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Polysaccharides PS-G and Protein LZ-8 from Reishi (*Ganoderma lucidum*) Exhibit Diverse Functions in Regulating Murine Macrophages and T Lymphocytes

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Bioactive components in *Ganoderma lucidum* mainly include polysaccharides (PS-G) and immunomodulatory protein Ling Zhi-8 (LZ-8). These components may have diverse regulatory functions in the immune system. However, the PS-G preparations from different procedures still contained partial LZ-8 residue, indicating that the specific target and regulating function of PS-G and LZ-8 were not fully understood. In the present study, PS-G was subjected to 15% TCA for removing proteins and the LZ-8 detection using anti-LZ-8 monoclonal antibodies showed a remarkable 89.7% protein reduction of the deproteinized PS-G (dpPS-G). The *Saccharomyces cerevisiae* which expressed recombinant LZ-8 protein (rLZ-8) without glycosylation was generated and then compared with dpPS-G in the induction toward murine primary macrophage and T lymphocytic cells. The peritoneal macrophages from TLR4-deficient and wild type mice revealed that TLR4 was a putative receptor of dpPS-G, mediating the TNF-α, IL-1β and IL-12p70 cytokine production and CD86, MHC II expression on macrophages, while rLZ-8 enhanced the production of IL-1β, IL-12p70, CD86, and MHC II expression by another obscure route. rLZ-8-treated macrophages enhanced the release of IFN-γ and IL-2 by murine $CD4^+$ and $CD8^+$ T cells, whereas dpPS-G treatment did not enhance the release of IFN-γ and IL-2. Furthermore, although the direct rLZ-8-treatment conduced dramatic CD154, CD44 expression on $CD3^+$ T cells and increased IL-2, IFN-γ secretion on $CD4^+$ and $CD8^+$ T cells, the dpPS-G was incapable of priming $CD3^+$, $CD4^+$ or $CD8^+$ T cells unitarily. Taken together, these results demonstrated that LZ-8 could activate murine macrophages and T lymphocytes but PS-G was merely the activator for macrophages, suggesting their diverse roles in activating the innate and adaptive immunity.

KEYWORDS: *Ganoderma lucidum*; immune modulation; polysaccharide; PS-G; Ling Zhi-8 (LZ-8); TLR4

INTRODUCTION

Ganoderma lucidum, a native biosidiomycetous fungus (also known as Reishi or Ling-Zhi), has long been used as a traditional Chinese herbal medicine in the Orient to prevent and treat various human diseases such as bronchitis, hepatitis, hypertension, neoplasia, and immunological disorders for over 2 millennia (*1*). General compounds extracted from mycelia, fruiting body and spores of *G. lucidum* (e.g., triterpenoids, proteins, alkaloids, polysaccharides) have been reported to be effective in modulating immune functions, promoting antitumor activity (*2*), and generating antiviral effects (*3*). Peptides isolated from *G. lucidum* have also been shown to possess potent antioxidant activity with little or no side effects (*4*).

Polysaccharide from *G. lucidum* (PS-G) which comprises backbone with (1→3)-β-D-glucan moiety has demonstrated antineoplastic activity and immunomodulation activity by enhancing the cytotoxic activity of $CD56^+$ natural killer (NK) cells and stimulating the expression of interleukin-2 (IL-2), interferon-γ (INF-γ), and interleukin-1β (IL-1β) within mouse spleen cells (*5*,*6*). PS-G has also been reported to inhibit spontaneous and Fas-mediated apoptosis in human neutrophils through the activation of Akt-regulated signaling pathways and induce immunoglobulin production through the TLR4/TLR2-mediated induction of transcription factor Blimp-1 within B cells (*7*, *8*). For the activation of antigen-presenting cells (APCs), PS-G could induce the maturation of human monocyte-derived dendritic cells (DCs) via NF-κB and p38 mitogen-activated protein kinase pathways and be involved in the pro-IL-1/IL-1 regulation of human blood-derived macrophages and mouse macrophage cell lines through TLR4 (*9*, *10*).

In addition to PS-G and triterpenoids, a fungal immunomodulatory protein, Ling Zhi-8 (LZ-8), had been isolated from the mycelia of *G. lucidum* and was regarded as one of the major bioactive substances of *G. lucidum* (*11*). LZ-8 is a polypeptide consisting of 110 amino acid residues, and the native form of LZ-8 showed a homodimer configuration with noncovalent linkage and a molecular mass of 24 kDa (*12*). In addition, LZ-8 had superior thermal (121 °C to −80 °C) and acid stabilities (pH 2) and a moderate resistance to alkali (pH 13) and dehydration treatments (*13*). Crystallization study of LZ-8 revealed an

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N-terminal dimerization domain and a C-terminal FNIII domain which belongs to the immunoglobulin-like β-sandwich fold structure (*14*). LZ-8 resembled lectin mitogenic capacity toward mouse spleen cells, human peripheral blood lymphocytes (PBL), and peripheral mononuclear cells (*11*, *15*, *16*). Within the human primary T cells and cultured Jurkat T cells, LZ-8 induced the expression of IL-2 gene via the Src-family protein tyrosine kinase (PTK), reactive oxygen species (ROS), and differential protein kinase-dependent pathways (*17*). It has also been demonstrated that LZ-8 could effectively promote the activation and maturation of immature DCs by the NF-κB/MAPK pathways and prefers a Th1 response within OVA-immunized mice model (*18*).

