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Characterization and Antitumor Effect of a Novel Polysaccharide from *Grifola frondosa*

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A novel polysaccharide, MZF, with a molecular mass of 23 kDa was isolated from *Grifola frondosa*. Results from methylation and ^{1}H NMR led to the conclusion that MZF is a heteropolysaccharide consisting of →6)-α-D-Gal*p*-(1→ (36.2%), →3)-α-L-Fuc*p*-(1→ (14.5%), →6)-α-D-Man*p*-(1→ (9.4%), →3)-β-D-Glc*p*-(1→ (10.1%), α-D-Man*p*-(1→ (23.2%), and →3,6)-β-D-Glc*p*-(1→ (6.5%). Although MZF did not affect the proliferation of colon-26 cells in vitro, it significantly inhibited tumor growth in BALB/cA mice inoculated with colon-26 cancer cells. Moreover, MZF significantly induced the proliferation of splenocytes and peritoneal macrophages. The mRNA expression of IL-12p40, IL-2 and IFN-γ were increased significantly in MZF-treated spleen. Furthermore, MZF augmented the percentage of IFN-γ-producing cells in both splenic $CD4^+$ and $CD8^+$ T cells and tumor infiltrating $CD4^+$ and $CD8^+$ T cells and enhanced the cytotoxic activity of NK cells and CTLs. These results indicate that MZF is a novel effective immunomodulator that has antitumor activity associated with induced cell-mediated immunity.

KEYWORDS: Polysaccharide; *Grifola frondosa*; immunomodulatory effect; antitumor effect

INTRODUCTION

Many studies have reported the immunomodulatory effects of polysaccharides isolated from various natural sources such as plants, fungi, yeasts, mushrooms, and sea organisms (*1*, *2*). In clinical cancer treatment, an immunomodulating substance, or biological response modifier (BRM), may be combined with surgical therapy, chemotherapy, and radiotherapy for suppressing decreased host resistance to bacterial and viral infections and enhancing the therapeutic effect. Many effective BRMs have been derived from a variety of mushrooms. Lentinan from *Lentinus edodes* and Krestin (PSK) from *Coriolus versicolor* have been used clinically for cancer therapy in Japan (*3*,*4*). Polysaccharides from mushrooms activate immune cells (*5*) and enhance hematopoiesis (*6*). These polysaccharides induce an antitumor effect without showing side effects. Moreover, they reduce leukopenia induced by chemotherapy.

Maitake D fraction (MD-fraction), β-(1,3) (1,6)-glucan, extracted from the edible mushroom *Grifola frondosa* induces antitumor activity by activating the host immune system. MD-fraction activates macrophages, dendritic cells (DCs), and natural killer (NK) cells and promotes T-helper (Th)1-type immune responses (*7*−*9*). In addition, MD-fraction enhances granulocyte-macrophage colony formation (CFU-GM) in umbilical cord blood cells and mouse bone marrow cells (*10*, *11*) and granulopoiesis and mobilization of granulocytes in cyclophosphamide-induced granulocytopenic model mice (*12*). The immunological effects of an extract from *G. frondosa* have also been demonstrated in a phase I/II trial (*13*). Additionally, various biologically active substances have been extracted from *G. frondosa* in addition to β-glucan; they have been reported to show antiviral activity or an antidiabetic effect (*14*−*16*).

In this study, we isolated a novel polysaccharide maitake Z fraction (MZF) from *G. frondosa*, which was different from the MD-fraction, and analyzed the chemical structure of MZF by methylation analysis and proton nuclear magnetic imaging (^{1}H NMR). We also investigated the immunomodulatory effect of MZF in vitro and in vivo.

MATERIALS AND METHODS

Isolation of Polysaccharide MZF from *G. frondosa*. MZF was isolated from a boiling water extract of the fruit bodies of *G. frondosa* provided by the Yukiguni Maitake Co. (Minami-Uonuma City, Japan). The crude polysaccharide fraction was obtained by precipitation with ethanol from the aqueous extract and applied on a diethylaminoethyl (DEAE)-cellulofine anion exchange column (12.5 i.d. × 10 cm; Seikagaku Biobusiness Co., Ltd., Tokyo, Japan) pre-equilibrated with 5 mM Tris-HCl (pH 8.0); the unabsorbed fraction was eluted with 5 mM Tris-HCl (pH 8.0). The supernatant was collected by the addition of ethanol to a 50% concentration, and then the precipitate was collected by the addition of ethanol to a concentration of 80%. The precipitate was dissolved in sterilized water and loaded onto a gel filtration column (1.5 i.d. × 138 cm) of Sepharose CL-6B (GE Healthcare, Piscataway, NJ). Two fractions were

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separated completely by gel filtration, and the first fraction was collected as MZF. Lipopolysaccharide (LPS) contamination was tested using an Endospecy ES-24S set (Seikagaku Biobusiness Co., Ltd.), but contamination was not detected. The concentrations of carbohydrate and protein were determined by the anthrone method and the BCA Protein Assay (Pierce, Rockford, IL), respectively.

Determination of Neutral Sugar. The composition of neutral sugars in the samples was determined by using a high-performance liquid chromatography (HPLC) system consisting of a Hitachi L-6000 pump (Hitachi Seisakusho Co., Tokyo, Japan), a Rheodyne 7725i loop injector (Rohnert Park, CA), and a fluorescence detector (Jasco FP-2025 Plus from Nihonbunko Co., Tokyo, Japan). A double-plunger pump (SPU-2.5NP) to deliver the postreaction reagents and a dry reaction bath (DB-3) were purchased from Shimamura Instruments Co., Tokyo, Japan. The water bath incubator (Thermo Minder Mini-80) was from Taitec Co., Tokyo, Japan. The neutral sugars were determined by anion exchange chromatography with postcolumn derivatization. The column used was a TSKgel Sugar AXI (Tosoh Corp., Tokyo, Japan, 4.6 mm i.d. × 150 mm), which was maintained at 70 °C. The mobile phase comprised 0.5 M boric acid (pH 8.5 adjusted with 2 M NaOH) delivered at a flow rate of 0.4 mL/min. The derivatizing reagents were 1 M NaOH and 0.5% 2-cyanoacetamide, each of which was delivered at a flow rate of 0.25 mL/min. The reaction bath was at 120 °C. The fluorescence of the reaction product was measured by excitation at 331 nm with the emission wavelength at 383 nm. The neutral sugar composition of the samples was calculated on the basis of the peak area response of each monosaccharide standard (*17*).

