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Characterization of a Novel Maitake (*Grifola frondosa*) Protein That Activates Natural Killer and Dendritic Cells and Enhances Antitumor Immunity in Mice

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Supporting Information

ABSTRACT: *Grifola frondosa*, also known as maitake, is a culinary mushroom with immune-enhancing and antitumor effects. Numerous studies have investigated the activity of maitake polysaccharide extracts, but studies of maitake proteins are scarce. In this study, we purified and characterized a new *G. frondosa* protein, GFP, from maitake fruiting bodies. GFP is a nonglucan heterodimeric 83 kDa protein that consists of two 41 kDa subunits. GFP induced interferon-γ secretion by murine splenocytes and natural killer cells and activated the maturation of bone marrow-derived dendritic cells (BMDCs) via a TLR4-dependent mechanism. GFP-treated BMDCs promoted a Th1 response and exhibited significant antitumor activity when transferred into tumor-bearing mice. In conclusion, we are the first to reveal the critical role of GFP in modulating the immune response and to link the immune-enhancing effects of maitake to its antitumor activities.

KEYWORDS: *antitumor, bone marrow-derived dendritic cells, Grifola frondosa, immune modulation, maitake, natural killer cells*

INTRODUCTION

Grifola frondosa, also known as maitake, is an edible mushroom that is widely used as a culinary material and dietary supplement in Asia. The polysaccharide extracts from maitake exhibit multiple health benefits, such as regulating blood glucose, blood pressure,[1−4] and lipid metabolism[5] and eliciting a hepatoprotective effect.[6,7] In addition, maitake extracts were shown to enhance T-cell activity,[8,9] to promote the Th1 response in tumor-bearing mice,[10,11] to induce natural killer cell (NK) activation,[12] to increase the tumor-killing rate of NKs,[13] and to stimulate dendritic cell (DC) maturation.[11,14] Furthermore, studies have also demonstrated the suppressive effects of maitake extracts against mammary,[15] lung,[13,16] and colon carcinomas.[9,14,17] These studies suggest links between the immunostimulatory properties and antitumor effects of maitake.

NKs are innate immune cells that play critical roles in tumor immune surveillance and can detect tumor cells in the absence of specific antigens.[18] NKs as well as T cells can mediate the antitumor response by directly inducing tumor cell death or by secreting cytokines such as TNF-α and IFN-γ.[19−21] In addition to T cells and NKs, DCs also participate in the uptake, processing, and presentation of tumor antigens and are indispensable for the generation of T-cell tumor antigen-specific response.[18,22]

Most studies describing the immune-enhancing and antitumor activities of maitake used polysaccharide extracts. Methods for isolating specific polysaccharide fractions have been proposed; however, these fractions contain mixtures of multiple compounds, and the chemical composition of these fractions has been difficult to define.[9,12,23] In addition to polysaccharides, fungal proteins have demonstrated significant immune-modulating and tumor-suppressing activities.[24] Our previous studies showed that proteins from *Ganoderma lucidium* and *Anoectochilus formosanus* exhibited functions different from those of the polysaccharide extracts from *Ga. lucidium* and *A. formosanus*.[25,26] Notably, maitake polysaccharide extracts such as the D and MD fractions also contained 1−20% proteins[27] that might possess unique activities.

In this study, we purified a novel *G. frondosa* protein, GFP, from the fruiting bodies of maitake, characterized its composition, and investigated its functions with murine splenocytes, T cells, NK cells, and DCs. Moreover, the tumor-suppressing properties of GFP-activated immune cells were demonstrated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Purification of GFP from the Fruiting Bodies of *G. frondosa*. Fresh fruiting bodies of *G. frondosa* (Fr.) S.F. Gray var. Grifon 120 were produced by the HOKTO Corp. (Nagano City, Japan) and purchased from a supermarket in Taipei, Taiwan. The fruiting bodies were immersed in extraction buffer (320 g of fruiting bodies/L) containing 5% (v/v) acetic acid, 0.1% (v/v) 2-mercaptoethanol, and 0.308 M sodium chloride. The samples were allowed to stand overnight at 4 °C and then homogenized using a Waring Laboratory blender (Waring Laboratory Science, Torrington, WY) followed by sonication using a Sonicator XL2015 (MISONIX, Farmingdale, NY). The homogenates were centrifuged at 7000*g* and 4 °C for 40 min, and the supernatant was collected and filtered with 5C 110 mm filter paper (Advantec MFS, Inc., Dublin, CA) to remove the insoluble fraction. The protein in the filtered supernatant was salted out by applying 90% saturation of ammonium sulfate and allowing the solution to stand for

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at least 24 h at 4 °C. The precipitates containing the crude protein were collected by centrifugation at 7000 g and 4 °C for 45 min, and the crude protein was suspended and dialyzed in 0.01 M Tris-HCl buffer (pH 8.2) for 72 h with at least six changes of the dialysis solution. The dialysate was then loaded onto a self-packed, DEAE 52 cellulose (Whatman, Maidstone, U.K.) column (2.5 cm × 20 cm) that was pre-equilibrated with 0.01 M Tris-HCl buffer (pH 8.2) for fractionation. The column was eluted with a linear gradient from 0.0 to 1.0 M NaCl, and the main active fractions were pooled for further purification using an AKTA fast protein liquid chromatography system (FPLC, Amersham Biosciences, Uppsala, Sweden). The pooled fractions were loaded onto a HitrapQ anion exchange column (GE Healthcare, Buckinghamshire, U.K.) that had been pre-equilibrated with 0.01 M Tris-HCl buffer (pH 8.2) and then eluted with a linear gradient from 0.0 to 1.0 M NaCl. The fractions containing purified GFP were collected, dialyzed in PBS, and then concentrated to the appropriate concentration (1 mg/mL).

Molecular Weight (MW) Determination of GFP Samples. Capillary electrophoresis and gel filtration were conducted to determine the MW of reduced and native GFP, respectively.

A commercial, sodium dodecyl sulfate-MW analysis kit (Beckman Coulter, Inc., Brea, CA) was used to prepare the samples for capillary electrophoresis. Briefly, to prepare the reduced GFP and MW standards, 85 μL of GFP (1 mg/mL) or sodium dodecyl sulfate-MW standards was treated with 10 μL of sodium dodecyl sulfate-MW sample buffer, 10 μL of 2-mercaptoethanol, and 2 μL of 10 kDa internal standards. The sample/standard solution was mixed thoroughly and then boiled for 5 min to reduce the level of any possible tertiary or quaternary structures. Data acquisition and analysis was conducted using a PA800 plus Pharmaceutical Analysis System (Beckman Coulter) with assistance from a technician following the manufacturer's instructions. The MW was calibrated by normalizing the migration time of the samples with the internal standard and with the standard curve that was constructed using the protein size standard.