Though the immunological properties of polysaccharides and immunomodulatory protein derived from *G. lucidum* have been studied respectively, the *G. lucidum* polysaccharides extracted from different procedures still contain partial (up to 10−20%) protein (*19*, *20*) which may include the LZ-8 residue. The effective dose of LZ-8 (1 μg/mL) reported was much lower than PS-G (50 μg/mL), thus, the minor LZ-8 content remaining within PS-G might give a misleading impression for researchers to understand the actual action of Reishi polysaccharide in regulating immunity. In the present study, the existence of LZ-8 within PS-G samples was demonstrated and reported for the first time. The deproteinized PS-G (dpPS-G) and yeast expressed recombinant LZ-8 (rLZ-8) without glycosylation were then prepared to clarify the major target cells and functions of dpPS-G and rLZ-8 . It was revealed that dpPS-G and rLZ-8 were both effective activators of murine macrophages, but obviously in diverse manners. TLR4 was involved in the recognition of PS-G and dpPS-G on macrophages, while rLZ-8 might be recognized by macrophages with another obscure route. Moreover, the capability of rLZ-8 to directly activate murine macrophage and $CD4^+/CD8^+$ T lymphocytic cells suggested the partial function contribution of LZ-8 in the PS-G preparation.

MATERIALS AND METHODS

Animal Experiments. C57BL/6J mice aged between 8 and 12 weeks old were used as wild-type (WT) control animals. The mice were obtained from the National Laboratory Animal Center, Taiwan. TLR4-deficient ($TLR4^{-/-}$, strain C57BL/10ScN) mice from Jackson Laboratory (Bar Harbor, ME) were maintained in animal facility under pathogen-free conditions. All animal studies performed were approved by the Institutional Animal Care and Use Committee of National Taiwan University.

Primary Cell Culture. Thioglycollate-elicited murine peritoneal macrophages were elicited by ip injecting 1.6 mL of 4% Brewer's thioglycollate medium (Sigma Aldrich, St. Louis) into the peritoneal cavities of the C57BL/6J and $TLR4^{-/-}$ mice. After three days, the peritoneal exudates cells were harvested by lavage of the peritoneal cavity with ice-cold phosphate buffered saline (PBS), then washed and resuspended in a complete culture medium (Dulbecco's modified Eagle medium (DMEM) (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (FBS), kanamycin (0.1 mg/mL), and gentamycin (0.1 mg/mL). Unless otherwise stated, cell suspensions were plated in 48- and 96-well culture plates (flat-bottom) at 1×10^6 cells/well and $3-5 \times 10^5$ cells/well, respectively. The plates were incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. Nonadherent cells were removed by repeated washing with warm Hanks' balanced salt solution, and the adherent cells (macrophages) were further cultured.

Functional Expression of rLZ-8. Full length of the LZ-8 gene (NCBI M58032.1) was amplified from cDNA of *G. lucidum* by PCR using forward primer 5′-AAAAAGAATTCATG**TCCGACACTGCCTTGATCT**-3′ and reverse primer 5′-AAAAAGAATTC**CTAGTTCCACTGGGCGATGATG**-3′, and restriction sites of *Eco*RI (underlined) were designed for flanking the PCR product at the 5′- and 3′-terminus, respectively. The PCR fragment was cloned into the *Eco*RI site of pYEX-S1 plasmid (Clontech, USA) to produce the expression plasmid pYEX-LZ-8. The LZ-8 expression plasmid was transformed into *Saccharomyces cerevisiae* DBY747 (MAT**a** *his3-1 leu2-3,-112 trp1-289 ura3-52*) according to the manufacturer's instructions (Yeastern Biotech Co., Taiwan). The transformant of *S. cerevisiae* was cultured in a 1 L flask containing 250 mL of YNBD medium at 30 °C with shaking at 250 rpm until the OD600 value reached 1.0. The rLZ-8 proteins were produced intracellularly, and the cultured mycelia were homogenized. Then, the cell lysate was collected by centrifugation at 13000*g* and 4 °C for 10 min. The rLZ-8 protein was further purified by AKTA fast protein liquid chromatography (FPLC) system with a HiTrap Q column (Amersham Biosciences, Uppsala, Sweden) as previously described (*11*). The purity of rLZ-8 was analyzed using 12% SDS−PAGE, and protein concentration was determined according to the method of Bradford with bovine serum albumin as a standard. Endotoxin levels of rLZ-8 were determined by *Limulus* amoebocyte lysate (LAL) assay (< 0.012 EU/μg) to exclude the possibility of LPS contamination.

Deproteinization of PS-G. Polysaccharide from *G. lucidum* (PS-G), a commercial product prepared from hot-water extracts of *G. lucidum* fruiting bodies, was obtained from Double-Crane Pharmaceutical Enterprise Co., Ltd. (Tainan Hsien, Taiwan). The PS-G powder was dissolved in deionized water at a concentration of 5 mg/mL and then added to an equal volume of 15% trichloroacetic acid (TCA) for deproteinization at 4 °C overnight as previously described (*21*). After centrifugation at 3500*g* for 15 min, supernatant was aspirated and the crude polysaccharide fraction was obtained through 75% ethanol precipitation and was considered as deproteinized PS-G (dpPS-G). The total carbohydrate concentration of dpPS-G was determined using the phenol−sulfuric acid test, and protein concentration was determined using a fluorescence method by adding *o*-phthalaldehyde (Pierce, Appleton, WI) with excitation at 360 nm and emission at 445 nm on a Perkin-Elmer VICTOR3 multilabel counter.

