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Immunomodulatory Properties of *Grifola frondosa* in Submerged Culture

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Maitake (*Grifola frondosa*) is a popular mushroom in Asia for its tasty flavor and immune-stimulating property. The aim of the study is to investigate the innate immunity augmentation effects of different extracts of mycelia and culture filtrate from *G. frondosa* in submerged cultures. The hot water extract of mycelia showed the strongest cytokine induction effect as a function of its concentration in human whole blood culture. The most potent fractions of hot water extract, Fr. I and II, were mainly composed of polysaccharides with molecular masses of 43−140 and 13−38 kDa, respectively. These fractions (0.025 mg/mL) showed marked activity in enhancing phagocytosis of human polymorphonuclear neutrophils (PMN). In parallel, the expression of CD11b, an early marker of PMN activation, was also up-regulated dose dependently. This result suggested that complement receptor 3 was primed by these fractions. In addition to activation of phagocytes, these bioactive fractions also increased human peripheral blood natural killer cell cytotoxicity. These results imply that the relatively low molecular mass polysaccharides isolated from mycelia of *G. frondosa* can enhance innate immunity in vitro and therefore may serve as biological response modifiers.

KEYWORDS: ***Grifola frondosa*; immunomodulatory effect; phagocytosis; cytokine; NK cytotoxicity; CD11b**

INTRODUCTION

Mushrooms have been valued as an edible and medical resource for thousands of years. Among them, maitake (*Grifola frondosa*) may be one of the most versatile and promising medicinal mushrooms for use as a dietary supplement. Polysaccharides of the *G. frondosa* fruiting body, grifolan (GRN), are the best known and most potent substances with antitumor and immunomodulating properties (*1*). These polysaccharides are of different chemical compositions, with most belonging to the group of β-glucans; these have β-1,3-linkages in the main chain of the glucan and additional β-1,6-branch points (*2*−*4*). The polysaccharide structures in cultured mycelia, however, are more diverse than those in the fruiting body and may depend on the composition of the nutrient medium used for cultivation (*5*). Some research demonstrated that polysaccharides from mycelia are heteromanans, heterofucans, and heteroxylans or their complexes with protein (*6*). However, other research revealed similar β-glucan structures in cultured mycelia (*7*, *8*).

The innate immunity plays key roles in the host defense system. The innate immune responses involve phagocytic cells (neutrophils, monocytes, and macrophages); cells that release inflammatory mediators (basophils, mast cells, and eosinophils); natural killer (NK) cells; and molecules such as complement proteins, acute phase proteins, and cytokines. The induction of cytokine synthesis is one of the methods commonly used to evaluate potentials for immunoaugmentors. Cytokines are intercellular signaling proteins released by both immune and nonimmune cells. They play important roles in controlling homeostasis of the whole organism by the induction of cell differentiation, proliferation, and apoptosis, as well as defense functions such as immune responses and inflammatory reactions. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-γ (IFN-γ), known as proinflammatory cytokines, modulate the acute phase response that involves potent systemic and local effects. Several publications (*9*, *10*) demonstrated that GRN, an antitumor β-glucan isolated from the *G. frondosa* fruiting body, activates macrophages to produce TNF-α, IL-1, and IL-6 in vitro. GRN administrated intraperitoneally (ip) also significantly elevated lipopolysaccharide (LPS)-elicited TNF-α production in the sera of animal models (*11*). In addition, they also reported that an insoluble as well as a high molecular mass-soluble form of GRN are required for TNF-α production in RAW 264.7 macrophages (*12*).

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Phagocytes form a vitally important front-line defense against infection and are a major part of innate immunity. Phagocytosis is the primary method to remove free microorganisms in the blood and tissue fluids and is mediated mainly by polymorphonuclear neutrophils (PMNs) and macrophages. Several biological response modifiers can increase the phagocytic activity of PMN and monocytes (*13*, *14*).

PMNs play a major role in innate immunity and host defense against infection, and an essential component of their activation is expression of CD11b on their cell surface. CD11b is a subunit of the CD11/CD18 heterodimeric complex, which is one of a subfamily of four cell surface integrin receptors sharing a common β-chain (β2 or CD18). CD11b is involved in neutrophil localization at inflammatory sites and phagocytosis of iC3b- or IgG-coated particles, and its expression is rapidly up-regulated on neutrophil activation (*15*). CD11b/CD18 (complement receptor 3, CR3) is therefore a critical complex for normal host defense (*16*).

The NK cells are lymphocytes of innate immunity and play a decisive role in the defense against virus-infected and malignant cells by virtue of cytotoxicity activity (*17*). They also produce cytokines such as IFN-γ, TNF-α, and GM-CSF, which can modulate natural and specific immune responses. Recent research showed that D-fraction, a commercial β-glucan prepared from the *G. frondosa* fruiting body, enhanced the cytotoxicity activity of NK cells in cancer patients and animal models (*18*, *19*).

To obtain bioactive components from *G. frondosa*, most investigators focused on fruiting body production rather than submerged cultures. The cultivation of *G. frondosa* to produce fruiting bodies is a long-term process requiring from one to several months for the first fruiting bodies to appear. Nowadays, many attempts are made through submerged culture to accelerate the process and to result in biomass yield within a few days (*20*−*22*).

The aim of this research was to study the immunomodulatory properties of *G. frondosa* in submerged cultures. *G. frondosa* TSRI01 was isolated locally and cultivated in a 100 L fermentor. Different fractions were prepared from mycelia and culture filtrates. The induction of cytokine synthesis in human whole blood was employed to evaluate the immunostimulating effects. The most potent fractions were then obtained and characterized. The phagocytic activities and cytotoxicity activity of peripheral blood NK cells were also analyzed to better understand the activities of these potent fractions. Finally, the effects of these bioactive fractions on the activation of PMN and possible cell surface receptors involved were also investigated in this study.

MATERIALS AND METHODS

Microorganism and Media. *G. frondosa* TSRI01 was isolated from a mountainous district in Taiwan. The stock medium was made up of potato dextrose agar (Merck KGaA, Darmstadt, Germany) containing 0.5% yeast extract (Difco Laboratory, Detroit, MI). The stock slants were incubated at 28 °C for 7 days and then stored at 4 °C. The seed culture medium was composed of 2% glucose, 0.15% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ (all from Merck), 0.4% yeast extract, 0.2% malt extract, and 0.2% peptone (all from Difco). The fermentation medium was made up of 1% glucose, 0.8% yeast extract, 0.15% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.2% $(NH_4)_2SO_4$.