Gel filtration was conducted on a high-performance liquid chromatography (HPLC) system (ECOM, Prague, Czech Republic) using a PROTEIN KW-802.5 gel filtration column (Shodex, Munich, Germany). The column was equilibrated and eluted with PBS at a flow rate of 1 mL/min. Native GFP was dialyzed in PBS and loaded onto the column, and the MW was determined using a gel filtration calibration kit (GE Healthcare). The calibration kit contained MW standards that included blue dextran, albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A (MWs of 2000, 67, 43, 25, and 13.7 kDa, respectively). The MW of GFP was calculated using the calibration curve that was established using the MW standards and following the manufacturer's protocol.

Periodic Acid Schiff (PAS) Staining To Determine the Carbohydrate Content of GFP. Purified GFP was analyzed by SDS−PAGE using a Bio-Rad Mini Protein III gel apparatus (Bio-Rad, Hercules, CA). The gel was stained with PAS reagent (Sigma, St. Louis, MO) to reveal carbohydrate-containing proteins, and a control gel was stained with Coomassie Brilliant Blue R250 (CBR) to visualize all protein bands.

N-Terminal Amino Acid Sequence Analysis of GFP. Purified GFP was separated by SDS−PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Cell System (Bio-Rad) in transfer buffer. A protein band corresponding to GFP was cut from the CBR-stained membrane and subjected to Edman degradation. Automated Edman degradation and sequence analyses were conducted using an Applied Biosystems Procise Sequencer (Life Technologies, Carlsbad, CA).

Mice and Primary Cell Acquisition. C57BL/6J and BALB/c mice between 6 and 8 weeks of age were purchased from the National Laboratory Animal Center in Taipei, Taiwan. TLR2 (B6.129-*Tlr2*^{tm1Kir}/J) and TLR4 (C57BL/10ScNJ) gene knockout mice were purchased from The Jackson Laboratory, and all mice were maintained in our animal facility under specific, pathogen-free conditions. All animal studies were approved (NTU-IACUC-98-112) and performed under the guidelines of the Institutional Animal Care and Use Committee of National Taiwan University.

Murine splenic cells were acquired as described previously.[28] Briefly, mouse spleens were excised from 6−10-week-old BALB/c mice after CO_2 euthanasia. Splenocytes were suspended in PBS, and red blood cells were lysed with hypotonic buffer. The cells were washed with and maintained in complete RPMI medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (GIBCO-BRL Life Technologies, New York, NY). Splenic T and NK cells were positively selected from total splenocytes using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-mouse CD90 and anti-mouse CD49b microbeads, respectively. Splenic cells were then seeded in 96-well flat-bottomed plates at a density of 2.5 × 10^5 cells/well.

Bone marrow (BM)-derived DC cells were generated following previously described protocols.[29] Briefly, femurs and tibiae were removed from 4-week-old BALB/c, C57BL/6, B6.129-*Tlr2*^{tm1Kir}/J, or C57BL/10ScNJ mice after CO_2 euthanasia, and the surrounding muscle tissue was rubbed off using Kleenex tissues. The cleaned bones were sterilized in 70% ethanol for 2−5 min and then washed with PBS. Both ends of the bones were cut, and the marrow was flushed out with PBS using a syringe and a 26G needle. The BM cells were washed once with PBS, and the red blood cells were lysed with hypotonic buffer. The BM cells were suspended in complete RPMI medium that was supplemented with 500 units/mL GM-CSF (PeproTech, Rocky Hill, NJ) and were seeded in six-well plates (Corning) at a density of 1 × 10^6 cells/mL per well. The cells were cultured for 6 days with one change of fresh, GM-CSF-supplemented, complete RPMI medium on day 3. Nonadherent cells were harvested for BMDC experiments, and the purity of the $CD11c^+$ BMDCs was determined to be >70% by flow cytometry. All cells were maintained in a HeraCell CO_2 incubator (Heraeus group, Hanau, Germany) at 37 °C with 5% CO_2 humidified air.

Cytokine Production Measurement of Murine Splenic Cells and BMDCs. Total splenocytes, purified splenic NK cells, or BMDCs (2.5 × 10^5 cells/well in a flat-bottomed 96-well plate) were cultured with GFP (0.25−2 μg/mL) for 24−72 h. For mixed leukocyte reactions, BMDCs that had been cultured with GFP (1 μg/mL) for 24 h were cocultured with naïve, purified T cells in the presence of purified NA/LE rat anti-mouse IL-12, p40/p70, or isotype-matched IgG2A control antibodies (0.1 or 1 μg/mL) for 72 h. Additionally, culture supernatants were collected for cytokine measurements using Mouse IFN-γ DuoSet, Mouse IL-12p70 DuoSet (R&D Systems, Minneapolis, MN), Mouse IL-4 ELISA Ready-SET-Go!, and Mouse IL-6 ELISA Ready-SET-Go! kits following the manufacturers' protocols (eBioscience, San Diego, CA).

Surface Marker Expression Analysis of Murine Splenic Cells and BMDCs by Flow Cytometry. Total splenocytes and BMDCs were cultured with or without GFP (0.25−2 μg/mL) or LPS (1 μg/mL) for 72 h. The cells were then harvested, resuspended, and washed with PBS containing 20% FBS and 1.25 M sodium azide. The cells were stained with FITC-conjugated anti-mouse CD3e, CD86, major histocompatibility complex (MHC) class I, or MHC class II or with phycoerythrin (PE)-conjugated anti-mouse CD11c, CD69, or CD314 (eBioscience) following the manufacturer's protocol. Data were acquired using a FACScan (Becton Dickson, Franklin Lakes, NJ), analyzed using FlowJo (Tree Star Inc., Ashland, OR), and presented as the geometric means of the fluorescence intensities.

Evaluation of the Tumor-Suppressive Activity of GFP-Stimulated Murine Splenic Cells and BMDCs. Lewis lung carcinoma cell line LLC1 (ATCC CRL-1642) was purchased from the American Type Culture Collection (Manassas, VA) and was maintained in DMEM medium with 10% FBS in a HeraCell incubator at 37 °C with 5% CO_2 humidified air.

