indeed, their blockade

by specific inhibitors of these pathways also reduced TMF-II-mediated NO production. Thus, stimulation of RAW264.7 cells with TMF-II strikingly upregulated the phosphorylation (a hallmark of activation) of Akt and Src (Fig. 4), 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. 3D), according to immunoblotting analysis. Strikingly, the upregulation pattern of MAPK and Akt activation seems to be different from that induced by LPS. Namely, LPS exposure to the macrophages showed remarkable phosphorylation levels of JNK and Akt (but not p38 and ERK) at the early phase (10 min), whereas TMF-II-mediated activation of p38 and Src but not JNK and ERK was clearly displayed at 10 min. However, both LPS and TMF-II increased the phosphorylation of all MAPKs, Src, and Akt at 30 min. This signaling pattern seems to suggest that TMF-II-induced activation signals may in turn share cellular signaling enzyme machinery with the LPS-evoked pathway. The fact that PP2, a Src inhibitor, LY29004, a PI3K inhibitor, and NF-κB inhibitors (cynaropicrin and Bay 11-7082) blocked both LPS- and TMF-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 TMF-II- and LPS-induced NO production, even though there is a time-dependent stimulation. Since zymosan has been reported to induce Src, phospholipase C (PLC), Akt, and Btk for releasing arachidonate in mast cells [42], a possibility to trigger the activation of PLC and Btk by TMF-II will be further examined. Although we cannot exactly address the difference between the two signals (LPS and TMF-II), 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 [9], several PRRs such as TLR-2, CR3 (CD11b), and CDw17 (lactosylceramide) are also reported to participate in regulating β-glucan-induced signaling [4, 18]. Therefore, whether these molecules were capable of acting as a receptor for TMF-II in modulating macrophage activation will be further investigated.

In summary, we found that TMF-II is able to upregulate functional activation-indicating parameters of macrophages such as NO production and cytokine expression, comparable to LPS exposure. The activation seems to be managed by NF-κB activation and its upstream signaling cascades such as PI3K/Akt and MAPK pathways with the distinct upregulation pattern from LPS, according to immunoblotting analysis and pharmacological dissection with specific inhibitors to the enzymes. Therefore, our data suggest that the polysaccharide fraction (TME-II) from *T. matsutake* may have macrophage-stimulating potency *via* activation of multiple signaling pathways, some that are shared with LPS.

Acknowledgment

This project was supported by an ARPC grant (2006) from the Korean Government.

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