Fermentation. *G. frondosa* TSRI01 was initially grown on stock medium in a Petri dish at 28 °C for 7 days and then transferred to the seed culture medium by punching out a 10 mm diameter of mycelia out of the agar plate culture with a sterilized cutter. The primary seed culture experiments were performed in 500 mL Hinton flasks containing 100 mL of the seed culture medium and incubated at 28 °C on a rotary shaker at 110 rpm for 6 days. The secondary seed culture experiments were performed in 1 L Hinton flasks containing 300 mL of fermentation medium after inoculation with 10% (v/v) of the primary seed culture under the conditions described above. Fermentation was then performed in a 100 L fermentor (Bioengineering AG, Wald, Switzerland) containing 50 L of fermentation medium after inoculation with 5% (v/v) of the secondary seed culture. The fermentation was performed at 28 °C, with an aeration rate of 1.0 vvm, an agitation speed of 150 rpm, and an initial pH of 5.4 for 10 days. Mycelia were then separated from the cultivation broth by vacuum filtration and then washed with distilled water. Finally, the mycelia were lyophilized to a powder form.

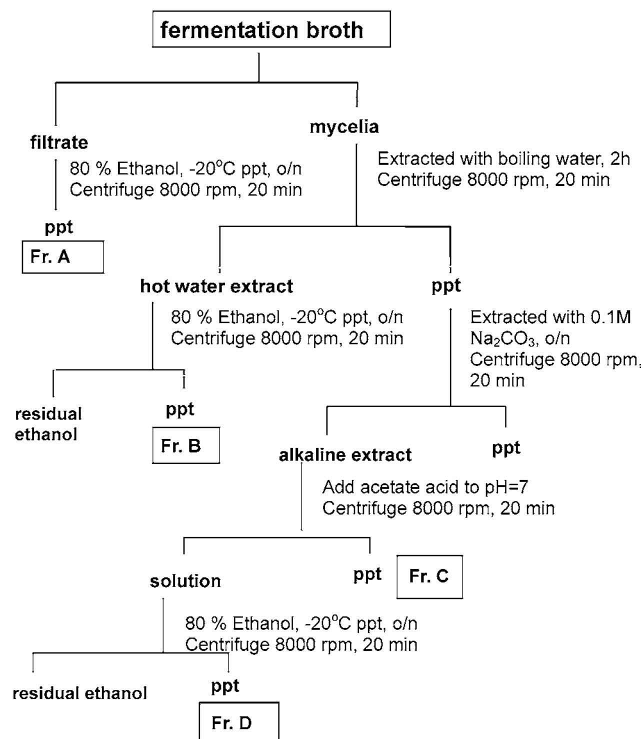

Figure 1. Flow diagram showing the steps in preparation of different fractions from submerged cultivated *G. frondosa*.

Preparation of Filtrate. One liter of the obtained culture filtrate was concentrated 6-fold under vacuum, and 80% ethanol (final concentration, v/v) was added and left overnight at −20 °C. The precipitate was collected after centrifugation and lyophilized to a powder form, which was designated as Fr. A.

Preparation of Extracts of Mycelia. The lyophilized mycelia from the submerged culture were homogenized at 7000 rpm for 2 min at room temperature (Kinematica polytron PT 3000, Lucerne, Switzerland) and then extracted with boiling water for 2 h. The hot water extract was centrifuged at 8000 rpm for 20 min (Sorval RC-5B, rotor SS-34), and 80% ethanol (final concentration, v/v) was then added to the supernatant and left overnight at −20 °C. The precipitate was collected after centrifugation and lyophilized to a powder form, which was designated as Fr. B.

The residual mycelia were further extracted with 0.1 M Na_2CO_3 at 25 °C overnight. The alkaline extract was centrifuged at 8000 rpm for 20 min to separate it from residual mycelia. Glacial acetic acid was then added to the supernatant until a neutral pH was obtained. The precipitate was collected after centrifugation and lyophilized to a powder form, which was designated as Fr. C.

From the remaining solution, 80% ethanol (final concentration, v/v) was added and left overnight at −20 °C. The precipitate was collected after centrifugation and lyophilized to a powder form, which was designated as Fr. D. The extraction flowchart is shown in **Figure 1**.

Fractionation of Hot Water Extract of Mycelia. Mycelia were extracted with boiling water as described above and then fractionated by stepwise precipitation with 50, 67, 75, and 80% of ethanol (final concentration, v/v). The obtained precipitates were designated as Fr. I, II, III, and IV, respectively. The fractionation process is shown in

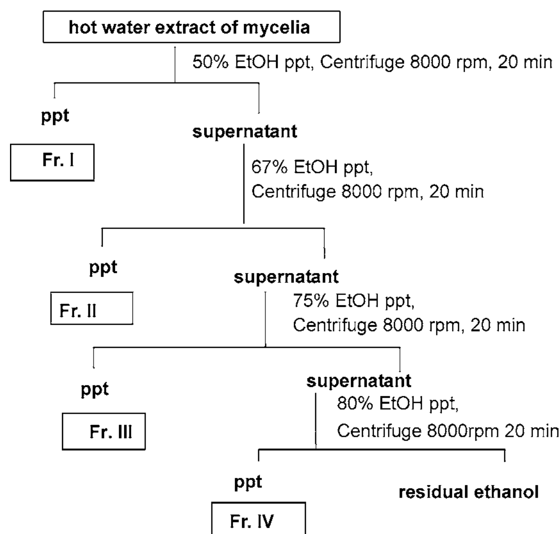

Figure 2. Fractionation of hot water extract of *G. frondosa* mycelia. *G. frondosa* TSRI01 mycelia from submerged culture were extracted with hot water as described in the Materials and Methods. Subsequent fractionation was performed by stepwise addition of increasing amounts of ethanol followed by precipitation.

Figure 2. The polysaccharide and protein contents as well as molecular mass distribution of each fraction were analyzed as described below.