For *in vitro* evaluation, LLC-1 cells were seeded in 96-well, U-bottomed plates at a density of 10^5 cells/mL and were cultured for 4 h in the presence of BrdU labeling reagent (Roche Applied Science, Mannhelm, Germany) at a working concentration that was recommended by the manufacturer. After BrdU labeling, the LLC1 cells were washed with PBS to remove excessive BrdU labeling reagent, and splenocytes that had been pretreated with GFP (2 μg/

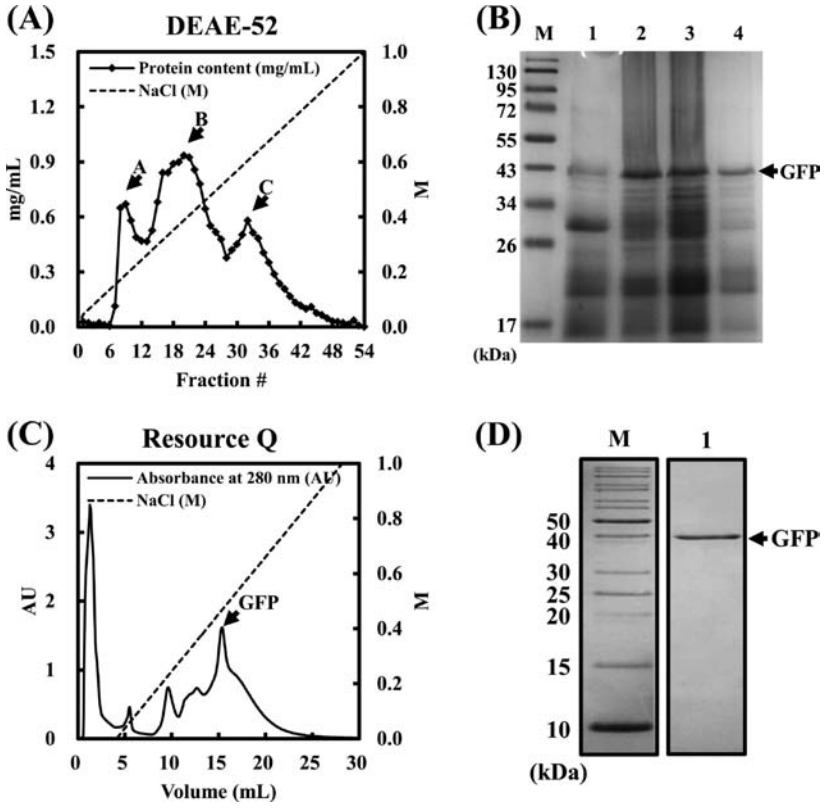

Figure 1. Purification of GFP. (A) The crude proteins that were extracted from fresh *G. frondosa* fruiting bodies were separated into three parts via DEAE anion exchange chromatography. (B) The DEAE-separated protein fractions in panel A were analyzed by SDS−PAGE, and the content in each lane is as follows: lane 1, PageRuler Prestained Protein Ladder SM0671; lane 2, fractions 28−30; lane 3, fractions 31−33; lane 4, fractions 34−36; and lane 5, fractions 37−39. (C) The fractions of part C, which consisted mainly of GFP, were pooled and further purified via a Resource Q anion exchange column. (D) The Resource Q-purified GFP sample was analyzed by SDS−PAGE, and the content in each lane is as follows: lane M, PageRuler Unstained Protein Ladder SM0661; and lane 1, purified GFP.

mL) for 48 h or without it were added to the LLC1 cultures at the indicated effector/target ratios (E/T; splenocytes/LLC1). The mixed cells were cocultured for 4 h, and adherent $BrdU^+$ LLC1 cells were detected using a Cell Proliferation ELISA BrdU colorimetric kit (Roche) following the manufacturer's protocol.

For *in vivo* evaluation, tumor antigen LLC1 lysates were prepared with four freeze−thaw cycles. Briefly, an autoclaved Eppendorf tube carrying 1×10^7 LLC1 cells was immersed in liquid nitrogen for 5 min, vortexed for 1 min, immersed in a 37 °C water bath for 5 min, and vortexed for 1 min. BMDCs were derived from BM cells of 5-week-old C57BL/6J mice. The cells were cultured in six-well plates (3×10^6 cells/well) in the presence of LLC1 lysates (equal to 1×10^6 cells/well) and were treated with GFP (0.625 μg/mL) or PBS as a vehicle control for 24 h. LLC1-primed cells were adoptively transferred by subcutaneous injection into the dorsal region near the thigh of 6-week-old C57BL/6J mice (3×10^5 cells/mouse). Another group of mice was treated with PBS and served as a control group. Seven days after cell transfer, LLC1 cells were inoculated into the mice (3×10^5 cells/mouse) in the same region where the adoptive transfer of BMDCs was conducted. Tumor size was monitored every other day from 10 to 20 days after tumor inoculation, and mouse serum was collected for tumor-specific immunoglobulin quantification by a standard enzyme-linked immunosorbent assay (ELISA) method using a 96-well ELISA microplate that was coated with LLC1 lysates and biotinylated anti-mouse IgA, IgG, or IgM (H+L) antibodies (KPL, Inc., Gaithersburg, MD). A schematic figure showing the time line of the model is presented in Figure S1 of the Supporting Information.

Statistical Analysis. All *in vitro* experiments were performed in triplicate (n = 3), and a representative result from at least two independent experiments is shown as the mean ± standard deviation (SD). The antitumor murine model was performed in pentaplicate (n = 5), and a representative result from three independent experiments is shown as the mean ± SD. One-way analysis of variance and a Student's t test were used for statistical comparisons, and the differences between the experimental groups were considered to be significant when the obtained P value was less than 0.05.

RESULTS

Purification of GFP. Crude proteins were extracted from the fruiting bodies of fresh *G. frondosa* (Figure S2 of the Supporting Information) and fractionated using DEAE-52 anion exchange chromatography. The crude proteins were divided into three major groups [parts A−C (Figure 1A)]. The fractions of each group were collected, pooled, and examined for potential immunomodulating activity. The part C fraction, which was eluted by 0.25−0.3 M NaCl, showed the most robust potential and was further purified (Figure S3 of the Supporting Information). Multiple proteins with MWs of 12, 16, 20, 30, and 40 kDa were identified in part C by SDS−PAGE analysis (Figure 1B). Using a Resource Q anion exchange column, a major peak isolated from the part C fraction (Figure 1C) was collected and analyzed by SDS−PAGE, and a single 40 kDa protein band was found (Figure 1D). The protein was named GFP (*G. frondosa* protein). The purity of the GFP that was used in this study was found to be >95% by SDS−PAGE, and the yield was 11.5 ± 2.5 mg/kg of fresh *G. frondosa* fruiting bodies. Polysaccharide contamination in the GFP preparation was excluded by the PAS staining test (Figure S4 of the Supporting Information), and the endotoxin level was found to be <0.011 EU/μg according to the chromogenic LAL assay.