Monoclonal Antibody Production. BALB/c mice were immunized by monthly intraperitoneal injections of 50 μg of native LZ-8 purified as described previously (*11*), which was mixed with an appropriate volume of aluminum potassium sulfate (10% w/v). Splenocytes from immunized mice were fused 3 days after an intraperitoneal injection of immunogen with mouse X63Ag8.653 myeloma cells from the American Type Culture Collection (ATCC; Manassas, VA) using 50% polyethylene glycol 1500 (Roche, Mannheim, Germany) as previously described (*22*). Hybridomas were selected in a hypoxanthine−aminopterin−thymidine medium (Gibco/BRL Life Technologies, Eggenstein, Germany), and the supernatants were screened by conventional immunoenzymatic assays using purified nLZ-8 as a tracer. The cells from the positive wells were cloned twice using limiting dilution method. Monoclonal antibodies were purified from the soup of hybridoma culture by protein A/G affinity chromatography (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Electrophoresis and Western Blotting Analysis. The recombinant LZ-8 proteins, PS-G, and dpPS-G were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) on a Bio-Rad mini protein III gel apparatus (Bio-Rad Laboratories, Hercules, CA). The apparent molecular mass was determined based on relative migration of protein standards (Amersham Biosciences), consisting of ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa). Protein bands in gel were visualized by staining with Coomassie Brilliant Blue R250 or periodic acid−Schiff (PAS) reagent (Merck, Darmstadt, Germany) for the detection of polysaccharide. To determine the existence of LZ-8 in PS-G preparation, PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) Immobilon P (Millipore) membrane by employing the Trans-Blot Cell system (Bio-Rad) in a transfer buffer and was subjected to Western blotting with anti-LZ-8 monoclonal antibodies.

Determination of Antigen Presentation Capability. Allogenic mixed leukocyte reaction was performed with WT and $TLR4^{-/-}$ derived adherent macrophages (5×10^5 cells/well) treated with rLZ-8 and dpPS-G for 24 h in 24-well culture microplates. Subsequently, macrophages were washed with DMEM three times to make sure there was no stimulator residue and were then incubated with 1×10^6 cells/well responder T cells (1:2 ratio). The murine responder $CD4^+$ and $CD8^+$ T cells were purified from murine splenocytes via negative selection technique. Non-CD4/CD8 T cells were subsequently labeled utilizing a cocktail of biotin-conjugated antibodies against CD8/CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (Miltenyi Biotec, Germany). These

cells were subsequently magnetically labeled with Anti-Biotin MicroBeads (Miltenyi Biotec, Germany) for depletion according to the manufacturer's instructions.

Determination of Cytokine Production. The cytokine levels in cell culture supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA), using TNF-α, IL-1 (eBioscience, San Diego, CA), IL-2 (BD, Biosciences, San Jose, CA) and IFN-γ (R&D systems, Minneapolis, MN) ELISA kits. Colorimetric absorbance was measured at 450 nm on a Bio-Rad 3550 microplate reader.

Quantitative Real-Time Polymerase Chain Reaction (qPCR). Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). The concentrations of RNAs were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The first-strand cDNA was synthesized from total RNA using ThermoScript RT-PCR system (Invitrogen) as a template for evaluating gene expression involving PS-G/rLZ-8 immunomodulation in macrophages. Differential expression for individual genes was assessed by quantitative real-time PCR (qPCR). Specific primer sets were designed for SYBR Green probes with Roche Probe Design Software (Roche) based on macrophage-related cytokine and immune factors sequences on NCBI. β-Actin (endogenous gene) was used to confirm the quality of reverse-transcripted cDNA. qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA). The expression level of each target gene was quantifyed by the relative levels of a given mRNA according to cycling threshold (Ct) analysis.

Macrophage Binding Study by Flow Cytometric Analysis. Samples were labeled with fluorescein isothiocyanate (FITC) using an FITC protein labeling kit (Pierce, Appleton, WI) according to the manufacturer's instructions. In evaluating the binding activity of LZ-8 to macrophages, cells from WT and $TLR4^{-/-}$ mouse were preincubated with 100 μg/mL of unlabeled LZ-8 and FITC-labeled LZ-8 in PBS containing 2% bovine serum albumin and 0.1% sodium azide (FACS buffer) at 4 °C for 30 min. Subsequently, the cells were washed twice with FACS buffer for removal of unbound proteins. Acquisition and analysis were performed with FACScan using CellQuest software (BD Biosciences, San Jose, CA). Results are expressed as the total mean fluorescence intensity (MFI).

Fluorescence-Activated Cell Sorting (FACS) Analysis. For surface maturation marker detection, the pretreated cells were resuspended in PBS containing 2% BSA and 0.1% sodium azide (FACS buffer). Suspended cells were washed twice in FACS buffer and stained with FITC-conjugated primary antibodies specific for MHC class I, MHC class II, CD80 and CD86 (eBioscience, San Diego, CA) at 4 °C for 30 min for macrophages or CD44 and C154 (CD40L) for splenocyte. Subsequently, cells were washed twice with FACS buffer to remove excess antibodies. After washing, the resuspended cells were acquired on a FACScan (Becton Dickinson, USA) and analyzed using CellQuest software (Becton Dickinson, USA). The results were expressed in total mean fluorescence intensity (MFI) and percentage of positive fluorescent cells. Cells incubated with medium only served as negative controls. Data analyses were performed by the comparison of peak fluorescence intensities between the control and treatment groups.