Permethylation Analysis. Permethylations were undertaken as described previously (*18*), using a NaOH−dimethyl sulfoxide (DMSO) suspension. The product was subjected to acetolysis in 80% AcOH containing 1 M HCl at 80 °C for 24 h, and the resulting partially O-methylated monosaccharides were reduced by 5% $NaBH_4$ in 10 mM NaOH. After removal of borate by evaporation as methylborate, partially O-methylated alditols were acetylated (*17*). Gas chromatography−mass spectrometry (GC-MS) analyses of the derivatized samples employed a Hewlett-Packard model 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) with an HP-5 ms fused silica capillary column (0.25 μm film thickness, 0.25 mm i.d. × 30 m) configured in electron impact mode. The oven temperature was ramped over a linear gradient from 150 to 260 °C at 10 °C/min. Mass spectra were recorded in positive-ion mode over the range m/z 30−500.

NMR. ^{1}H NMR spectroscopy was performed as follows: 3 mg samples were treated repeatedly with 0.5 mL of ^2H_2O, followed by desiccation over P_2O_5 in vacuo to exchange the labile protons with deuterons. Then, the thoroughly dried samples were redissolved in 0.5 mL of ^2H_2O and transferred to the NMR tube (5.0 mm o.d. × 25 cm; PP-528, Wilmad Glass Co., Buena, NJ). All spectra were obtained by a JNM-ECP600 spectrometer (JEOL, Tokyo, Japan). The operation conditions for the one-dimensional (1D) spectra were as follows: frequency, 600 MHz; sweep width, 8 kHz; flip angle, 90° (10.8 μs); sampling points, 32000; accumulation, 500 times; and temperature, 333 K.

Molecular Weight Determination of the Polysaccharide Using High-Performance Size-Exclusion Chromatography. The average molecular weight of the polysaccharide was determined using high-performance size exclusion chromatography (HPSEC) with reference to the calibration curve obtained using dextran standards (MW 6, 10, 40, 100, and 150 kDa; Wako Pure Chemicals Co. Ltd., Osaka, Japan). The HPSEC system consisted of a Jasco 980-PU pump (Jasco, Los Angeles, CA), a Rheodyne 7725i loop injector, and a refraction index detector (Shimamura Seisakusho, Tokyo, Japan). Two AsahiPak GF510HQ (4.6 × 150 mm; Shodex, Kawasaki, Japan) columns connecting in-line were used at 30 °C. The eluate containing 10 mM ammonium bicarbonate was used at a flow rate of 0.5 mL/min. A Hitachi D-2500 integrator (Tokyo, Japan) was used to acquire and analyze the data.

Mice and Antibodies. Female BALB/cA SPF mice were purchased from CLEA Japan Inc. (Higashimaya, Japan), and 6-week-old mice were used in the study. Animal care and processing were performed in accordance with the guidelines for proper conduct of animal experiments by the Science Council of Japan and approved by the animal care committee at this institution. The following antibodies (Abs) from BD Bioscience (San Jose, CA) were used: fluorescein isothiocyanate (FITC) anti-CD49b (clone DX5), R-phycoerythrin (PE) anti-CD3ε (clone 145-2C11), Cy-Chrome anti-CD4 (clone RM4-5), Cy-Chrome anti-CD8 (clone 53-6.7), FITC anti-IFN-γ (clone XMG 1.2), R-PE anti-IL-4 (clone 11B11), and anti-CD16/CD32 (clone 2.4G2).

Proliferation Assay. Murine peritoneal resident macrophages and spleen cells were collected using a previously described procedure (*19*) from BALB/cA mice. A colon-26 carcinoma cell line was provided by Kitasato University. Macrophages (1×10^5 cells/well), spleen cells (1×10^6 cells/well), and colon-26 tumor cells (2×10^4 cells/well) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; GIBCO, Invitrogen, Carlsbad, CA), L-glutamine (0.03 mg/mL), penicillin (100 units/mL), streptomycin (100 μg/mL), and MZF at various concentrations in 5% CO_2 at 37 °C for 24 h in 96-well plates (100 μL/well). Cell proliferation was measured with WST-8 reagent by using Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer's instructions.

Quantitation of Cytokine Production. Supernatants were collected to measure cytokine production after 24 h of stimulation of peritoneal macrophages. TNF-α and IL-12 levels were determined by sandwich ELISA as previously described (*9*). Monoclonal antibodies used for ELISA were as follows: monoclonal anti-mouse IL-12 (clone C17.8) (BioLegend, CA), biotinylated anti-mouse IL-12 (Pepro Tech, Rocky Hill, NJ), monoclonal anti-mouse (clone TN3-19.12) (Santa Cruz Biotechnology, Santa Cruz. CA), biotinylated anti-mouse TNF-α (R&D Systems, Minneapolis, MN).

In Vivo Antitumor Activity. Colon-26 carcinoma cells (1×10^5 cells/mouse) were subcutaneously (sc) inoculated into 6-week-old female BALB/cA mice on day 0. Tumor-inoculated mice were injected intraperitoneally (ip) with either saline or MZF (8 mg/kg/day) for 17 consecutive days after tumor inoculation. Tumors were measured in two dimensions perpendicular to each other every other day from day 8 after tumor challenge. Tumor volume was calculated using the formula (cm^3 = longest diameter × shortest diameter^2/2). Final tumor weight was measured on day 18.

Quantitative Reverse Transcription Polymerase Chain Reaction. Total RNA was isolated from spleen using the QuickGene RNA Tissue Kit S II (Fujifilm Corp., Tokyo, Japan). Total cellular RNA (1 μg/20 μL reaction) was reverse transcribed using Oligo dT primer and ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. Real-time PCR analysis was performed using iQTM SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions using specific primers. The specific primers were designed on the basis of the qPrimerDepot database (GAPDH, IFN-γ, IL-2, IL-4, and TNF-α) (*20*) and Primer Bank (IL-12p40) (*21*). The following primer pairs were used: GAPDH right primer, 5-TTGATGGCAACAATCTCCAC-3, GAPDH left primer, 5-CGTCCCGTAGACAAAATGGT-3; IFN-γ right primer, 5-TGAGCTCATTGAATGCTTGG-3, IFN-γ left primer, 5-ACAGCAAGGCGAAAAAGGAT-3; IL-2 right primer, 5-CGCAGAGGTCCAAGTTCATC-3, IL-2 left primer, 5-AACTCCCCAGGATGCTCAC-3; IL-4 right primer, 5-CGAGCTCACTCTCTGTGGTG-3, IL-4 left primer, 5-TGAACGAGGTCACAGGAGAA-3; TNF-α right primer, 5-AGGGTCTGGGCCATAGAACT-3, TNF-α left primer, 5-CCACCACGCTCTTCTGTCTAC-3; IL-12 p40 right primer, 5-ACAGGTGAGGTTCACTGTTTCT-3, IL-12 p40 left primer, 5-TGGTTTGCCATCGTTTTGCTG-3.