GFP Exhibits Characteristics of a Heterodimeric Protein. Gel filtration was conducted to determine the precise MW of GFP. Interestingly, the MW of native GFP was 83.4 kDa (Figure 2A), which was approximately twice the MW of

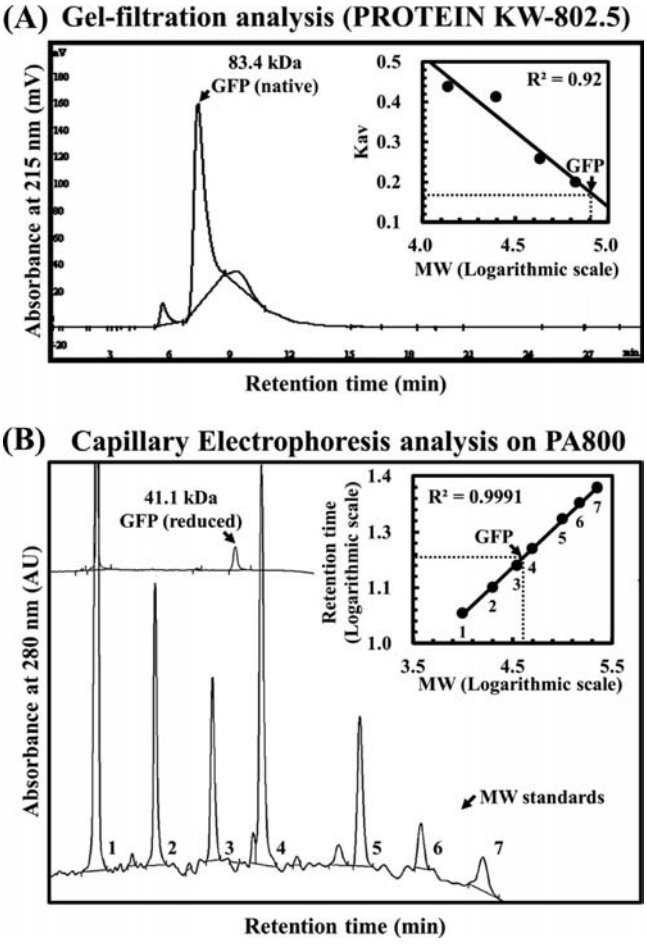

Figure 2. Molecular weight determination of native and SDS- and heat-treated GFP. (A) The MW of the native form of GFP was determined by gel filtration using a PROTEIN KW-802.5 SHODEX column in an ECOM high-performance liquid chromatography system. (B) The MW of GFP that was denatured by SDS and heat treatment was determined by capillary electrophoresis using an SDS-MW analysis kit. Data were acquired using a PA800 plus Pharmaceutical Analysis System.

SDS-treated GFP. The precise MW of SDS-treated GFP was verified by capillary electrophoresis, and as in the SDS−PAGE analysis, the MW of SDS-treated GFP was 41.1 kDa (Figure 2B). These results suggested that GFP could consist of two subunits with similar MWs. Furthermore, because 2-mercaptoethanol is a reducing agent that breaks down disulfide linkages, it is possible that the two subunits of GFP are connected via disulfide bonds. N-Terminal amino acid sequence analysis of GFP also revealed intriguing information. The first six and the eighth N-terminal amino acid signals, which were obtained from the Edman reaction, showed two possible amino acid signals at each position: [A and G] at residues 1 and 2, [R and H] at residue 3, [V and A] at residue 4, [A and P] at residue 5, [H and S] at residue 6, [V] at residue 7, and [T and P] at residue 8. In addition, the area under the curve of the dual signals was indistinguishable at each position, which suggested that GFP might consist of two different subunits (Figure S5 of the Supporting Information).

GFP Activates Secretion of IFN-γ by Murine Splenocytes and NK Cells. The ability of GFP to modulate the splenocyte response was evaluated. It was reported that extracts of *G. frondosa* could induce Th1-dominant responses in mice.[30] We found that GFP induced murine splenocytes to secrete IFN-γ, which is a key cytokine that mediates the Th1 response. This effect was dose-dependent, and at a concentration of 2 μg/mL, GFP stimulated IFN-γ secretion more than 10-fold compared with that of the control group (Figure 3A). To further identify the cell types that responded to GFP, we analyzed GFP-stimulated splenocytes by flow cytometry. GFP increased the population of DX-5$^+$ NK cells from 4.0 to 6.1% (Figure 3B) and enhanced the expression of NKG2D, which is a receptor that is strongly expressed in mature NK cells, by 2.6-fold compared to that of the control cells (Figure 3C). These results indicated that NK cells were activated by GFP. Using MACS-purified DX5$^+$ splenic NK cells, we demonstrated that GFP could directly induce a 45-fold increase in the level of IFN-γ secretion by NK cells without aid from other immune cells (Figure 3D). Despite the fact that T and NK cells are the major sources of IFN-γ secretion,[31] GFP failed to stimulate MACS-purified T cells to secrete IFN-γ (Figure 3D).

GFP Induces the Maturation and Th1-Driving Capacity of Murine BMDCs. We also evaluated the ability of GFP to activate BMDCs. When stimulated by GFP (0.25−2 μg/mL), the expression levels of MHC I, MHC II, and CD86, which are surface markers that are associated with DC maturation, were enhanced by 1.7-, 1.4-, and 2.4-fold, respectively (Figure 4A). The secretion of cytokine IL-6 and IL-12 by BMDCs also showed a clear dose response upon stimulation with GFP (0.25−2 μg/mL), and IL-6 and IL-12 production was increased by 32- and 297-fold, respectively (Figure 4B,C).

DCs play critical and indispensable roles in mediating the innate and adaptive T-cell immune responses. Therefore, we performed a mixed leukocyte reaction to examine whether GFP-induced BMDCs could activate T-cell responses. We demonstrated that IFN-γ secretion was activated when MACS-purified murine CD90$^+$ T cells were cocultured with GFP-induced BMDCs (Figure 4D). Furthermore, we found that adding IL-12-neutralizing antibodies into the coculture system significantly reduced the level of IFN-γ secretion, whereas the isotype control antibodies showed no such effect (Figure 4D). These findings suggested that GFP could activate the T-cell response by stimulating BMDCs to secrete Th1 cytokines such as IL-12.

TLR4 Dependency of GFP-Induced BMDC Maturation. To elucidate the putative mechanism of GFP-induced BMDC maturation, we used B6.129-*Tlr2*^{tm1Kir}/J (TLR2$^{-/-}$) and C57BL/10ScNJ (TLR4$^{-/-}$) TLR gene knockout mice. We discovered that knockout of the TLR2 gene did not alter the ability of GFP to activate BMDCs. GFP significantly induced the secretion of IL-12 and IL-6 from TLR2$^{-/-}$ BMDCs by 29- and 64-fold, respectively (Figure 5A,B). However, TLR4 gene knockout completely abrogated the ability of GFP to activate BMDCs, and GFP did not induce secretion of IL-12 or IL-6 by TLR4$^{-/-}$ BMDCs (Figure 5A,B). These findings revealed that TLR4 could be the major receptor for GFP and that GFP-induced BMDC maturation might be mediated through the TLR signaling pathway.