Analysis of Polysaccharide Content. The free reducing sugar of each fraction was measured before it was completely hydrolyzed with 2 N HCl in a 100 °C water bath for 1 h. The total and free reducing sugar contents were measured by the DNS (3,5-dinitrosalicylic acid) method (*23*) using glucose as a standard. The polysaccharide contents were calculated by deduction of free reducing sugar from total reducing sugar content.

Analysis of Protein Content. The protein content of each fraction was analyzed by the Bradford assay (*24*) utilizing the Protein Assay Reagent (Amresco, Solon, OH). A595 was determined using a spectrophotometer (Beckman Coulter, Fullerton, CA). Bovine serum albumin (Sigma) served as a standard.

Molecular Mass Determination. The molecular mass of polysaccharides was estimated by a Waters 2690 GPC (gel permeation chromatography) system (Waters Co., Milford, MA) with an Ultrahydrogel linear column (7.8 mm × 300 mm). The mobile phase was 0.1 M $NaNO_3$ (containing 0.02% NaN_3), the column temperature was 48 °C, the flow rate was 0.1 mL/min, and the injection volume was 10 μL. The elute was monitored by a refractive index detector (Waters 2410 refractometer). A set of pullulan standards P-400, P-200, P-100, P-50, P-20, P-10, and P-5 (Shodex Standard, P-82, Macherey-Nagel, Germany) were used for calibration of the columns. Therefore, the molecular mass parameters shown are relative to the pullulan reference.

Evaluation of Cytokine Inducing Capacity. Blood was collected from healthy volunteers who had not recently undergone either antibiotic or antimycotic treatment. Diluted human whole blood (1:10 dilution in RPMI-1640 medium, Sigma Chemical, St. Louis, MO) was incubated with various fractions of polysaccharides from *G. frondosa* culture, LPS (0.25 μg/mL; *Escherichia coli* O55:B5, Sigma), or phytohemagglutinin (PHA, 10 μg/mL, Sigma) in a humidified incubator at 37 °C in 5% CO_2. To rule out a possible contamination by the endotoxin of our polysaccharide samples, polysaccharides with added polymyxin B (10 μg/mL, Sigma) were tested in parallel (*25*).

Cytokine Measurements. Cytokine concentrations in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) kits that were specific against human cytokines. Levels of TNF-α and IL-6 were measured after culture for 24 h, and levels of IFN-γ were measured after 72 h using ELISA sets from Pierce Endogen (Rockford, IL). Assays were performed according to the manufacturer's instructions.

Effect of Polysaccharides on Phagocytosis. Various fractions (0.025 mg/mL) were added into 100 μL aliquots heparinized whole blood drawn from healthy volunteers and agitated in a closed water bath at 37 °C and 60 rpm. The reaction was stopped after 30 min by putting the test tubes on ice. To rule out a possible contamination by the endotoxin of our polysaccharide samples, polysaccharides with added polymyxin B (10 μg/mL, Sigma) were tested in parallel. Phagocytic activities were analyzed as described below.

Phagocytosis Analysis. The phagocytic activities of leukocytes, monocytes, and PMNs in heparinized whole blood were determined with Phagotest (Orpegen Pharma, Heidelberg, Germany) using a modified protocol. Briefly, 100 μL of heparinized whole blood was treated with polysaccharides as described above. The blood was then mixed with 2.5 μL of precooled, fluorescein-labeled opsonized *E. coli* bacteria (10^9/mL) and incubated for 10 min at 37 °C in a closed water bath and agitated constantly at 110 rpm to allow phagocytosis. Control samples remained on ice. Phagocytosis was stopped by placing all samples on ice simultaneously and adding 100 μL of quenching solution (0.4% of trypan blue) for suppression of signals from adherent, nonphagocytized bacteria. Samples were then washed twice with 3 mL of washing solution (PBS). Erythrocytes were lysed by 2 mL of diethylene glycol/formaldehyde solution during 20 min of incubation at room temperature. After the samples were washed with 3 mL of washing solution, cells were stained by addition of 200 μL of DNA-staining solution (40 μg/mL of propidium iodide, 100 μg/mL of RNase). Each sample was investigated in duplicate by flow cytometry (Coulter EPICS XL, Beckman Coulter), a standard technique for the examination of phagocytosis (*26*). Calibrite beads were used to adjust instrument settings and to set fluorescence compensation. The live gate was set in the red fluorescence histogram on those events that have at least the same DNA content as a human diploid cell, and the analysis was performed on 15000 leukocytes. WinMDI 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA) was used for the determination of phagocytic activity. The monocyte or PMN cluster was gated in the scatter diagram (FSC vs SSC), and its green fluorescence histogram (FL1) was analyzed. Phagocytic activities were determined as percentages of phagocytizing leukocytes, monocytes, and PMNs.

Effect of Polysaccharides on NK Cell Cytotoxicity Activity. The NK activity against K562 target cells was assessed by a flow cytometry assay (*27*) using the DIO membrane dye (Molecular Probes, Eugene, OR) to stain live K562 cells and propidium iodide nuclear dye to stain dead cells. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden). Various fractions were incubated with isolated PBMC for 1 h in a humidified incubator at 37 °C in 5% CO_2. DIO-labeled K562 cells (effector cells: target cells = 50:1) and counterstaining solution (propidium iodide) were then added, and the mixture was continuously incubated at 37 °C for 4 h. Each sample was investigated in duplicate by flow cytometry, a standard technique for the examination of NK activity. During data acquisition, a live gate was set in the FL1 histogram on the green fluorescent target cells in order to discriminate effector and target cells. At least 5000 target cells (events) per sample were collected.

Effect of Polysaccharides on CD11b Expression. Heparinized whole blood (100 μL) was incubated with various amounts of each fraction or LPS (0.25 μg/mL) at 37 °C in 5% CO_2 for 90 min. After incubation, tubes were placed on ice, and 20 μL of phycoerythrin (PE)-conjugated murine anti-human CD11b mAb (clone ICRF44; BioLegend, San Diego, CA) or a PE-conjugated isotype-matched, nonreactive negative control mAb (BioLegend) was added. Samples were incubated on ice for 60 min before they were washed twice with PBS (250*g*, 5 min). Erythrocytes were lysed using Erythrolyse Lysis Buffer (Serotec, Oxford, United Kingdom). Samples were analyzed by flow cytometry. PMNs exhibit characteristic light scatter properties that can be identified on a FSC vs SSC light scatter plot. The PMN population was located using these parameters, and a live gate was set around this population. Data were acquired from 15000 cells (events), and the fluorescent intensity of CD11b expression on the gated cells was determined and expressed as the mean fluorescence intensity (MFI) (*28*).