Statistical Analysis. All values presented are the mean ± SD of three independent experiments performed in triplicate. Statistical comparisons were performed by analysis of variance (ANOVA). Results were considered significant when p values <0.01.

RESULTS

LZ-8 Contamination within PS-G. PS-G and LZ-8 were both regarded as the major bioactive substance of *G. lucidum*. SDS−PAGE demonstrated a clear Coomassie-brilliant-blue staining of the rLZ-8 protein band but faintly stained the PS-G fraction (**Figure 1A**). To investigate the presence of LZ-8 protein residual in the PS-G sample, mouse anti-LZ-8 mAbs, which could successfully recognize the native LZ-8 (Supplementary Figure 1 in the Supporting Information) and rLZ-8 proteins (**Figure 1C**, lane 2, and **2C**), was prepared. Immunoblot analysis demonstrated that PS-G polysaccharide fraction stained with brilliant purple by PAS staining (**Figure 1B**, lane 4) could also be detected by anti-LZ-8 monoclonal antibodies (**Figure 1C**, lane 4).

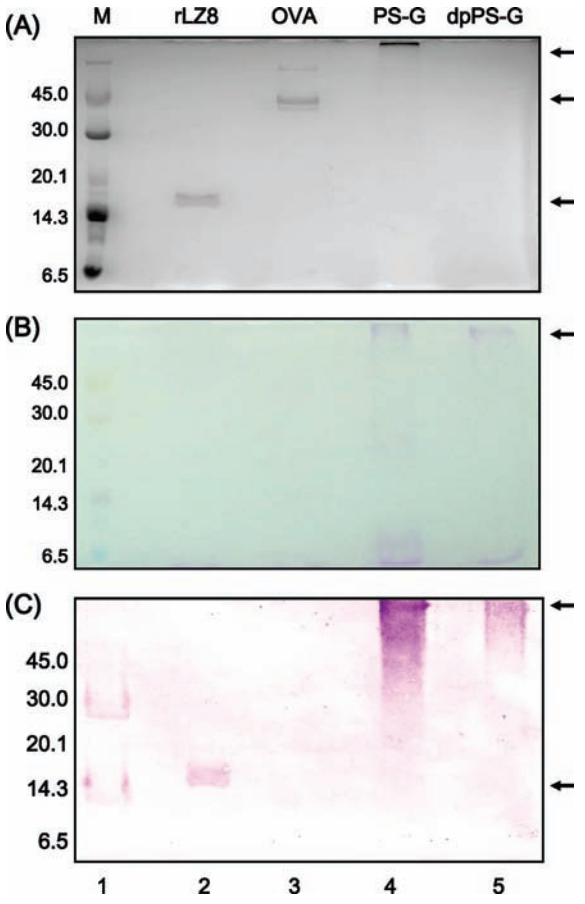

Figure 1. Deproteinization of PS-G showed significant reduction of LZ-8. (**A**) SDS−PAGE, (**B**) Schiff's staining, and (**C**) Western blotting analysis of rLZ-8, PS-G, and dpPS-G. Western blotting used monoclonal antibodies against nLZ-8. Lane 1 contains molecular weight markers consisting of ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa).

These results revealed that the PS-G sample which was prepared by ethanol precipitation of the hot-water extract of *G. lucidum* fruiting bodies was naturally contaminated with LZ-8 protein. In addition, the interaction between PS-G and LZ-8 was stable even when they were pretreated with SDS sample buffer in the presence of reducing agents (DTT and 2-mercaptoethanol). To rule out interference between PS-G and LZ-8 with respect to each other and to clarify their individual function, we further prepared recombinant LZ-8 (rLZ-8) expressed by *S. cerevisiae* with free glycosylation and deproteinized PS-G (dpPS-G) with trace LZ-8 as the materials for further experiments.

Deproteinization of PS-G Showed Significant Reduction of LZ-8. To eliminate the contaminated LZ-8 protein, PS-G was deproteinized with the TCA/protein precipitation method. As shown in **Table 1**, dpPS-G protein concentration exhibited markedly 89.7% reduction (from 6% of total PS-G weight to 0.6%). According to the phenol−sulfuric acid test and HPLC gel filtration analysis, TCA treatment did not affect the carbohydrate quantity of dpPS-G (**Table 1** and Supplementary Figure 2 in the Supporting Information), while Western blotting detection with anti-nLZ-8 monoclonal antibodies was remarkably decreased (**Figure 1C**, lane 5). These results indicated that native PS-G might reside with LZ-8 and could be removed after a TCA deproteinization process.