Flow Cytometric Analysis. For intracellular cytokine detection, whole spleen cells (1×10^6 cells/mL) were incubated with ionomycin (750 ng/mL), phorbol-12-myristate-13-acetate (50 ng/mL), and Goldistop (BD Biosciences) at 37 °C in 5% CO_2 for 4 h. The cells were then harvested, stained for extracellular Cy-Chrome-conjugated CD4 or CD8a Ab, and then stained for P-Phycoerythrin-conjugated IL-12 Ab with the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences).

NK Cell Cytotoxicity. Splenic NK cytotoxicity against YAC-1 target cells was assessed by the calcein-AM cytotoxicity assay. Eighteen days after tumor inoculation, spleen cells were collected as effector cells. YAC-1 target cells (1×10^6 cells/mL) were labeled with calcein-AM (15 μM) for 45 min at 37 °C. The labeled target cells were washed and resuspended to a mixture of 2×10^5 cells/mL in RPMI-1640 containing 10% FBS; target cells (50 μL) were mixed with an equal volume of the effector cells at various ratios in 96-well U-bottom plates. After incubation for 2 h at 37 °C in 5% CO_2, supernatants were harvested, and the fluorescence was read at 485/538 nm (excitation/emission) (*22*). The percentage of specific

calcein-AM release was calculated as 100 × ([experimental release] − [spontaneous release])/([maximal release] − [spontaneous release]) (%). Spontaneous release was determined using target cells without effectors, whereas maximal release was determined with target cells exposed to 2% Triton X-100 (Nacalai Tesque Inc.).

Cytotoxic T Lymphocyte (CTL) Assay. Spleen cells (5×10^6 cells/mL) were removed from mice 18 days after tumor inoculation, mixed with mitomycin C (MMC)-treated colon-26 cells (5×10^5 cells/mL), and incubated for 72 h at 37 °C in 5% CO_2 in the presence of rIL-2 (50 ng/mL) to induce antigen-specific CTLs. Live cells were isolated on a Lympholyte M density gradient (Cedarlane, Hornby, ON, Canada). The CTL activity of antigen-stimulated spleen cells was determined using calcein AM-labeled colon-26 cells (1×10^5 cells/mL) as target at different effector-to-target (E:T) ratios. After incubation for 2 h at 37 °C in 5% CO_2, supernatants were harvested, and the fluorescence was read at 485/538 nm (excitation/emission).

Table 1. Partially Methylated Alditol Acetates of MZF

O-methylalditol acetate	mode of linkage	molar %
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	36.2
2,4-Me_2-Fuc	→3)-Fuc*p*-(1→	14.5
2,3,4-Me_3-Man	→6)-Man*p*-(1→	9.4
2,4,6-Me_3-Glc	→3)-Glc*p*-(1→	10.1
2,3,4,6-Me_4-Man	Man*p*-(1→	23.2
2,4-Me_2-Glc	→3,6)-Glc*p*-(1→	6.5

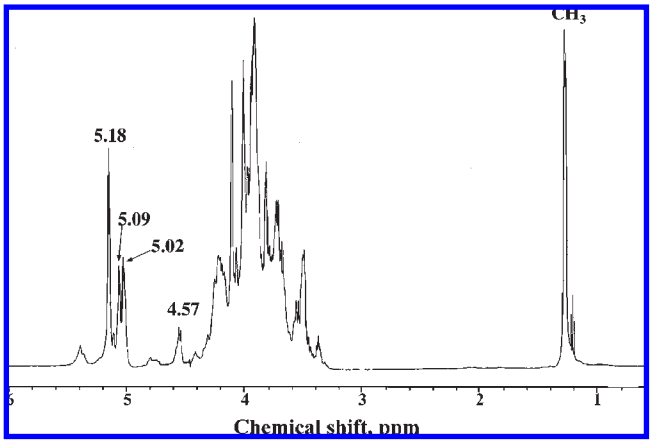

Figure 1. 600 MHz ^{1}H NMR spectrum (D_2O, 333 K) of MZF.

Preparation of Tumor-Infiltrating Lymphocytes (TIL). Tumors were removed from the lateral flank of each mouse, and the tissues were fragmented with scissors and incubated in RPMI medium containing collagenase (70 units/mL; Wako Pure Chemical Industries, Osaka, Japan) for 30 min at 37 °C. Suspended tumor cells and TILs were placed on Lympholyte-M (Cedarlane) and centrifuged at 1750*g*. TILs were analyzed by flow cytometry.

Statistical Analysis. Values are presented as means ± standard error (SE). Student's *t* test was used to analyze significance. Tumor volumes of different groups were analyzed using a nonparametric two-tailed test (Mann−Whitney test) for unpaired samples. A p value of < 0.05 was considered to be significant.

RESULTS

Structure of the Polysaccharide MZF from *G. frondosa*. The hot water extract from *G. frondosa* contained approximately 46% carbohydrates and 54% proteins. The polysaccharide MZF containing up to 98.7% carbohydrates was obtained following DEAE-Cellulofine isolation, ethanol precipitation, and gel filtration column chromatography. The HPSEC profile showed a single peak, which indicated that MZF was a homogeneous polysaccharide, with an average molecular mass of 23.0 kDa (not shown). The composition of neutral sugars in MZF was determined by using a TSKgel Sugar AXI column. MZF contained galactose (Gal), mannose (Man), fucose (Fuc), and glucose (Glc). The molar ratio of the neutral sugars (Gal:Man:Fuc:Glc) was 1.24:1:0.95:0.88.

To investigate the mode of linkage, the MZF polysaccharide was methylated, hydrolyzed, reduced, and acetylated. The partially methylated alditol acetates were analyzed and identified by GC-MS, which revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol; 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol; and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in 36.2, 14.5, 9.4, 10.1, 23.2, and 6.5 molar %, respectively (**Table 1**).