Statistical Analysis. The *t*-test was used to compare the effects of various fractions on the cytokine production, phagocytic activity, NK cytotoxicity, and CD11b expression with a negative control group. Data

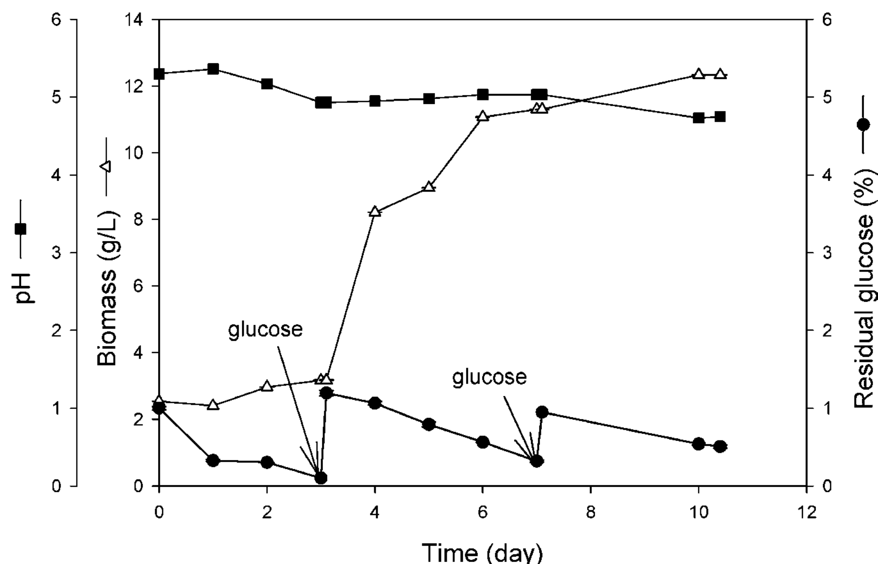

Figure 3. Time profiles of mycelia production in a 100 L stirred-tank fermentor. *G. frondosa* TSRI01 was cultivated in fermentation medium under the following conditions: initial pH, 5.4; 28 °C; aeration rate, 1.0 vvm; and agitation speed, 150 rpm. Samples were taken at indicated time points, and mycelia were then separated from the cultivation broth by vacuum filtration. The quantity of the mycelial biomass was noted after washing with distilled water and drying at 105 °C in a hot oven until a constant weight was obtained.

are expressed as the means ± standard errors of the mean (SEM), and a $p < 0.05$ was considered to indicate statistically significant differences.

RESULTS

Mycelial Growth in a 100 L Stirred Tank Bioreactor. The primary seed culture of *G. frondosa* was cultivated in 500 mL Hinton flasks, which contained 100 mL of the seed culture medium for 6 days at 28 °C and 110 rpm. The secondary seed culture was then performed in 1 L Hinton flasks, which contained 300 mL of fermentation medium for 6 more days at 28 °C and 110 rpm, as described in the Materials and Methods. The fresh seed culture (5%, v/v) was then transferred to a 100 L fermentor, which contained 50 L of fermentation medium. **Figure 3** shows the typical time profiles of mycelial growth of *G. frondosa* TSRI01 in the 100 L stirred tank bioreactor. During the first 3 days of submerged culture of *G. frondosa*, the glucose content decreased from an initial 1 to 0.1% while mycelia only increased slightly. A glucose solution (35%, w/v) was then fed into the fermentor until the glucose content went back to about 1%. Log-phase mycelial growth with the parallel decrease of residual glucose was observed since then until the sixth day of culture. When the residual glucose dropped to 0.3% on the seventh day, another glucose feeding (35%, w/v) was performed until the glucose content went back to about 1%. The rate of mycelial growth slowed since then. It indicated that the stationary phase was approached after the sixth day of submerged culture. The biomass reached 12 g/L after 10 days of culture. After cultivation, the mycelia were filtered and washed. Various fractions were prepared from the culture filtrate and mycelia as described in the Materials and Methods (**Figure 1**).

Hot Water Extract of Mycelia Induced Proinflammatory Cytokine Production. Because we were interested in investigating the influence of different fractions of *G. frondosa* on the activities of innate immune effector cells, in addition to a traditional macrophage cell line system, we chose the in vitro cytokine release from human whole blood as a convenient and simple surrogate approach to characterize changes in immune functions of humans. **Figure 4A** demonstrated the effects of Fr. A−D on the release of TNF-α from diluted human whole blood. It showed that the treatment of diluted human blood with Fr. B (hot water extract, 0.05 and 0.1 mg/mL) for 24 h was capable of augmenting TNF-α production from an undetectable basal level up to 160 ± 44 and 741 ± 260 pg/mL, respectively. LPS (0.25 μg/mL) served as a positive control, which induced 4561 ± 540 pg/mL of TNF-α release after 24 h of culture.

Effects of Fr. A−D on IL-6 production are shown in **Figure 4B**. Only Fr. B (0.025, 0.05, and 0.1 mg/mL) significantly induced IL-6 release (57.0 ± 21.0, 403.2 ± 148.5, and 1396.8 ± 553.1 pg/mL, respectively) as compared with the negative control (13.0 ± 4.7 pg/mL) after 24 h of incubation ($p < 0.01$). LPS (0.25 μg/mL) also served as a positive control, which induced IL-6 levels to 5185 ± 1391 pg/mL after 24 h of culture.

Similarly, only Fr. B (0.1 mg/mL) had an induction potency in IFN-γ production (150.0 ± 23.8 pg/mL) as compared with the negative control (19.3 ± 1.6 pg/mL) ($p < 0.01$) after 72 h of incubation. PHA (10 μg/mL) was employed as a positive control, which exhibited a strong induction capacity of IFN-γ (1250 ± 94 pg/mL) (**Figure 4C**). In parallel, Fr. A−D were also tested in the presence of polymyxin B (10 μg/mL) to eliminate the possible contamination by the endotoxin of our samples (*25*). The stimulatory activity of each fraction was not affected in the presence of polymyxin B to the cultures indicating that none of the samples was contaminated with the LPS (data not shown). In addition, none of the tested fractions was able to stimulate IL-10 release in the tested range (data not shown).