Functional Expression and Biochemical Characterization of rLZ-8. To obtain the bioactive LZ-8, we used the *S. cerevisiae*

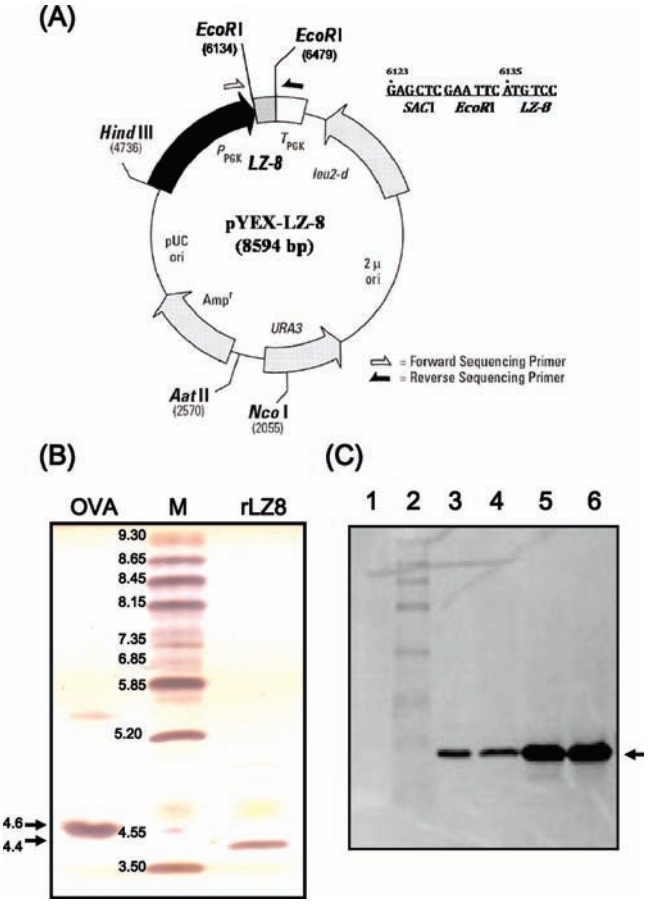

Figure 2. Functional expression and characteristics of rLZ-8. (**A**) Schematic diagram of pYEX-LZ-8 plasmid construct for *S. cerevisiae* protein expression system. The 336bp *lz8* cDNA was cloned into the pYEX-S1 plasmid using the *Eco*R1 restriction sites, and expressed in yeast strain DBY747. (**B**) Isoelectronic focusing of rLZ-8 and OVA. The PI value of rLZ-8 is approximately 4.4. (**C**) Western Blot analysis for rLZ-8. The *S. cerevisiae* expressed rLZ-8 was recognized by anti-LZ-8 mAbs (lanes 5, 6) as well as nLZ-8 (lanes 3, 4). Lane 2 contains molecular weight markers (175, 83, 62, 47.5, 32.5, 25, 16.5, and 6.5 kDa).

Table 1. Determination of Protein, Total Carbohydrate, and LZ-8 Contents in the PS-G and dpPS-G

	PS-G	dpPS-G[a]
total carbohydrate content[b] (%)	18.8	18.2
protein content[c] (%)	6	0.6
carbohydrate/protein ratio	3.1	30.3
protein reduction (%)		89.7

[a] dpPS-G: TCA deproteinated PS-G. [b] Total carbohydrate concentration was determined by the phenol−sulfuric acid test. [c] Protein concentration was determined by reacting with *o*-phthaladehyde and the fluorescence of each sample.

protein expression system for efficient production. Full length LZ-8 gene amplified from cDNA of *G. lucidum* was cloned into pYEX-S1 plasmid with *Eco*RI restriction sites to generate the pYEX-LZ-8 construct (**Figure 2A**). This expression plasmid was transformed into *S. cerevisiae* DBY747 to express rLZ-8. The rLZ-8 proteins were then purified using the chromatographic method of Kino (*11*). Various types of electrophoresis were further used to examine the biochemical characteristics of purified rLZ-8. The polyacrylamide gel stained with periodic acid/Schiff reagent after SDS−PAGE under reducing conditions showed that 15 kDa subunit gave a negative result (**Figure 1B**, lane 2). This indicated that rLZ-8 had no detectable carbohydrates. Western blotting showed that rLZ-8 was recognized by anti-LZ-8 monoclonal antibodies (**Figure 1C**, lane 2, and **Figure 2C**). Isoelectric focusing electrophoresis in the pH range of 3−10 was further used to examine the isoelectric characteristics of purified rLZ-8. As shown in **Figure 2B**, the isoelectic point of rLZ-8 was 4.4, consistent with the native LZ-8.

Diverse Activation Effects of PS-G and LZ-8 on Mouse Peritoneal Macrophage. Macrophages play a central role in innate immunity as well as in helping to initiate the adaptive immunity of vertebrate animals. To determine the different immune regulating activities of each *G. lucidum* bioactive compound, secretion of specific cytokines, including TNF-α, IL-1β, and IL-12, by mouse peritoneal macrophages was investigated. Genes with relevance for the role of PS-G and rLZ-8 in cytokine induction were further analyzed by quantitative real-time PCR. Meanwhile, for clarifying the involvement of TLR4 receptors in the interaction of macrophages with PS-G and LZ-8, peritoneal macrophages obtained from TLR4$^{-/-}$ C57BL/10SCN mice were incubated with each sample and LPS were used as TLR4 agonist in vitro. In our preliminary experiments, the possibility of endotoxin contamination by LAL gel-clot assay was excluded as no gel formation was found in any of 4 samples in the concentrations which were applied (Supplementary Table 1 in the Supporting Information).