The 1D ^{1}H NMR spectrum of MZF showed four resonances in the anomeric proton region (**Figure 1**). The chemical shift of the resonance at δ 4.57 (doublet) was assigned to the β-anomeric proton of Glc. The resonances at δ 5.18 (singlet), 5.09 (s), and 5.02 (s) were assigned to α-anomeric protons of Man, Gal, and Fuc, respectively. These assignments are consistent with reports in the

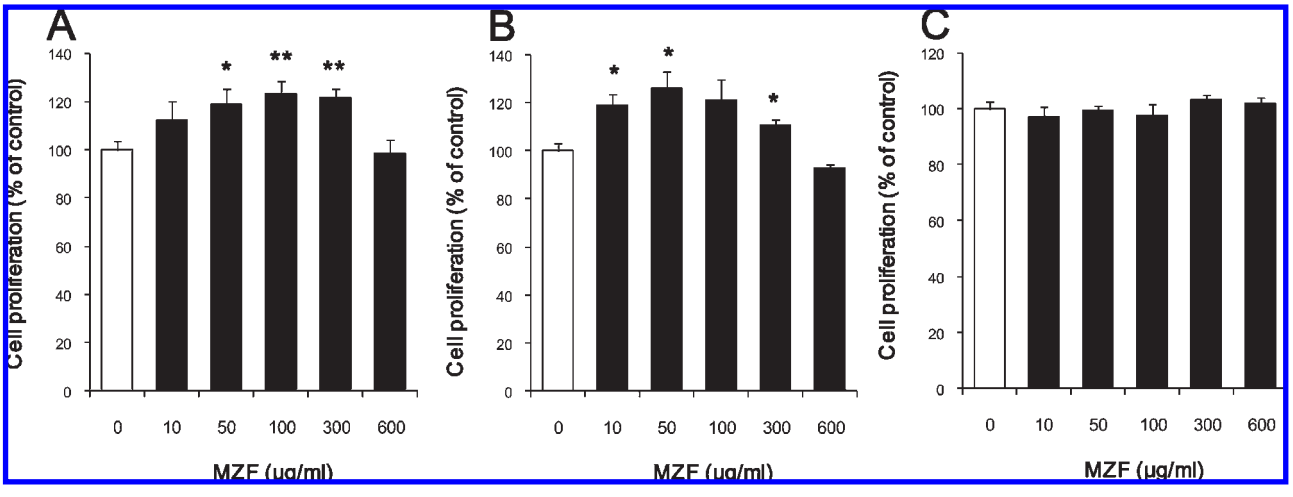

Figure 2. Effect of MZF on cell proliferation in vitro: (**A**) macrophages (1×10^5 cells/well), (**B**) spleen cells (1×10^6 cells/well), and (**C**) colon-26 tumor cells (2×10^4 cells/well) were cultured with MZF at various concentrations for 24 h in 96-well plates (100 μL/well). After incubation, WST-8 solution was added to each well, and the cells were incubated for 2 h at 37 °C. The absorbance of the culture was measured at 450 nm. Results represent one of two independent experiments with similar results. Values are expressed as mean ± SE ($n = 5$). ∗, $p < 0.05$, and ∗∗, $p < 0.01$, as compared to control (MZF 0 μg/mL).

Table 2. Effect of MZF on TNF-α and IL-12 Production by Mouse Peritoneal Macrophages in Vitro[a]

treatment	TNF-α (pg/mL)	IL-12 (pg/mL)
control	32.95 ± 6.92	19.66 ± 2.59
MZF (250 μg/mL)	100.76 ± 3.96**	22.05 ± 5.71
MZF (500 μg/mL)	169.71 ± 5.14***	39.66 ± 6.07*

[a] Peritoneal macrophages (1×10^5 cells/well) were cultured with MZF for 24 h in 96-well plates (100 μL/well). After incubation, supernatants were collected, and levels of TNF-α and IL-12 were measured by ELISA. Values are expressed as mean ± SE ($n = 3$). *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, as compared to control (MZF 0 μg/mL).

literature (*23*), and our results suggest that MZF is a heteropolysaccharide consisting of →6)-α-D-Gal*p*-(1→ (core structure 36.2%), →3)-α-L-Fuc*p*-(1→ (core structure 14.5%), →6)-α-D-Man*p*-(1→ (core structure 9.4%), →3)-β-D-Glc*p*-(1→ (core structure 10.1%), α-D-Man*p*-(1→ (nonreducing end 23.2%), and →3,6)-β-D-Glc*p*-(1→ (branched 6.5%).

Effect of MZF on Cell Proliferation and Cytokine Production in Vitro. To confirm the immunomodulatory effect of MZF in vitro, we evaluated the proliferation of immune cells. As shown in **Figure 2A**,**B**, MZF significantly induced the proliferation of spleen cells and resident peritoneal macrophages. MZF significantly affected immune cell proliferation only at a certain optimal concentration, and a high concentration of MZF resulted in a low efficacy. On the other hand, MZF did not affect the proliferation of colon-26 cells in vitro (**Figure 2C**).

Next, we tested whether MZF enhances cytokine production by peritoneal macrophages. As shown in **Table 2**, MZF significantly induced the production of TNF-α and IL-12 by macrophages.

In Vivo Antitumor Activity. We next examined the ability of MZF to modulate tumor growth in vivo. The ip administration of MZF (8 mg/kg/day) significantly inhibited tumor growth by 47.6% as compared with the control group in BALB/cA mice inoculated with colon-26 cancer cells (**Figure 3**). Our in vitro and in vivo data suggest that MZF can efficiently stimulate an antitumor immune response and induce tumor regression in mouse tumor models.

Cytokine mRNA Expressions in Spleen. Colon-26 cell-bearing BALB/cA mice were injected ip with MZF for 17 consecutive days, and spleens were collected on day 18. Total RNA was prepared from the spleens, and mRNA expressions of TNF-α, IL-12 p40, IL-2, IFN-γ, and IL-4 were evaluated by using quantitative real-time PCR. As shown in **Figure 4**, MZF showed a significant 2.19-fold increase in mRNA expression of IL-12 p40, which stimulates NK cells and induces cell-mediated immunity. Additionally, mRNA expressions of IL-2 and IFN-γ, Th1 cytokines, increased 2.86- and 2.32-fold, respectively, in MZF-treated spleens. On the other hand, mRNA expression of IL-4, a Th2 cytokine, was not affected by MZF treatment. Although MZF directly enhanced TNF-α production by peritoneal macrophage in vitro (**Table 2**), there was no significanct difference between the control and MZF-treated spleens on day 18 in vivo. TNF-α is a soluble pro-inflammatory cytokine produced by leukocytes of the innate immunity arm, namely, macrophages and neutrophils. Therefore, more investigations at earlier time points are required to determine the expression of TNF-α in spleen in vivo.