To characterize the active principals among hot water-soluble substances of mycelia, a stepwise precipitation with an increasing ratio of ethanol was performed as shown in **Figure 2**. Fr. I−IV were obtained from stepwise precipitation with 50, 67, 75, and 80% of ethanol (final concentration, v/v), respectively. The cytokine-augmenting capacities-guided approach led us to identify the most potent fractions. **Figure 5** shows that Fr. I and II (0.025−0.1 mg/mL) dose dependently induced elevated levels of TNF-α, IL-6, and IFN-γ as compared with negative controls ($p < 0.01$). Fr. I at a concentration as low as 0.025 mg/mL could induce TNF-α, IL-6, and IFN-γ production to 690 ± 88, 2199 ± 561, and 57.9 ± 27.2 pg/mL, respectively. The stimulatory potencies of Fr. I and II were much higher than that of Fr. B indicating that Fr. I and II contained the major

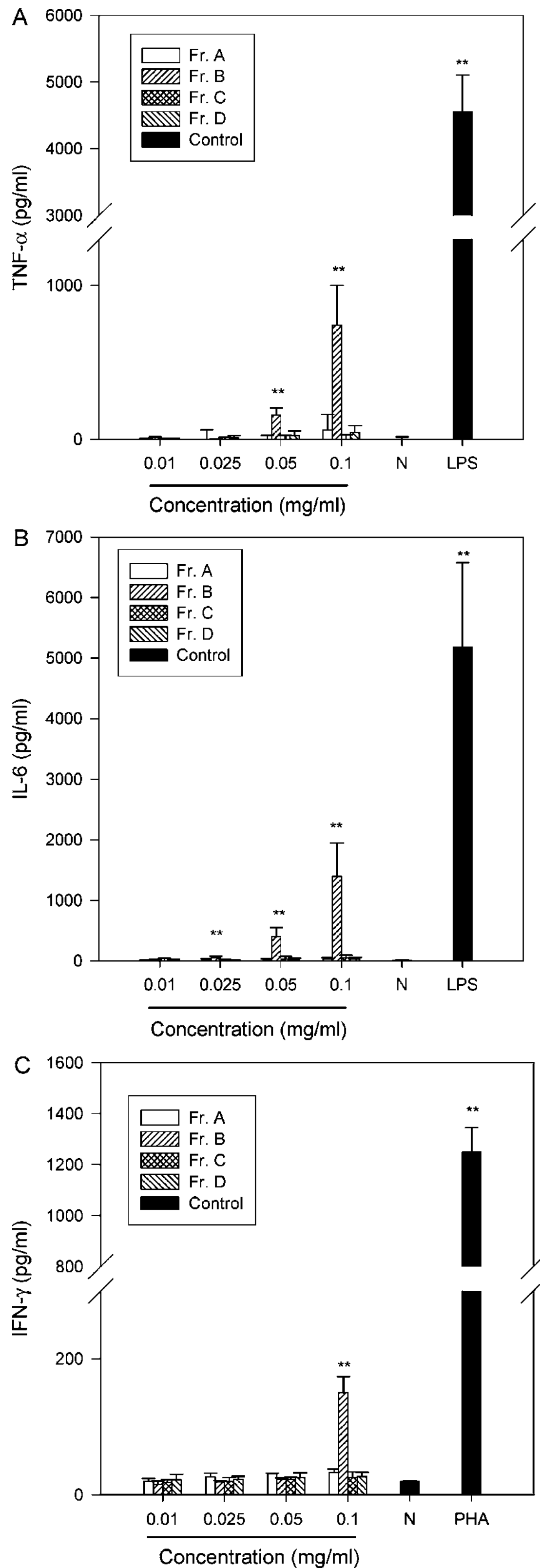

Figure 4. Induction of TNF-α, IL-6, and IFN-γ release in human whole blood treated with different fractions of polysaccharides produced by a submerged culture of *G. frondosa*. Diluted human whole blood (1:10 in RPMI 1640) was stimulated with Fr. A–D, LPS (0.25 μg/mL), or PHA (10 μg/mL) as indicated in the figure. Supernatants were collected after 24 h of culture for the analyses of TNF-α and IL-6 production (**A** and **B**) and 72 h for IFN-γ release (**C**). Data are represented as the means ± SEM of four independent experiments. *$p < 0.05$ and **$p < 0.01$ represent significant differences as compared to the negative control (PBS).