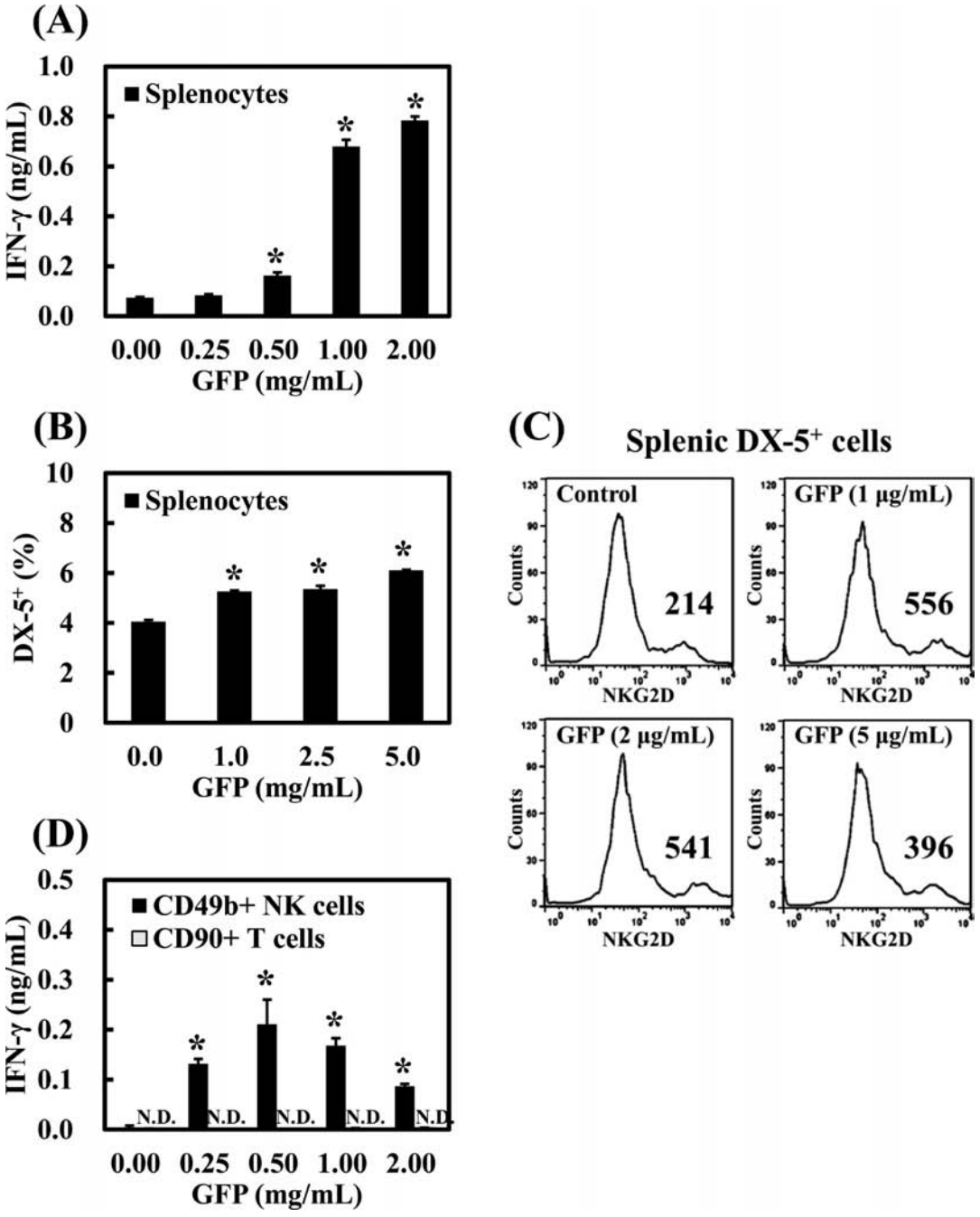

Figure 3. GFP activates IFN-γ production by murine splenic NK cells. (A–C) Splenocytes from BALB/c mice were cultured with GFP (0.25–2 μg/mL) for 72 h or without it. Subsequently, the cell-free culture supernatants were collected for (A) IFN-γ measurements via an ELISA, and the cells were harvested for (B) DX5 and (C) NKG2D expression analysis via flow cytometry. (B) Percentages of DX-5$^+$ cells that were present among total splenocytes. (C) Geometric means of the fluorescence intensities of NKG2D in DX5$^+$ cells. (D) MACS-purified CD49$^+$ NK and CD90$^+$ T cells from BALB/c mice cultured with GFP (0.25–2 μg/mL) for 72 h or without it. The cell-free culture supernatants were collected for IFN-γ measurements via an ELISA. Asterisks indicate data that were significantly ($p < 0.05$) different from those of the control group, and the N.D. sign indicates that the value was below the detection limit of the standard curve that was established following the manufacturer's protocol.

GFP-Induced Splenocytes and BMDCs Elicit an Antitumor Effect *in Vitro* and *in Vivo*. DC-based immunotherapy has been developed to treat cancer.[32] On the basis of the finding that GFP was capable of inducing cell maturation and the Th1-promoting activity of BMDCs, we established an *in vitro* evaluation system to test whether GFP-induced splenocytes could exert an antitumor effect. Murine Lewis lung cancer cell line LLC1 was grown and labeled with BrdU. Then, the BrdU-labeled cells were cocultured with murine splenocytes that had been treated with either 2 μg/mL GFP or medium alone for 4 h. Subsequently, the tumoricidal effect of the cells was measured by a BrdU ELISA.

The amount of BrdU that was detected steadily decreased with an increased E/T ratio in the GFP-treated splenocyte (effector cells) and LLC1 (target cells) coculture system. This finding indicated that GFP-treated splenocytes reduced the cell number of the surviving LLC1 cells and that this effect was dependent on the amount of effector cells that was added