MZF Induces Cell-Mediated Immunity. Cell-mediated cytotoxicity can be influenced by a variety of immune system cells, including CTLs, NK cells, and macrophages. The increases in mRNA expression levels of IL-12 p40, IL-2, and IFN-γ suggest that MZF induces cell-mediated immunity. Whole spleen cells were collected on day 18 after tumor inoculation, and the

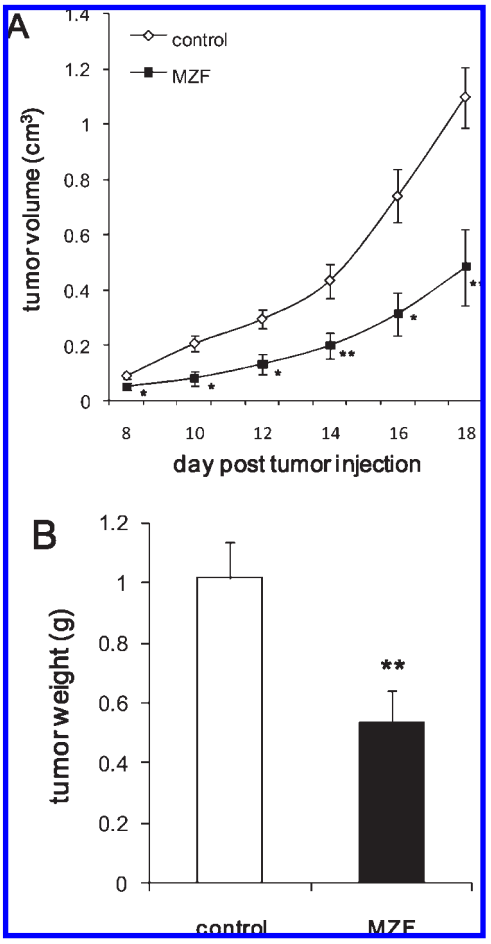

Figure 3. Effect of MZF on colon-26 tumor growth in vivo: (**A**) tumor volume; (**B**) tumor weight on day 18 after tumor inoculation. Colon-26 cells (1×10^5 cells/mouse) were inoculated subcutaneously into BALB/cA mice on day 0. Tumor-inoculated mice were injected intraperitoneally with either saline or MZF (8 mg/kg/day) for 17 consecutive days after tumor inoculation. At the end of the experiment, tumor weight was recorded. Data ($n = 5$) are presented as means ± SE. Similar results were obtained in at least three independent experiments. ∗, $p < 0.05$, and ∗∗, $p < 0.01$, by the Mann−Whitney *U* test.

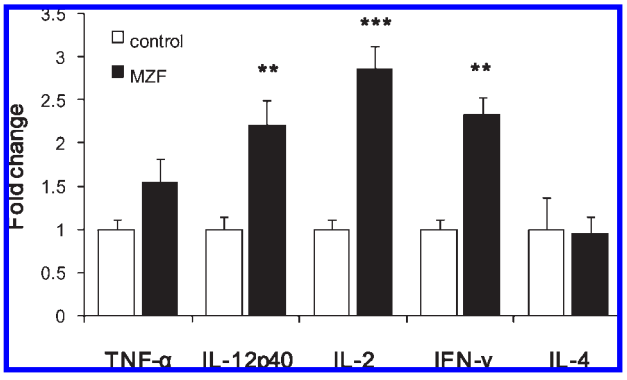

Figure 4. Expression of mRNA for various cytokines in spleen. Eighteen days after tumor inoculation, total RNA was prepared from the spleens, and mRNA expressions of TNF-α, IL-12p40, IL-2, IFN-γ, and IL-4 were evaluated by using quantitative real-time PCR. Cytokine mRNA expression was normalized to GAPDH expression. Results represent one of two independent experiments with similar results. Values are expressed as mean ± SE ($n = 5$). ∗∗, $p < 0.01$, and ∗∗∗, $p < 0.001$, versus control.

percentages of NK cells and T cells were examined. MZF significantly increased the percentage of splenic $CD49b^+CD3^-$ NK cells in MZF-treated mice (**Figure 5**). However, the number of splenic T cells was not affected by MZF treatment (not shown).

IFN-γ is the hallmark cytokine of Th1-type $CD4^+$ T cells. NK cells and $CD8^+$ CTLs also produce IFN-γ. Therefore, we investigated the percentages of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in MZF-treated mice by using flow cytometry. As shown in **Figure 6**, the percentage of IFN-γ-producing cells in both $CD4^+$ and $CD8^+$ T cells from MZF-treated mice was significantly higher than that from control mice. The percentages of IL-4-producing cells in $CD4^+$ and $CD8^+$ T cells were not affected by MZF treatment. These results are consistent with the pattern of mRNA expression in spleen.

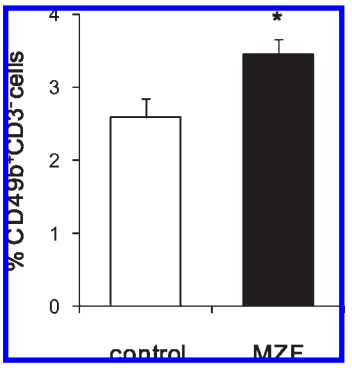

Figure 5. MZF administration increases the number of NK cells in spleen. Whole spleen cells were collected after 18 days of MZF administration, and the percentage of NK cells ($CD49b^+CD3^-$ cells) was analyzed by flow cytometry. The percentages shown are representative of two independent experiments with similar results. Values are expressed as mean ± SE (n = 5). *, $p < 0.05$, versus control.

To investigate NK cell activity, the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed. Compared to the MZF-untreated control, MZF treatment significantly enhanced NK cell-mediated cytotoxicity at all E:T ratios (E:T = 50, 100, and 150) (**Figure 7A**). We further examined the effect of MZF on the induction of tumor-specific CTL responses. Spleen cells were removed 18 days after tumor inoculation and cultured with MMC-treated colon-26 cells in the presence of rIL-2 (50 ng/mL), and the CTL activity against calcein AM-labeled colon-26 cells was determined. As shown in **Figure 7B**, MZF treatment significantly induced CTL activity compared with the MZF-untreated control at an E:T ratio of 100.

MZF Promoted the Infiltration of T Cells into the Tumor Site. We examined whether MZF enhanced the infiltration of T cells in the tumor site. On day 18 after tumor inoculation, the tumor-bearing mice were sacrificed, and the tumors were removed. The TILs were separated and analyzed by flow cytometry. As shown **Figure 8**, MZF treatment increased the percentage of the tumor infiltrating $CD4^+$ and $CD8^+$ T cells compared with the control group. These results indicated that accumulation of T cells in the tumor sites was augmented by MZF administration.