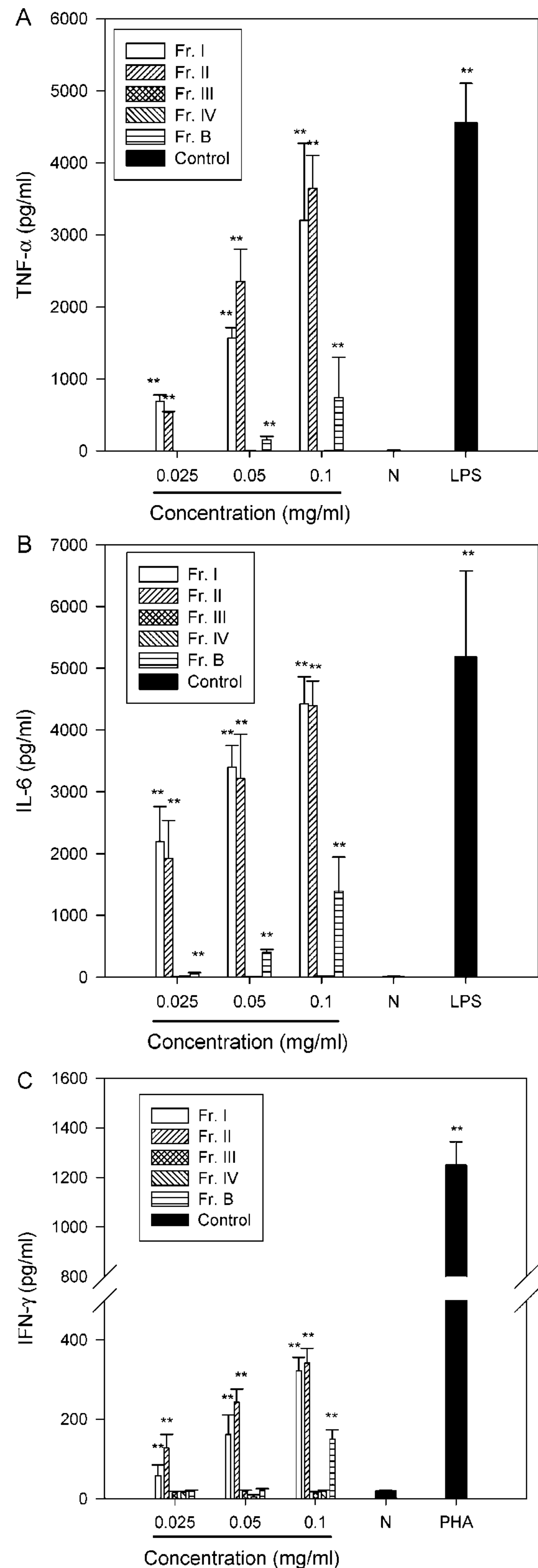

Figure 5. Induction of TNF-α, IL-6, and IFN-γ release in human whole blood treated with hot water-soluble fractions of *G. frondosa* mycelia. Diluted human whole blood (1:10 in RPMI 1640) was stimulated with Fr. I–IV or LPS (0.25 μg/mL), or PHA (10 μg/mL) as indicated in the figure. Supernatants were collected after 24 h of culture for the analyses of TNF-α and IL-6 production (**A** and **B**) and 72 h for IFN-γ release (**C**). Data are represented as the means ± SEM of four independent experiments. *$p < 0.05$ and **$p < 0.01$ represent significant differences as compared to the negative control (PBS).

Table 1. Characterization of Hot Water-Soluble Fractions Prepared from a Submerged Culture of *G. frondosa* Mycelia

	%, w/w			
	polysaccharide[a]	protein[b]	molecular mass distribution[c] (kDa)	yield (g/100 g mycelia)
Fr. B	67.3	0.11	>100, 15–36	3.00
Fr. I	∼100	0.24	43–140	0.72
Fr. II	91.9	0.21	13–38	0.65
Fr. III	20.1	0.11	16.5–36	1.44
Fr. IV	63.2	0.42	2–8	0.25

[a] Polysaccharide content was determined by subtracting free reducing sugars from total reducing sugars after hydrolysis with HCl as described in the Materials and Methods. [b] Protein content was determined by the Bradford method. [c] Molecular mass distribution was determined by GPC using pullulan as a reference.

immunostimulating constituents of hot water extract. On the other hand, no significant induction could be found in the groups treated with Fr. III or IV. Similar results were also observed in the presence of polymyxin B (10 μg/mL) indicating that all of the tested samples were free of endotoxin contamination (data not shown). None of the tested fractions exhibited IL-10 inducing potency in the tested range (data not shown).

Characterization of Hot Water-Soluble Fractions of Mycelia. The yields and characteristics of Fr. B and I−IV, including polysaccharide and protein contents as well as molecular mass distributions, were analyzed as described in the Materials and Methods. **Table 1** shows that Fr. B was composed of 67.3% of polysaccharides with two major groups, one with a molecular mass higher than 100 kDa and the other with a molecular mass of 15−36 kDa. Fr. I and II contained ∼100 and ∼92% of polysaccharides with 0.24 and 0.21% of protein contamination, respectively. Their molecular mass distributions were 43−140 and 13−38 kDa, respectively, as determined by GPC with a refractive index detector using pullulan as a reference.

Hot Water-Soluble Fractions of Mycelia Enhanced Phagocytosis. Various immunomodulators can enhance phagocytic activity in vitro or ex vivo. To test whether *G. frondosa* in submerged cultures can augment the phagocytic ability of human leukocytes, the putative potent fractions, Fr. B, I, and II, were tested. Following incubation of human whole blood with 0.025 mg/mL of Fr. B, I, and II for 30 min at 37 °C, the phagocytic activities were measured using Phagotest as described in the Materials and Methods. The monocyte or PMN cluster was further gated in the scatter diagram (FSC vs SSC). The percentages of phagocytizing leukocytes, monocytes, and PMNs are measured and summarized in **Figure 6**. Blood incubated with 0.025 mg/mL of Fr. I and II exhibited significantly higher phagocytic activities in PMNs (60.1 ± 2.1 and 53.9 ± 1.7%, respectively) as compared with negative control groups (40.7 ± 4.3%). The induction potencies of Fr. I and II were also higher than that of Fr. B indicating that the functional components of hot water-soluble extracts had molecular masses of 13−140 kDa.

Hot Water-Soluble Fractions of Mycelia Enhanced NK Cell Cytotoxicity. The *G. frondosa* fruiting body has been traditionally used to treat breast and other tumors in Asia. To test whether *G. frondosa* in submerged cultures also has antitumor potential, the NK cell cytotoxicity was measured as described in the Materials and Methods. **Figure 7** shows that preincubation of PBMC with 0.025 mg/mL of Fr. I and II for 1 h significantly increased the lysis of K562 erythroleukemic cells (55.9 ± 7.9 and 60.2 ± 1.6%, respectively) as compared with a negative control (46.5 ± 0.4%). Marked induction of

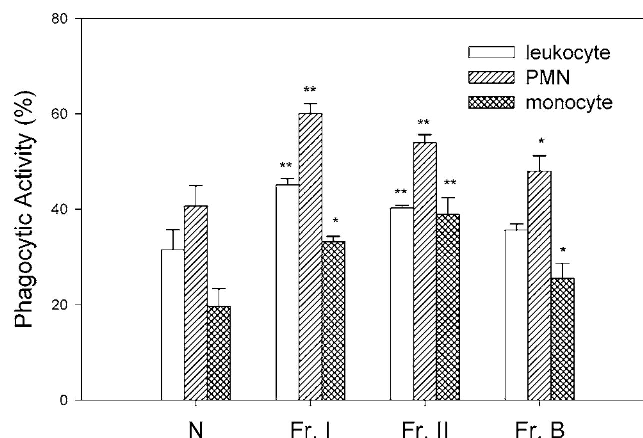

Figure 6. Hot water-soluble fractions of *G. frondosa* mycelia enhanced phagocytic activity. Human whole blood was incubated with indicated fractions (0.025 mg/mL) for 30 min before phagocytosis analysis. Phagotest kit was employed to analyze the phagocytic activity as described in the Materials and Methods. The percentages of phagocytising leukocytes, monocytes, and PMN in various groups were determined by flow cytometry. Data are from three independent experiments and are presented as means ± SEM. *$p < 0.05$ and **$p < 0.01$ represent significant differences as compared to the negative control (PBS).