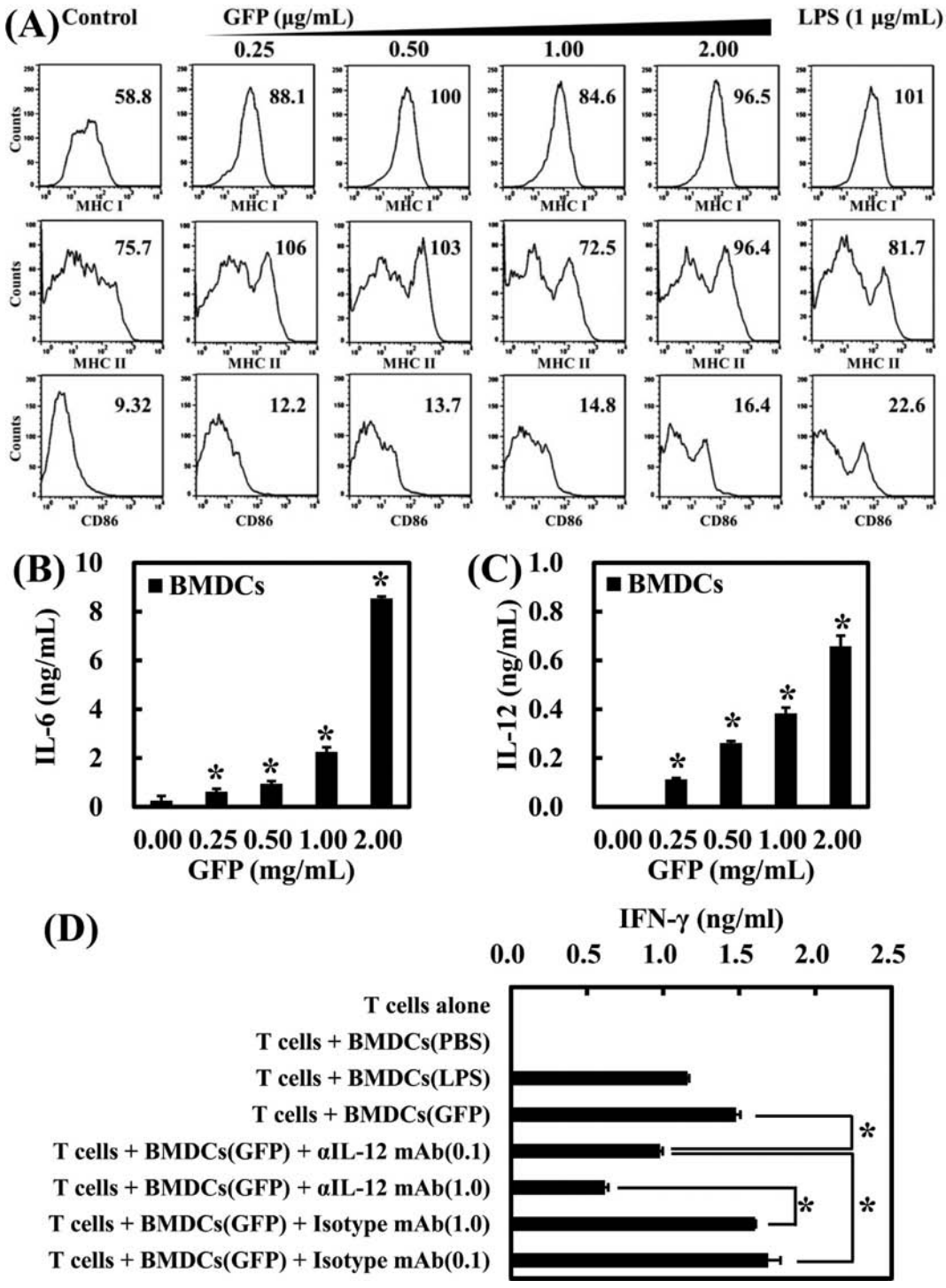

Figure 4. GFP stimulates the maturation and Th1-inducing ability of BMDCs. (A–C) BMDCs from BALB/c mice were cultured with GFP (0.25–2 μg/mL) or LPS (1 μg/mL) for 24 h or without it. Then, the cells were harvested for (A) surface marker MHC-I, MHC-II, and CD86 expression analysis via flow cytometry, and the numbers indicate the geometric means of the fluorescence intensities. Cell-free culture supernatants were collected for (B) IL-6 and (C) IL-12 p70 measurements via an ELISA. Asterisks indicate data that were significantly ($p < 0.05$) different from those of the control group. (D) BMDCs from BALB/c mice were cultured with GFP (1 μg/mL) or LPS (1 μg/mL) for 24 h or without it. The cells were then cocultured with MACS-purified $CD90^+$ T cells from BALB/c mice in the presence or absence of IL-12-neutralizing antibodies (0.1 or 1 μg/mL) for 72 h. Then, cell-free culture supernatants were collected for IFN-γ measurement via an ELISA. Asterisks indicate that the differences between the groups were significant ($p < 0.05$).

(Figure 6A). Conversely, the extent of BrdU detection only mildly decreased with an increased E/T ratio in the control splenocyte and LLC1 coculture system (Figure 6A). To calculate the tumoricidal rate, we determined the number of LLC1 cells using a calibration curve (Figure S6 of the Supporting Information) and then divided the cell number of the remaining LLC1 cells by the cell number of the seeded LLC1 cells. We showed that at E/T ratios of >40 effector cells

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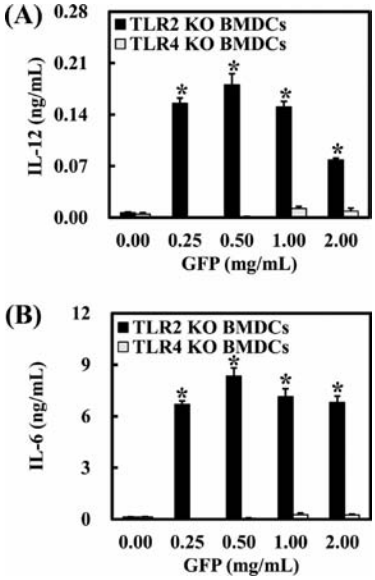

Figure 5. GFP-induced BMDC cytokine production is TLR4-dependent. BMDCs from TLR2 (B6.129-*Tlr2*^{tm1Kir}/J) and TLR4 (C57BL/10ScNJ) gene knockout mice were incubated with GFP (0.25−2 μg/mL) for 24 h or without it. Cell-free culture supernatants were then collected for (A) IL-12 p70 and (B) IL-6 measurements via an ELISA. Asterisks indicate that the data were significantly ($p < 0.05$) different from those of the control group.

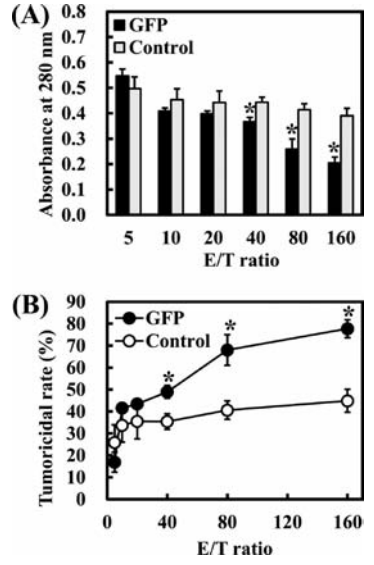

Figure 6. GFP-activated splenocytes exert tumoricidal activity *in vitro*. (A) LLC1 murine Lewis lung cancer cells were seeded in a 96-well U-bottomed plate and labeled with BrdU labeling reagent for 4 h. Then, the BrdU-labeled LLC1 cells were cocultured with splenocytes that had been treated for 48 h with GFP (2 μg/mL) or PBS as a control. The cells were cocultured at the indicated E/T (splenocytes/LLC1) ratios for 4 h, and adherent BrdU$^+$ LLC1 cells were detected using a Cell Proliferation ELISA BrdU colorimetric kit. (B) The cell number of the remaining BrdU$^+$ LLC1 was calculated using a calibration curve (Figure S5 of the Supporting Information), and the tumoricidal rate was calculated by dividing the cell number of the remaining LLC1 cells by the cell number of the seeded LLC1 cells. Asterisks indicate data that were significantly ($p < 0.05$) different from those of the control group.

per target cell, GFP-treated splenocytes exerted a significantly higher tumoricidal rate than control splenocytes. The tumoricidal rates at E/T ratios of 40, 80, and 160 were 49, 68, and 78%, respectively, for the GFP group, whereas the tumoricidal rates at E/T ratios of 40, 80, and 160 were 35, 41, and 45%, respectively, for the control group (Figure 6B). The relatively high E/T ratio might be due to the rapid growth rate of LLC1 tumor cells and the fact that the GFP-stimulated splenocytes exert predominantly the nonspecific tumoricidal effect, which was less efficient than the tumor-specific immune response exerted by effector lymphocytes, such as tumor-specific cytotoxic T lymphocytes. These results revealed the potential of GFP to activate the antitumor immune response that inhibits cancer cell growth by direct killing.