For cytokine secretion ELISA assay, cells cultured with PS-G, dpPS-G and rLZ-8 were incubated for up to 24 h. Peritoneal macrophages treated with 50 μg/mL of PS-G or dpPS-G showed similar significant increases ($P < 0.01$) in TNF-α, IL-1β, and IL-12p70 cytokine secretion from the medium control (**Figure 3**, hollow). The highest peaks of TNF-α and IL-1β gene expression were observed at 4 h, and other cytokines appeared at 8 h after PS-G and dpPS-G treatment (Supplementary Figure 3 in the Supporting Information). Further investigation revealed that there was a significant reduction of cytokine secretion on TLR4$^{-/-}$ macrophages in response to PS-G stimulation, while dpPS-G-induced activities were almost absent (**Figure 3C**, solid). The deproteinization process did not affect the immunomodulatory property of PS-G toward WT macrophages, but did abolish dpPS-G activation on TLR4$^{-/-}$ macrophages. 1.25−5 μg/mL rLZ-8 was capable of enhancing cytokine secretion (IL-1β and IL-12p70) on WT and TLR4$^{-/-}$ macrophages, but no such detection was observed in TNF-α assay (**Figure 3A**). To investigate whether rLZ-8, PS-G, and dpPS-G could also modulate the development of peritoneal macrophages in vitro, a comparison was made among the phenotype of macrophages treated with medium alone, LPS, rLZ-8, PS-G or dpPS-G for 24 h. The data demonstrated that both rLZ-8 and dpPS-G could increased the surface expression of CD86 and MHC class II molecules on macrophages, while rLZ-8 also activate CD80 and MHC class I moderately (**Figure 4**). These results indicated that dpPS-G and rLZ-8 were both effective activators of murine macrophages, but obviously in diverse manner. TLR4 was involved in the recognition of PS-G and dpPS-G on macrophages; nevertheless, rLZ-8 might prime macrophages with another obscure route.

Enhancement of T Cell Activation by rLZ-8-Treated Mouse Peritoneal Macrophages. Primed macrophages have the capacity to induce activation and polarization in allogenic T cells (*23*). In mouse peritoneal macrophages, we found that dpPS-G and rLZ-8 treatments increased IL-12 production (**Figure 3C**) and induced the activation of inflammatory cytokines. To clarify whether these stimulations were sufficient to promote activation of naive T cells, macrophages were treated with dpPS-G and rLZ-8 for activating the allogenic naive CD4$^+$ or CD8$^+$ T cells.