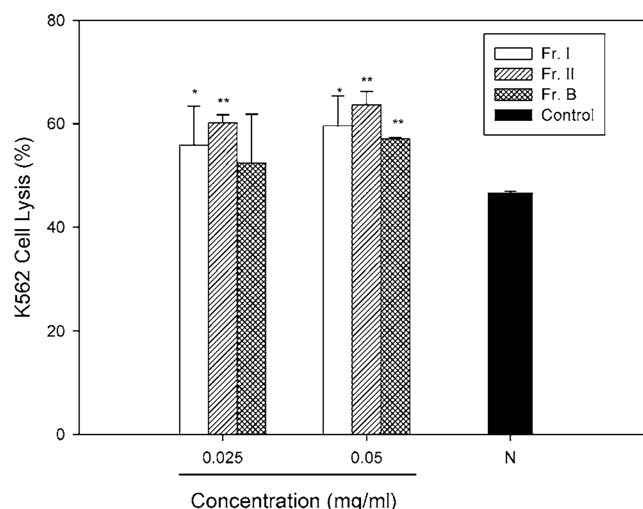

Figure 7. Effect of hot water-soluble fractions of *G. frondosa* mycelia on human peripheral blood NK cell cytotoxicity. Various fractions were incubated with isolated PBMC for 1 h at 37 °C. DIO-labeled K562 tumor cells were added afterward, and the NK activity was determined by flow cytometry after an incubation time of 4 h at an E:T ratio of 50:1 as described in the Materials and Methods. Data are from three independent experiments and are presented as means ± SEM. *$p < 0.05$ and **$p < 0.01$ represent significant differences as compared to the negative control (PBS).

NK cytotoxicity was also observed for 0.05 mg/mL of Fr. B (57.2 ± 0.2%, $p < 0.01$).

Hot Water-Soluble Fractions of Mycelia Up-Regulated CD11b. To determine whether the immunostimulating effects exerted by the potent fractions are related to PMN activation, CD11b expression, the activation marker, was measured using flow cytometry as described in the Materials and Methods. **Figure 8** shows that a 90 min incubation with Fr. I, II, or B (0.025−0.1 mg/mL) increased CD11b expression of PMNs in a dose-dependent manner. The MFIs of groups treated with 0.025 mg/mL of Fr. I, II, and B were 215.1 ± 0.2, 181.3 ± 38.9, and 177.9 ± 0.5, respectively. These were significantly higher than that of the negative control (134.0 ± 2.3) ($p < 0.01$).

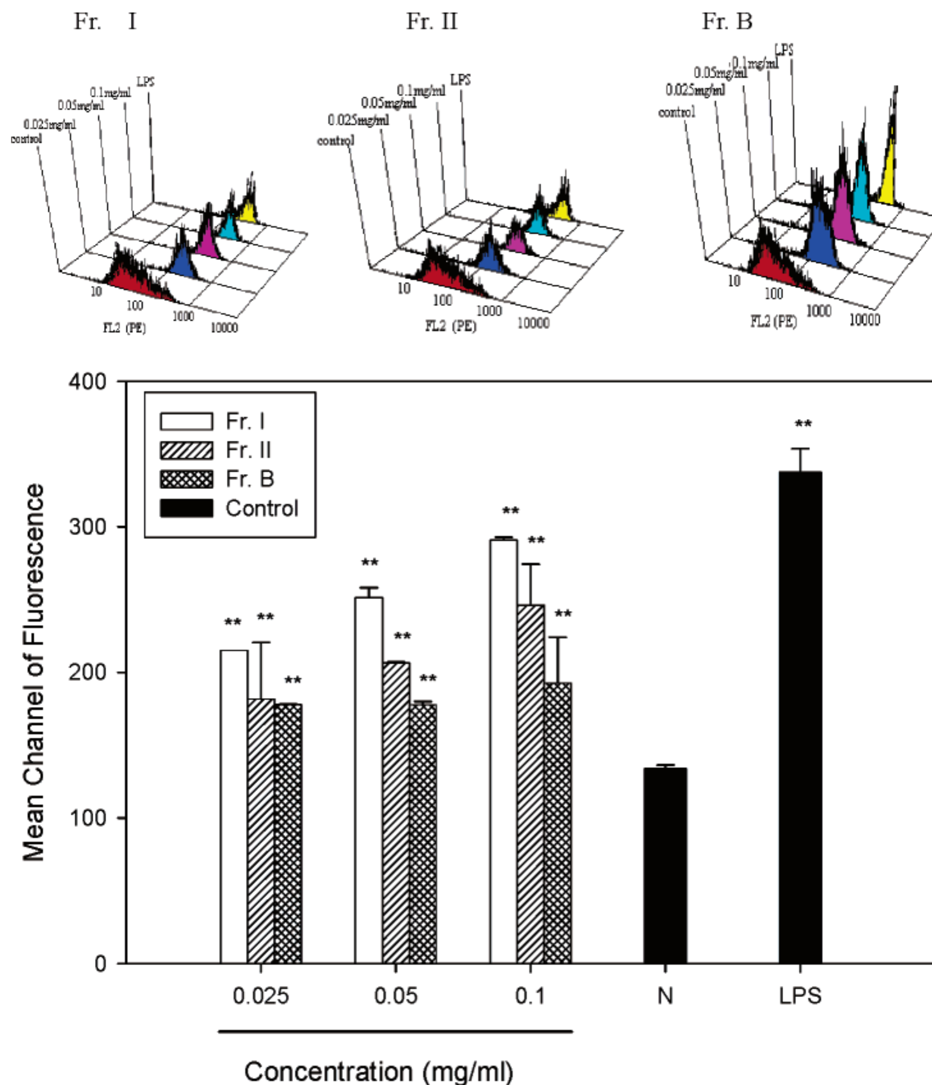

Figure 8. Effect of hot water-soluble fractions of *G. frondosa* mycelia on the CD11b expression of PMN. LPS (0.25 μg/mL) was used as a positive control, and PBS was used as a negative control. Samples were incubated with whole blood for 90 min, and CD11b expression was then determined as described in the Materials and Methods. Data are from six independent experiments and are presented as means ± SEM. *p < 0.05 and **p < 0.01 represent significant differences as compared to the negative control (PBS). Inset, representative fluorescent histogram.