Next, we examined the tumor-suppressing efficacy of *ex vivo*, GFP-stimulated BMDCs using a murine LLC1 tumor model. C57BL/6J mice received an adoptive transfer of PBS-treated antigen-primed BMDCs (pBMDCs), GFP-treated pBMDCs, or PBS as a control. Seven days after cell transfer, the mice were inoculated with Lewis lung cancer cell line LLC1, and tumor growth was permitted for 20 days. Then, the antitumor activity of the cells was evaluated by measuring tumor growth and by calculating the levels of tumor-specific antibodies in mouse serum. The sizes of the tumors were measured every other day beginning at day 10 after LLC1 inoculation. At the end of the experiment, the tumor sizes of the mice that received GFP-treated pBMDCs, PBS-treated pBMDCs, and PBS were 251 ± 89, 2708 ± 418, and 2092 ± 672 mm^3, respectively (Figure 7A). Tumor growth was significantly inhibited by 91% in mice that received GFP-treated pBMDCs versus that in mice that received PBS-treated pBMDCs (Figure 7A,B). Furthermore, the rate of tumor growth of mice that received PBS-treated pBMDCs was higher than that of mice that received PBS; however, the difference was insignificant. The levels of tumor-specific antibodies in the serum of the mice that received GFP-treated pBMDCs, PBS-treated pBMDCs, and PBS were 0.59 ± 0.15, 0.58 ± 0.12, and 0.87 ± 0.31, respectively [absorbance at 450 nm (Figure 7C)]. Altogether, we confirmed that GFP could inhibit tumor progression by activating antitumor immunity *in vitro* and in mice.

■ DISCUSSION

G. frondosa (maitake) is a culinary mushroom that has health-promoting effects. Studies of maitake extracts showed that the polysaccharide fractions contained promising immunostimulatory and tumor-suppressive agents.[33] However, maitake fractions consisted of mixtures of β-glucan and other components rather than a pure molecule, and the most widely studied D and MD fractions contained 1−20% protein.[27] In this study, through ammonium sulfate precipitation and anion exchange chromatography, we isolated a pure protein, GFP, from fresh maitake fruiting bodies, and polysaccharide contamination in the purified GFP preparation was excluded by PAS staining. To the best of our knowledge, this is the first report of a highly purified maitake protein that exhibits Th1-stimulating and antitumor activity.

In a previous study, Kodama et al.[15] isolated a low-molecular weight protein fraction from a dried powder of maitake fruiting bodies by a different approach. The maitake powder was extracted with hot water, and the substances with MWs that were <5 kDa in the extracts were collected for DEAE and gel filtration chromatography. The resulting fraction was designated as the MLP fraction. In this study, through a two-step chromatographic purification, the yield of GFP was 11.5 mg/kg of fruiting bodies; however, it was difficult to compare the

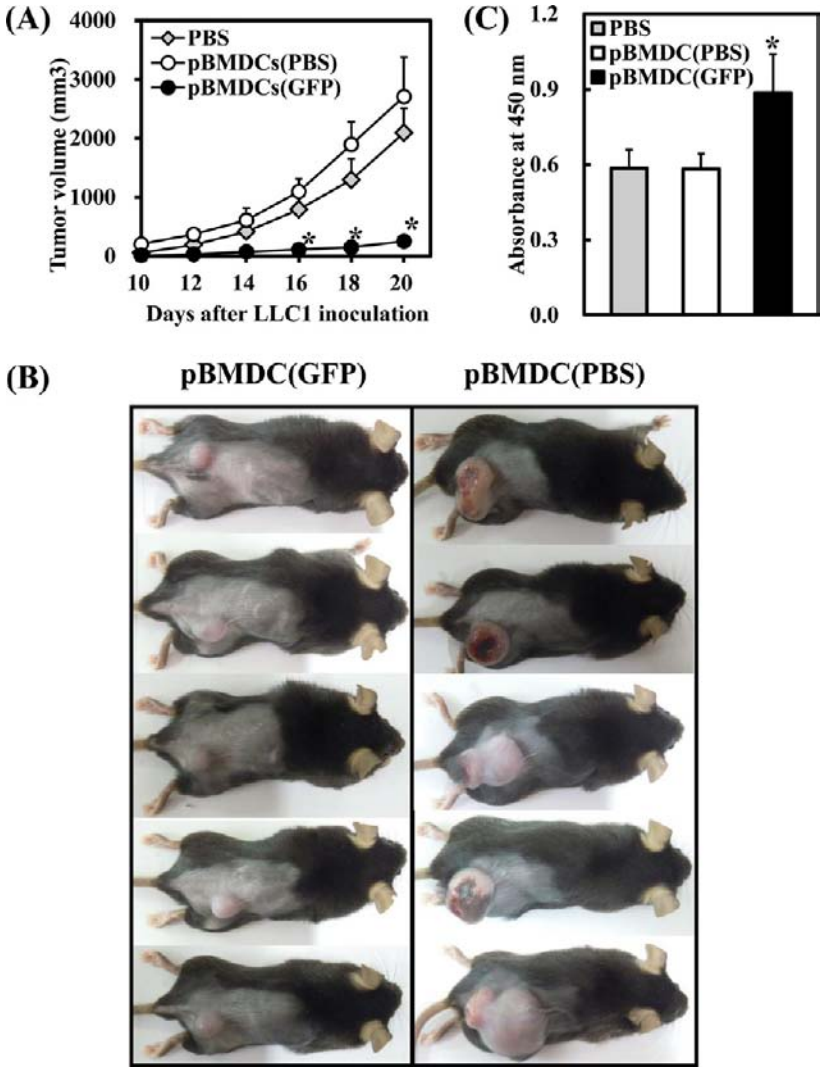

Figure 7. GFP-activated BMDCs inhibit tumor growth and increase the level of tumor antigen-specific antibody production in mice. BMDCs from C57BL/6J mice were cultured with tumor-antigen LLC1 lysates and treated with GFP (0.625 μg/mL) or PBS for 24 h. The LLC1-primed cells were then transferred into 6-week-old C57BL/6J mice by subcutaneous injection, and a control group was injected with PBS. One week after cell transfer, LLC1 cells were inoculated into the mice at the transfer site. (A and B) Ten days after LLC1 inoculation, the tumor volume was measured every other day until the end of the experiment on day 20. (A) Severity of tumor growth 20 days after LLC1 inoculation. (B) Progression of tumor volume from 10 to 20 days after LLC1 inoculation. (C) Mouse serum was collected to quantify tumor antigen-specific antibodies by a standard ELISA. Asterisks indicate data that were significantly ($p < 0.05$) different from those of mice that received PBS or PBS-treated BMDCs.

yields of GFP and MLP because the yield of MLP from maitake fruiting bodies was not available.