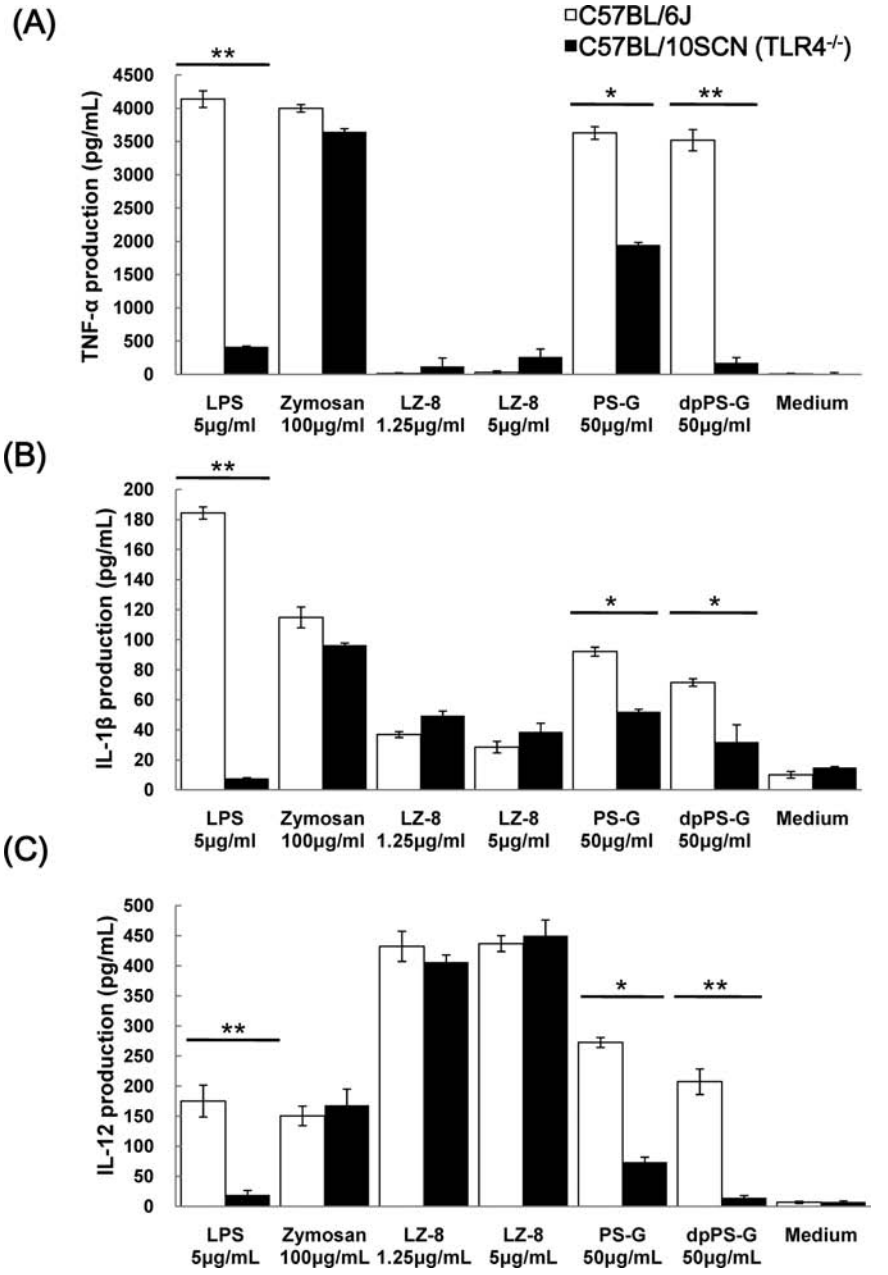

Figure 3. Diverse activation effects of PS-G, dpPS-G and LZ-8 on mouse peritoneal macrophage inflammatory cytokine secretion. Culture supernatants were harvested from the incubation treated with rLZ-8, PS-G and dpPS-G and subjected to ELISA assay for (**A**) TNF-α, (**B**) IL-1β and (**C**) IL-12p70. (* $p < 0.01$ among the different groups.) Solid bars represent the wild type C57BL-6 mice, and open bars represent the TLR4 knockout C57BL/10SCN mice.

As evidenced by culture supernatant cytokine production, dpPS-G treated-macrophages did not affect the IL-2 and INF-γ production level of naive $CD4^+$ (**Figure 5A**,**B**) and $CD8^+$ T cells (**Figure 5C**,**D**), whereas rLZ-8-treated macrophages could significantly ($P < 0.01$) enhance the activation of naive T cells at WT and $TLR4^{-/-}$ mouse. This implied that only rLZ-8 treatment could elevate macrophages' regulatory ability to further elicit IL-2 and IFN-γ secretion from alloreactive lymphoid cells.

Irrelevance of TLR4 and rLZ-8 in Mouse Peritoneal Macrophages. To study further the interaction between TLR4 and rLZ-8, the binding of rLZ-8 to the cell surface of peritoneal macrophages was demonstrated by comparing the flow cytometric analysis of $TLR4^{-/-}$ and WT control mice. These analyses indicated that FITC-conjugated rLZ-8 was bound to the cell surface of WT macrophages as demonstrated by a clear shift in fluorescence intensity as compared to nonconjugated rLZ-8 control (**Figure 6**, left panel). An identical level of rLZ-8 binding was also observed in $TLR4^{-/-}$ cells (**Figure 6**, right panel). In the present study, rLZ-8 was demonstrated to activate macrophages in a TLR4 independent manner.

LZ-8 Unitarily Activate Naive T Lymphocytes. We further estimated whether splenic $CD3^+$ T cell populations were altered during the induction of rLZ-8, PS-G, and dpPS-G which were characterized by flow cytometry analysis. Remarkable increases of CD154 and CD44 expression were observed in splenocyte upon the rLZ-8 stimulation, however only slight or no activation was noted with the PS-G and dpPS-G treatment (**Figure 7**). Among different types of T lymphocytes, $CD4^+$ T cells play an important role in establishing and supporting the capabilities of adaptive immune system, while the $CD8^+$ subgroup is capable of inducing the death of pathogen-infected or tumor cells. T cells may be activated by a variety of substances including specific antigens and nonspecific stimulating agents (e.g., plant lectins). To examine whether dpPS-G or rLZ-8 could unitarily activate