The elevated CD11b expression of PMN correlated well with enhanced phagocytic activities induced by these bioactive polysaccharides as shown in **Figure 6**. LPS (0.25 μg/mL) also served as a positive control, which induced a high level of CD11b expression with MFIs of 337.8 ± 16.0.

DISCUSSION

Numerous studies have confirmed that polysaccharides isolated from maitake (*G. frondosa*) fruiting bodies have prominent beneficial effects on immune function (*9*, *29*−*32*). They promote the action of not only macrophages but also a variety of other immune-related cells, such as NK cells that can attack tumor cells. In this research, we focused on the production of functional substances from *G. frondosa* mycelia through submerged cultures. We found that cultivation of *G. frondosa* TSRI01 in a 100 L bioreactor produced more than 12 g/L of mycelia in 10 days. Therefore, submerged cultures appear to be an efficient and economic way to produce a high yield of biomass.

To obtain the functional fractions from fermentation products, we used a cytokine induction potential-guided approach. The hot water-soluble fractions of *G. frondosa* mycelia, Fr. B, I, and II, dose dependently stimulated the release of TNF-α, IL-6, and IFN-γ, rather than IL-10, in human whole blood culture. These potent fractions also enhanced phagocytic activities of leukocytes, PMNs, and monocytes. It is well-known that INF-γ can work as an immunomodulator to enhance phagocytic activity. Herein, we found that 0.025 mg/mL of Fr. B could enhance phagocytic activity of PMN without potentiating IFN-γ release in human whole blood. This result indicates that the elevated phagocytic activity may be caused by direct interaction of functional polysaccharides with leukocytes rather than being activated by IFN-γ indirectly.

NK cells have multiple roles in the innate immune response including tumor surveillance and response to viral infections. It has been reported that cancer patients who received D-fraction showed elevated levels of NK cytotoxicity (*33*). Administration of tumor-bearing mice with D-fraction intraperitoneally also markedly suppressed tumor growth, corresponding with the increase in TNF-α and IFN-γ released from spleen cells and the activation of NK cells (*18*). In this report, we found that hot water-soluble fractions of *G. frondosa* mycelia significantly increased peripheral blood NK cytotoxicity against K-562 cells, indicating that they might have antitumor potentials as well.

It has been reported that there was a correlation between immunological activity and molecular mass, and glucans with a molecular mass of about 550 kDa possessed the greatest induction potency (*34*). Ishibashi et al. reported that an insoluble and a high molecular mass soluble form of GRN, polysaccharides of the *G. frondosa* fruiting body, are required for TNF-α production in macrophage cell line RAW264.7 (*12*). Recently, Lee et al. demonstrated that the polysaccharides prepared from mycelia or culture filtrate of submerged cultivated *G. frondosa* had a molecular mass that ranged from 470 to 1650 kDa (*21*). However, in this report, the most potent fractions prepared from *G. frondosa* mycelia, Fr. I and II, have molecular masses that range from 43−140 to 13−38 kDa, respectively, as determined by GPC using pullulan as a standard. The contradiction between our result and others' may be due to the differences in the compositions of the culture media and fraction preparation processes. It has been suggested that the polysaccharide structure in cultured *G. frondosa* mycelia depends on the composition of the medium used for cultivation (*5*). It has also been indicated that the β-glucan with a molecular mass higher than 100 kDa is hard to dissolve in water (*12*). This may well explain why only low molecular mass polysaccharides were obtained from the hot water extract of *G. frondosa* mycelia.

PMNs play a major role in innate immunity and host defense against infection, and an essential component of their activation is expression of CD11b on their cell surface. Under physiological conditions, circulating neutrophils are in the resting state. CD11b, a component in the CD11b/CD18 adhesion protein complex, is a sign of PMN activation by up-regulation of its expression. The presence of this adhesion complex allows PMN to migrate from the blood circulation into target tissue to perform their duty (*35*). As a result, CD11b serves as a marker for phagocytic cell activation. Our results demonstrated that these polysaccharides up-regulated the CD11b expression of PMN dose dependently. A previous study suggested that the capacity of leukocyte CR3 (CD11b/CD18) to recognize natural microbial surface components, such as β-glucan, LPS, and lipophosphoglycan, is the critical event to stimulate phagocytes and leads to the elimination of pathogens, toxic debris, and apoptotic cells (*36*). It has also been shown that incubation of NK cells and PMN with small soluble β-glucans primed the CR3 to enhance the cytotoxicity (*37*, *38*). As mentioned above, these functional low molecular mass polysaccharides enhanced cytokine release, phagocytosis, and NK cytotoxicity. The direct cytokine induction appears to be common among immunomodulators and therefore implies that a common receptor and signaling pathway is involved. All of these results indicated that CR3 may serve as a candidate receptor for these hot water-soluble polysaccharides and their attachment to CR3 can lead to the augmentation of innate immunity.

In conclusion, historically, hot water-soluble fractions from medicinal mushrooms were used as a traditional medicine in the Far East, where knowledge and practice of mushroom use primarily originated. In this report, we first demonstrated that the hot water-soluble fractions (polysaccharides) of *G. frondosa* mycelia from submerged fermentation can effectively induce innate immunity and therefore enhance proinflammatory cytokine release, phagocytosis, and NK cytotoxicity activity in vitro. These results are useful for the production of functional *G. frondosa* polysaccharides on a large scale and can be widely applied to other mushroom fermentations.

ABBREVIATIONS USED

CR, complement receptor; Fr., fraction; GPC, gel permeation chromatography; IFN-γ, interferon-γ; IL-6, interleukin-6; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NK cells, natural killer cells; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PHA, phytohemagglutinin; PMN, polymorphonuclear neutrophils; TNF-α, tumor necrosis factor-α.

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