When comparing the size of GFP with the sizes of proteins in other maitake protein fractions, we showed that GFP belongs to neither the M and MD nor the MLP fractions because the MWs of proteins in those fractions were approximately 1000 and <5 kDa, respectively. GFP had a MW of 83 kDa in its native form; however, when GFP was denatured via reducing treatments, its MW was reduced to 41 kDa. In addition, the results of an Edman degradation reaction showed dual amino acid signals at the N-terminus of GFP. These findings suggested that GFP could be a heterodimeric protein consisting of two subunits with similar MWs. Notably, we previously isolated a *Poria cocos* protein (PCP) that could activate murine macrophages through ligation to TLR4. Similar to GFP, PCP was a heterodimeric protein consisting of 14.3 and 21.3 kDa subunits.[34] Another example of a dimeric protein is fungal immunomodulatory protein-fve (FIP-fve), which can stimulate IFN-γ secretion by murine splenocytes[24] and human peripheral blood mononuclear cells.[35,36] The structure of FIP-fve was defined, and it was demonstrated that it formed a homodimer in nature.[37] It was also shown that the structures of these fungal proteins played critical roles in their immune-modulating activities.[38] Thus, whether the dimeric structure of GFP is essential to its function requires further study.

The immunostimulatory effects of maitake extracts have been extensively studied. In tumor-bearing mice, maitake extracts enhanced the production of IFN-γ by splenocytes, NKs, BMDCs, and activated lymph node T cells and promoted the Th1-dominant response.[10−12,39] In this study, we tested the function of GFP in normal mouse immune cells. GFP also stimulated splenocyte IFN-γ production and induced the activation and proliferation of splenic NKs. Furthermore, we revealed that GFP had the ability to activate IFN-γ production in purified NKs, which suggests the existence of potential GFP-responsive receptors in NKs that remain to be investigated. We

also showed that GFP was capable of inducing secretion of IL-12 by BMDCs and that GFP-treated BMDCs could induce the IL-12-dependent Th1 response. Nevertheless, this finding also suggested the possibility that Th1-cell-produced IFN-γ should also contribute significantly in the GFP-induced immune activation as well as antitumor activity. The observation that GFP-induced splenocytes produced a larger amount of IFN-γ than did the purified NKs under the same culture conditions also supported this hypothesis. Interestingly, another heteropolysaccharide maitake Z-fraction (MZF) that was isolated by Masuda et al.[14] showed a similar ability to enhance BMDC maturation and to induce IL-12-dependent antigen-specific secretion of IFN-γ by $CD4^+$ T cells.[14] Altogether, GFP and other maitake extracts share some similar immune-modulating properties; however, because the protein content in these maitake extracts was less appreciated, it would be interesting to elucidate the contribution of the maitake polysaccharides and proteins.

The ability of fungal polysaccharides and protein/peptides to stimulate immune responses largely occurs via interactions between pattern recognition receptors that are presented on immune cells. Our study and others have demonstrated the important roles played by the TLRs, especially TLR4. By taking advantage of TLR4-neutralizing antibodies, Lin et al. confirmed that the ability of *Ga. lucidium* polysaccharide extract F3 to activate B cell differentiation[40] and the ability of *Ga. lucidium* protein LZ-8 to induce DC maturation[41] were both TLR4-dependent. Our previous work also indicated the involvement of TLR4 in the APCs activation that was stimulated by proteins purified from *A. formosanus*, *P. cocos*, and *Trametes versicolor*.[26,34,42] Here, we showed that TLR4 also played an indispensable role in GFP-induced BMDC maturation because production of IL-6 and IL-12 was completely abrogated by TLR4 knockout.

The ability of maitake extracts to inhibit tumor growth has been demonstrated by murine models of tumor grafts, where NK- and T-cell activation and enhanced Th1 cytokine production were linked to an enhanced antitumor response in the host.[10−12,39] In this study, we used various approaches to evaluate the capacity of GFP-stimulated immune cells to repress tumors. First, we demonstrated *in vitro* that GFP-treated splenocytes, which were activated to secrete IFN-γ, exhibited superior tumoricidal activity when compared to normal splenocytes. Second, in a murine tumor model, we showed that the adoptive transfer of GFP-treated tumor-antigen-primed BMDCs significantly reduced the rate of tumor growth. We also tried to evaluate the systematic immune response induced by adoptive transfer of BMDCs by measuring the cytokine production of splenocytes acquired from tumor-bearing mice. However, we failed to detect any significant alteration on splenic cytokine production (Figure S7 of the Supporting Information). The mechanism by which GFP exerted antitumor effect remains to be elucidated. Masuda et al. also showed that DCs that were primed with tumor lysates in the presence of MZF exhibited therapeutic and preventive effects on tumor development in mice.[14] Our data showed the ability of GFP to exert an antitumor effect and provided direct evidence of a link between the enhanced immune function and improved antitumor activity.

In conclusion, we have introduced the first characterization of purified maitake protein GFP that is free of polysaccharide content. GFP naturally formed an 83 kDa heterodimer that was reduced to two 41 kDa subunits after SDS denaturation. GFP also induced secretion of IFN-γ by NKs and activated BMDCs via a TLR4-dependent mechanism. GFP-treated BMDCs activated an IL-12-dependent Th1 response and exhibited a significant tumor-suppressive effect *in vivo*. This study accentuates the critical contribution of proteins in the pharmacological function of maitake and demonstrates the link between its immune-stimulating and antitumor effects.

■ ASSOCIATED CONTENT

Supporting Information

Schematic figure that shows the time line of the LLC1 murine tumor model (Figure S1), one representative photograph of a fresh maitake (*G. frondosa*) fruiting body that was used in this study (Figure S2), results of an MTT assay on the proteins of the part C fractions that were isolated through DEAE chromatography (Figure S3), results of PAS and CBR staining (contents of lanes: M, PageRuler Prestained Protein Ladder SM0671; PCP, *P. coco* protein, which is a known carbohydrate-containing protein;[33] TFP, *Tremella fuciformis* protein, which is a non-carbohydrate-containing protein that was purified in our laboratory; and GFP, the *G. frondosa* protein that was used in this study) (Figure S4), graphs of the results of amino acid sequence analysis of the first eight N-terminal residues of GFP (Figure S5), calibration curve of the cell number of the $BrdU^+$ LLC1 cells versus their absorbance at 450 nm (Figure S6), and results of splenic cytokine production of LLC1 tumor-bearing mice (Figure S7). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

APC, antigen-presenting cell; BMDC, bone marrow-derived dendritic cell; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; Ig, immunoglobulin; LPS, lipopolysaccharide; MACS, magnetic-activated cell sorting; MHC, major histocompatibility complex; MW, molecular weight; NK, nature killer cell; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLR, Toll-like receptor; TNF, tumor necrosis factor

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