DISCUSSION

The maitake mushroom (*G. frondosa*) has become a very familiar edible mushroom in Japan because it became possible to produce its fruiting body artificially. We previously reported on the mechanism of antitumor action by MD-fraction β-(1, 3) (1, 6)-glucan (1000−2000 kDa) extracted from *G. frondosa* (*7*−*9*). In the present study, we isolated a novel polysaccharide, MZF, from *G. frondosa* and demonstrated its structure and immunomodulatory

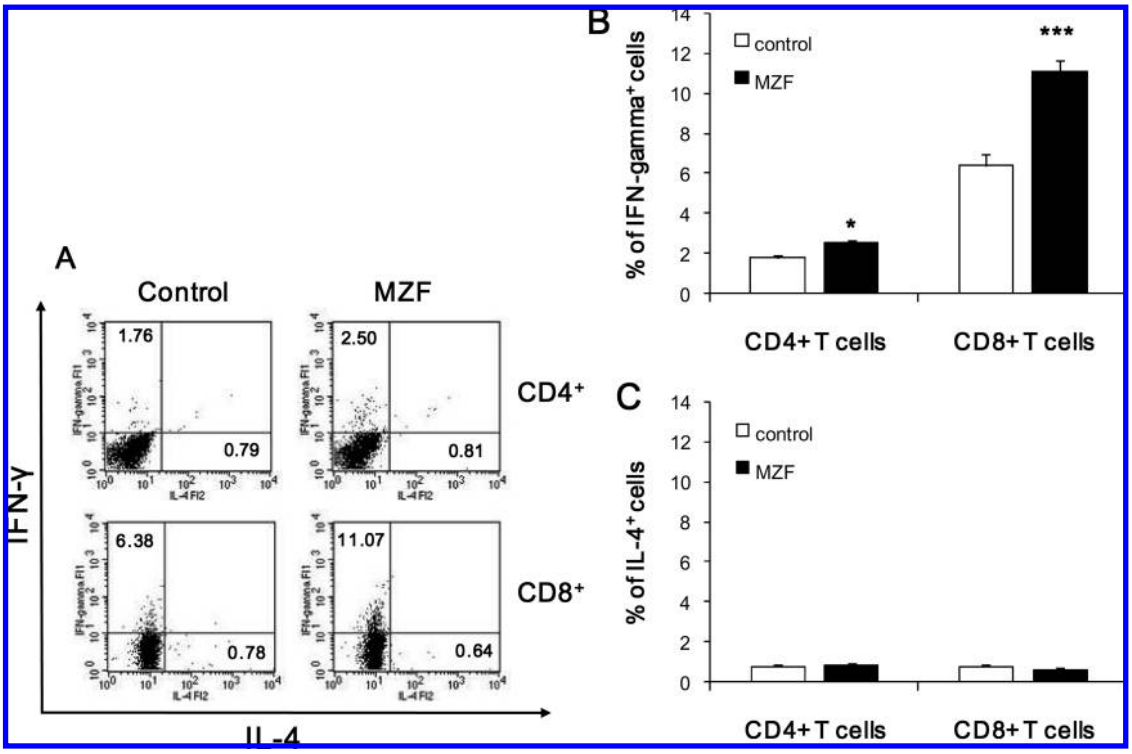

Figure 6. MZF administration induces IFN-γ-producing $CD4^+$ and $CD8^+$ T cells: (**A**) representative plots; (**B**) percent IFN-γ^+ cells in $CD4^+$ and $CD8^+$ cells; (**C**) percent IL-4^+ cells in $CD4^+$ and $CD8^+$ cells. Whole spleen cells (1×10^6 cells/mL) were collected after 18 days for MZF administration and incubated with ionomycin, phorbol-12-myristate-13-acetate, and Goldistop at 37 °C in 5% CO_2 for 4 h. After incubation, the cells were stained with anti-CD4, anti-CD8, and anti-IFN-γ Abs and analyzed by flow cytometry. The percentages shown are representative of two independent experiments with similar results. Values are expressed as mean ± SE (n = 5). *, $p < 0.05$, and ***, $p < 0.001$, versus control.

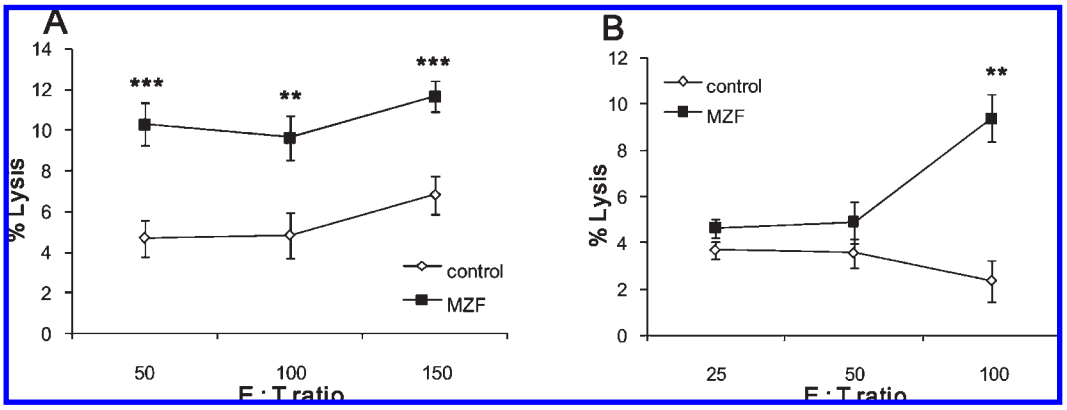

Figure 7. MZF induces increased lysing capacity of NK cells and CTLs. (**A**) Cytotoxicity of NK cells was assessed by measuring the lysis of the target YAC-1 cells mediated by splenocytes from 18 days following tumor challenge. The calcein AM-labeled target cells were mixed with the splenocytes at various effector-to-target (E:T) ratios in 96-well U-bottom plates. After incubation for 2 h at 37 °C in 5% CO_2, supernatants were harvested, and the fluorescence was read. (**B**) Cytotoxicity of tumor-specific CTLs in MZF-treated and control mice. Spleen cells were removed from mice on day 18 after the tumor inoculation, mixed with MMC-treated colon-26 cells, and incubated for 72 h at 37 °C in 5% CO_2 in the presence of rIL-2 (50 ng/mL) to induce antigen-specific CTL. The CTL activity was determined using calcein AM-labeled colon-26 cells as target at different E:T ratios. After incubation for 2 h at 37 °C in 5% CO_2, supernatants were harvested, and the fluorescence was read at 485/538 nm. The percentages shown are representative of two independent experiments with similar results. Values are expressed as mean ± SE ($n = 5$). **, $p < 0.01$, and ***, $p < 0.001$, versus control.

activity. The polysaccharide MZF was eluted as a single peak corresponding to an average molecular mass of 23.0 kDa, as determined by HPSEC chromatogram. MZF consisted of D-galactose, D-mannose, L-fucose, and D-glucose. Using the results from methylation and NMR experiments, it can be concluded that MZF is a complex heteropolysaccharide consisting of →6)-α-D-Gal*p*-(1→, →3)-α-L-Fuc*p*-(1→, →6)-α-D-Man*p*-(1→, →3)-β-D-Glc*p*-(1→, α-D-Man*p*-(1→, and →3,6)-β-D-Glc*p*-(1→ (**Figure 1**; **Table 1**). However, further studies, such as 2D NMR, will be required to establish the primary structure of MZF.

Immunomodulatory effects of polysaccharides extracted from natural sources have been previously reported. β-Glucans extracted from yeast and mushrooms show antitumor effects and enhance hematopoiesis. Glucans are recognized by dectin-1 and complement receptor 3 (CR3), and recent studies of those receptors have revealed the mechanism of the immunomodulatory effects of glucans (*24*−*26*). Mannans extracted from *Saccharomyces cerevisiae* and *Candida albicans* are recognized by several receptors, including Toll-like receptors, the mannose receptor, and DC-SIGN (*27*−*29*). These receptors—the pattern recognition receptors (PRRs)—are expressed on macrophages and DCs and recognize highly conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) (*30*). Polysaccharides extracted from fungi are recognized by PRRs as PAMPs. Following recognition, the polysaccharide can enhance the host immune system. MZF significantly induced the proliferation of resident peritoneal macrophages, as did polysaccharides derived from other natural products (**Figure 2A**). These facts suggest that MZF initially acts on antigen-presenting cells (APCs) such as macrophages and dendritic cells. MZF consists of many sugar linkages containing →6)-α-D-Man*p*-(1→; α-D-Man*p*-(1→; →3)-β-D-Glc*p*-(1→; and →3,6)-β-D-Glc*p*-(1→. These results suggest the possibility that MZF may be recognized by PRRs on APCs. However, because of its complex structure, MZF might be recognized by APCs via several pathways. Further investigation is required to clarify the details of recogination of MZF by APCs. Although MZF did not affect the proliferation of colon-26 cells in vitro, it significantly inhibited tumor growth in BALB/cA mice inoculated with colon-26 cancer cells (**Figures 2C** and **3**). In this study, we demonstrated that MZF could efficiently stimulate an antitumor immune response and induce tumor regression.

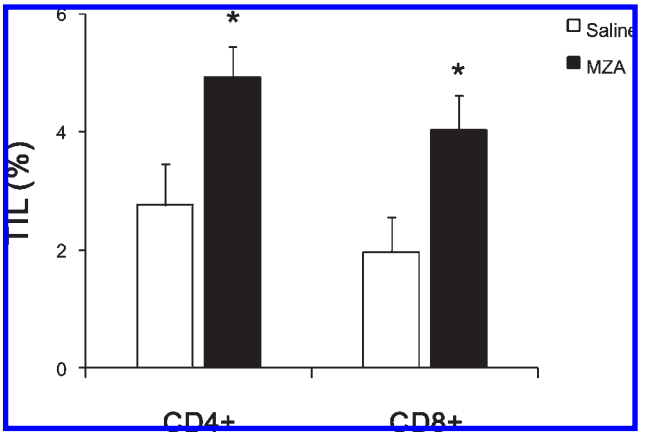

Figure 8. MZF promoted the percentage of the TILs. Tumors were removed from mice on day 18 after tumor inoculation, and the TILs were separated and analyzed by flow cytometry. Values are expressed as mean ± SE ($n = 4$−5). *, $p < 0.05$, versus control.

$CD8^+$ T cells (CTLs) play a major role as effector cells in cancer immunity. In addition, NK cells and macrophages are effector cells that attack tumor cells. IL-12 augments NK cell- and T cell-mediated cytotoxicity and stimulates IFN-γ and IL-2 production by Th1 cells. MZF increased the expression of IL-12, IFN-γ, and IL-2 significantly in tumor-bearing mice (**Figure 4**). MZF also induced a Th1 response by increasing the percentage of IFN-γ-producing cells in both splenic $CD4^+$ and $CD8^+$ T cells and cytotoxic activity in NK cells and CTLs (**Figures 5**−**7**). These results suggest that NK cells and CTLs contribute to the antitumor effects induced by MZF. Futhermore, MZF promoted the infiltration of $CD4^+$ and $CD8^+$ T cells in tumor sites (**Figure 8**). It is considered that the accumulation of tumor-infiltrating T cells positively correlated with the antitumor response caused by MZF treatment.

The immunological effects of an extract from *G. frondosa* have already been demonstrated in human clinical trials (*13*). In this study, we reported the immunomodulatory activity of the novel water-soluble heteropolysaccharide MZF isolated from *G. frondosa*. MZF administration suppressed colon-26 tumor growth in

BALB/cA mice to a level similar to that effected by the existing β-glucan, MD-fraction (*9*). The antitumor activity of MZF was thought to be associated with induced cell-mediated immunity. These findings support the clinical findings of the extract from *G. frondosa*.

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LITERATURE CITED

(1) Nair, P. K.; Rodriguez, S.; Ramachandran, R.; Alamo, A.; Melnick, S. J.; Escalon, E.; Garcia, P. I. Jr.; Wnuk, S. F.; Ramachandran, C. Immune stimulating properties of a novel polysaccharide from the medicinal plant *Tinospora cordifolia*. *Int. Immunopharmacol.* **2004**, *4* (13), 1645–1659.

(2) Bittencourt, V. C.; Figueiredo, R. T.; da Silva, R. B.; Mourao-Sa, D. S.; Fernandez, P. L.; Sassaki, G. L.; Mulloy, B.; Bozza, M. T.; Barreto-Bergter, E. An α-glucan of *Pseudallescheria boydii* is involved in fungal phagocytosis and Toll-like receptor activation. *J. Biol. Chem.* **2006**, *281* (32), 22614–22623.

(3) Tsukagoshi, S.; Hashimoto, Y.; Fujii, G.; Kobayashi, H.; Nomoto, K.; Orita, K. Krestin (PSK). *Cancer Treat. Rev.* **1984**, *11* (2), 131–155.

(4) Chihara, G.; Hamuro, J.; Maeda, Y.; Arai, Y.; Fukuoka, F. Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Res.* **1970**, *30* (11), 2776–2781.

(5) Hoshi, H.; Yagi, Y.; Iijima, H.; Matsunaga, K.; Ishihara, Y.; Yasuhara, T. Isolation and characterization of a novel immunomodulatory α-glucan-protein complex from the mycelium of *Tricholoma matsutake* in basidiomycetes. *J. Agric. Food Chem.* **2005**, *53* (23), 8948–8956.

(6) Harada, T.; Miura, N.; Adachi, Y.; Nakajima, M.; Yadomae, T.; Ohn, N. Effect of SCG, 1,3-β-D-glucan from *Sparassis crispa* on the hematopoietic response in cyclophosphamide induced leukopenic mice. *Biol. Pharm. Bull.* **2002**, *25* (7), 931–939.

(7) Nanba, H.; Hamaguchi, A.; Kuroda, H. The chemical structure of an antitumor polysaccharide in fruit bodies of *Grifola frondosa* (maitake). *Chem. Pharm. Bull.* **1987**, *35* (3), 1162−1168.

(8) Harada, N.; Kodama, N.; Nanba, H. Relationship between dendritic cells and the D-fraction-induced Th-1 dominant response in BALB/c tumor-bearing mice. *Cancer Lett.* **2003**, *192* (2), 181–187.

(9) Masuda, Y.; Inoue, M.; Miyata, A.; Mizuno, S.; Nanba, H. Maitake β-glucan enhances therapeutic effect and reduces myelosupression and nephrotoxicity of cisplatin in mice. *Int. Immunopharmacol.* **2009**, *9* (5), 620–626.

(10) Lin, H.; Cheung, S. W.; Nesin, M.; Cassileth, B. R.; Cunningham-Rundles, S. Enhancement of umbilical cord blood cell hematopoiesis by maitake β-glucan is mediated by granulocyte colony-stimulating factor production. *Clin. Vaccine Immunol.* **2007**, *14* (1), 21–27.

(11) Lin, H.; She, Y. H.; Cassileth, B. R.; Sirotnak, F.; Cunningham Rundles, S. Maitake β-glucan MD-fraction enhances bone marrow colony formation and reduces doxorubicin toxicity *in vitro*. *Int. Immunopharmacol.* **2004**, *4* (1), 91–99.

(12) Ito, K.; Masuda, Y.; Yamasaki, Y.; Yokota, Y.; Nanba, H. Maitake β-glucan enhances granulopoiesis and mobilization of granulocytes by increasing G-CSF production and modulating CXCR4/SDF-1 expression. *Int. Immunopharmacol.* **2009**, *9* (10), 1189–1196.

(13) Deng, G.; Lin, H.; Seidman, A.; Fornier, M.; D'Andrea, G.; Wesa, K.; Yeung, S.; Cunningham-Rundles, S.; Vickers, A. J.; Cassileth, B. A phase I/II trial of a polysaccharide extract from *Grifola frondosa* (maitake mushroom) in breast cancer patients: immunological effects. *J. Cancer Res. Clin. Oncol.* **2009**, *135* (9), 1215–1221.

(14) Kubo, K.; Aoki, H.; Nanba, H. Anti-diabetic activity present in the fruit body of *Grifola frondosa* (maitake). I. *Biol. Pharm. Bull.* **1994**, *17* (8), 1106–1110.

(15) Gu, C. Q.; Li, J. W.; Chao, F.; Jin, M.; Wang, X. W.; Shen, Z. Q. Isolation, identification and function of a novel anti-HSV-1 protein from *Grifola frondosa*. *Antiviral Res.* **2007**, *75* (3), 250–257.

(16) Nishina, A.; Kimura, H.; Sekiguchi, A.; Fukumoto, R. H.; Nakajima, S.; Furukawa, S. Lysophosphatidylethanolamine in *Grifola frondosa* as a neurotrophic activator via activation of MAPK. *J. Lipid Res.* **2006**, *47* (7), 1434–1443.

(17) Amornrut, C.; Toida, T.; Imanari, T.; Woo, E. R.; Park, H.; Linhardt, R.; Wu, S. J.; Kim, Y. S. A new sulfated β-galactan from clams with anti-HIV activity. *Carbohydr. Res.* **1999**, *321* (1−2), 121–127.

(18) Ciucanu, I.; Kerek, F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **1984**, *131* (2), 209–217.

(19) Masuda, Y.; Murata, Y.; Hayashi, M.; Nanba, H. Inhibitory effect of MD-fraction on tumor metastasis: involvement of NK cell activation and suppression of intercellular adhesion molecule (ICAM)-1 expression in lung vascular endothelial cells. *Biol. Pharm. Bull.* **2008**, *31* (6), 1104–1108.

(20) Cui, W.; Taub, D. D.; Gardner, K. qPrimerDepot: a primer database for quantitative real time PCR. *Nucleic Acids Res.* **2007**, *35* (database issue), D805−D809.

(21) Wang, X.; Seed, B. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res.* **2003**, *31* (24), e154.

(22) Neri, S.; Mariani, E.; Meneghetti, A.; Cattini, L.; Facchini, A. Calcein-acetyoxymethyl cytotoxicity assay: standardization of a method allowing additional analyses on recovered effector cells and supernatants. *Clin. Diagn. Lab. Immunol.* **2001**, *8* (6), 1131–1135.

(23) Vliegenthart, J. F. G.; Dorland, L.; Halbeek, H. High-resolution, ^{1}H-nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 209–374.

(24) Yan, J.; Vetvicka, V.; Xia, Y.; Coxon, A.; Carroll, M. C.; Mayadas, T. N.; Ross, G. D. β-Glucan, a "specific" biologic response modifier that uses antibodies to target tumors for cytotoxic recognition by leukocyte complement receptor type 3 (CD11b/CD18). *J. Immunol.* **1999**, *163* (6), 3045–3052.

(25) Brown, G. D.; Taylor, P. R.; Reid, D. M.; Willment, J. A.; Williams, D. L.; Martinez-Pomares, L.; Wong, S. Y.; Gordon, S. Dectin-1 is a major β-glucan receptor on macrophages. *J. Exp. Med.* **2002**, *196* (3), 407–412.

(26) Li, B.; Allendorf, D. J.; Hansen, R.; Marroquin, J.; Ding, C.; Cramer, D. E.; Yan, J. Yeast β-glucan amplifies phagocyte killing of iC3b-opsonized tumor cells via complement receptor 3-Syk-phosphatidylinositol 3-kinase pathway. *J. Immunol.* **2006**, *177* (3), 1661–1669.

(27) Tada, H.; Nemoto, E.; Shimauchi, H.; Watanabe, T.; Mikami, T.; Matsumoto, T.; Ohno, N.; Tamura, H.; Shibata, K.; Akashi, S.; Miyake, K.; Sugawara, S.; Takada, H. *Saccharomyces cerevisiae*- and *Candida albicans*-derived mannan induced production of tumor necrosis factor α by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. *Microbiol. Immunol.* **2002**, *46* (7), 503–512.

(28) Sheng, K. C.; Pouniotis, D. S.; Wright, M. D.; Tang, C. K.; Lazoura, E.; Pietersz, G. A.; Apostolopoulos, V. Mannan derivatives induce phenotypic and functional maturation of mouse dendritic cells. *Immunology* **2006**, *118* (3), 372–383.

(29) Taylor, P. R.; Gordon, S.; Martinez-Pomares, L. The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol.* **2005**, *26* (2), 104–110.

(30) Brown, G. D. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* **2006**, *6* (1), 33–43.

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