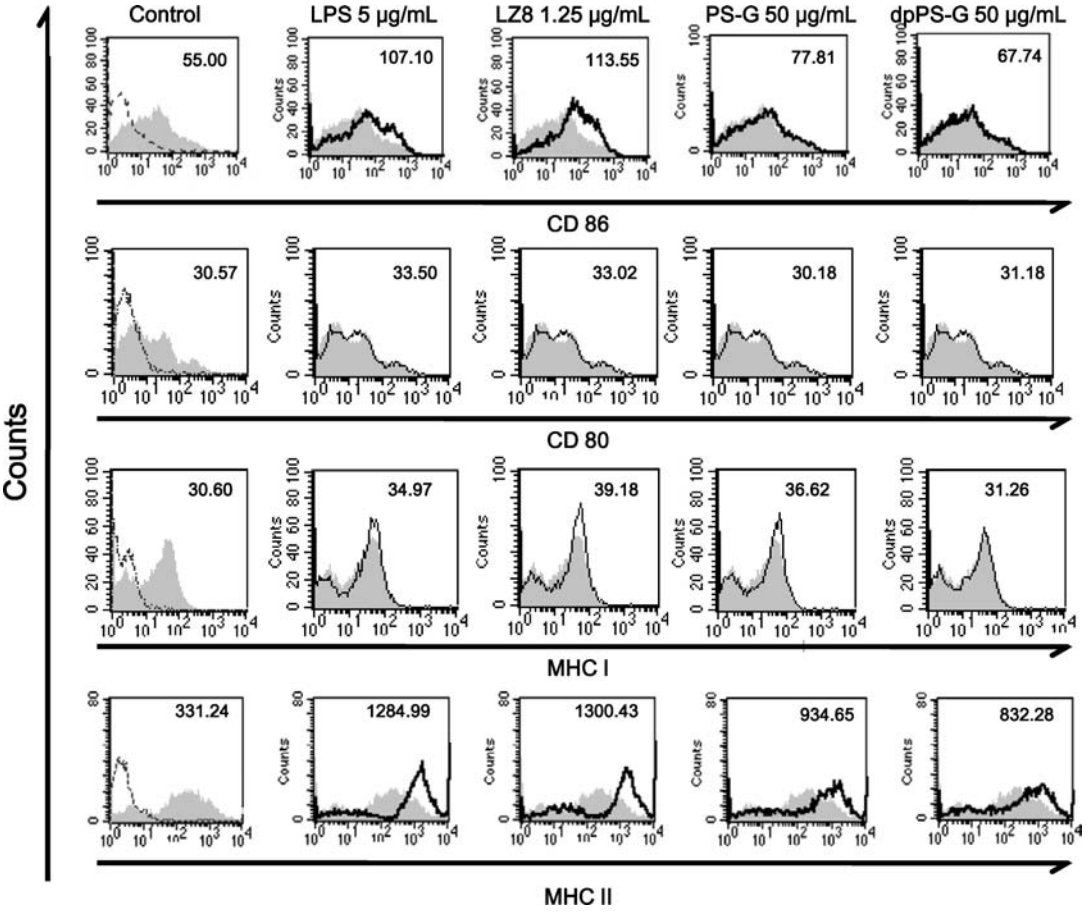

Figure 4. LZ-8, PS-G, and dpPS-G induced MHC class I, MHC class II, and costimulatory molecule expression on mouse peritoneal macrophages. Peritoneal macrophages from C57BL/6J mice were isolated and stimulated with rLZ-8 1.25 (μg/mL), PS-G (50 μg/mL), dpPS-G 50 (μg/mL) or LPS (5 μg/mL) for 12 h. The cells were harvested and stained with FITC-conjugated monoclonal antibodies against MHC I, MHC II, CD80, and CD86, followed by the analysis with flow cytometry. Data are representative results of two replicates. The shaded area shows the medium control. The dotted line represents the isotype antibody control. The solid line indicates the cell surface expression of MHC I, MHC II, CD80, and CD86 on peritoneal macrophages.

mouse naive T lymphocyte, purified $CD4^+$ and $CD8^+$ T cell (>95% by MACS) were cultured with 50 μg/mL dpPS-G and 1.25 μg/mL rLZ-8, respectively. In comparison with medium control, dpPS-G treatments did not elicit significant IL-2 and INF-γ production difference. This indicated that the dpPS-G-induced T cell activation was dependent on the cooperation with other leukocytes (**Figure 8**). On the other hand, rLZ-8-treated $CD4^+$ and $CD8^+$ T cells exhibited a dramatic increase in IL-2 and IFN-γ secretion. Taken together, these results implied ConA-like mitogenic property of rLZ-8 on mouse T lymphocyte.

DISCUSSION

Reishi is very rare in nature, rather insufficient for commercial exploitation and therapeutic applications. The isolation of native LZ-8 depends mainly on the extraction of mycelia from *G. lucidium* (*11*). In order to produce sufficient and high-purity of LZ-8 protein for substantial studies, a number of reports describing the heterologous expression of rLZ-8 have been performed. A previous study reported that LZ-8 has been expressed in *Escherichia coli*, but the splenocyte proliferation activity was remarkably lower than that of the native LZ-8 (*24*). Similar results were also observed when the FIP-*fve* and FIP-*gts* expressed in *E. coli* (*25*, *26*); these indicated that fungous origin protein may not have been folded properly and/or may have improper correct glycosylation in the *E. coli* expression system. Extended reports tend to express active rLZ-8 in *Pichia pastoris* secretory expression system for medicinal applications and genetic study (*17*, *18*). Codon-optimized LZ-8 was formerly expressed extracellularly in the GRAS host *Bacillus subtilis* and *Lactococcus lactis* and has provided a useful source of recombinant LZ-8 as a safe, orally administered agent (*27*). In the present study, we have developed a large-scale *S. cerevisiae* expression system for rLZ-8 expression. In common with *P. pastoris, S. cerevisiae* is a lower eukaryote as well as *G. lucidum*, and provides a suitable environment for the recombination of functional rLZ-8. While the SDS−PAGE and Schiff's staining showed that there is none or little glycosylation within the *S. cerevisiae* expressed rLZ-8 (**Figure 1B**, lane 2), the splenocyte proliferation activity and isoelectric point of rLZ-8 expressed in *S. cerevisiae* were similar to those of the native LZ-8 in vitro (*11*). *S. cerevisiae* has been awarded GRAS (generally recognized as safe) status for use in human consumables. Thus, this food-grade rLZ-8 transformant could be applied safely in feed and food research.

The immunological properties of polysaccharides and immunomodulatory protein derived from Ling Zhi (*G. lucidum*) have been studied (*6*, *9*, *19*, *28*), however, effective polysaccharides extracted from different procedures still contain partial protein residue. *Gl*-PS isolated from the boiling water-soluble polysaccharides of *G. lucidum* has a molecular weight of 584,900, with a ratio of polysaccharides to peptides of 14.1 to 1 (*19*). The GLPS obtained by the liquid fermentation of the mycelia of *G. lucidum* was found to contain 86.4% polysaccharides and 8.3% proteins (10.4:1) by the phenol−sulfuric acid method and Lowry−Folin

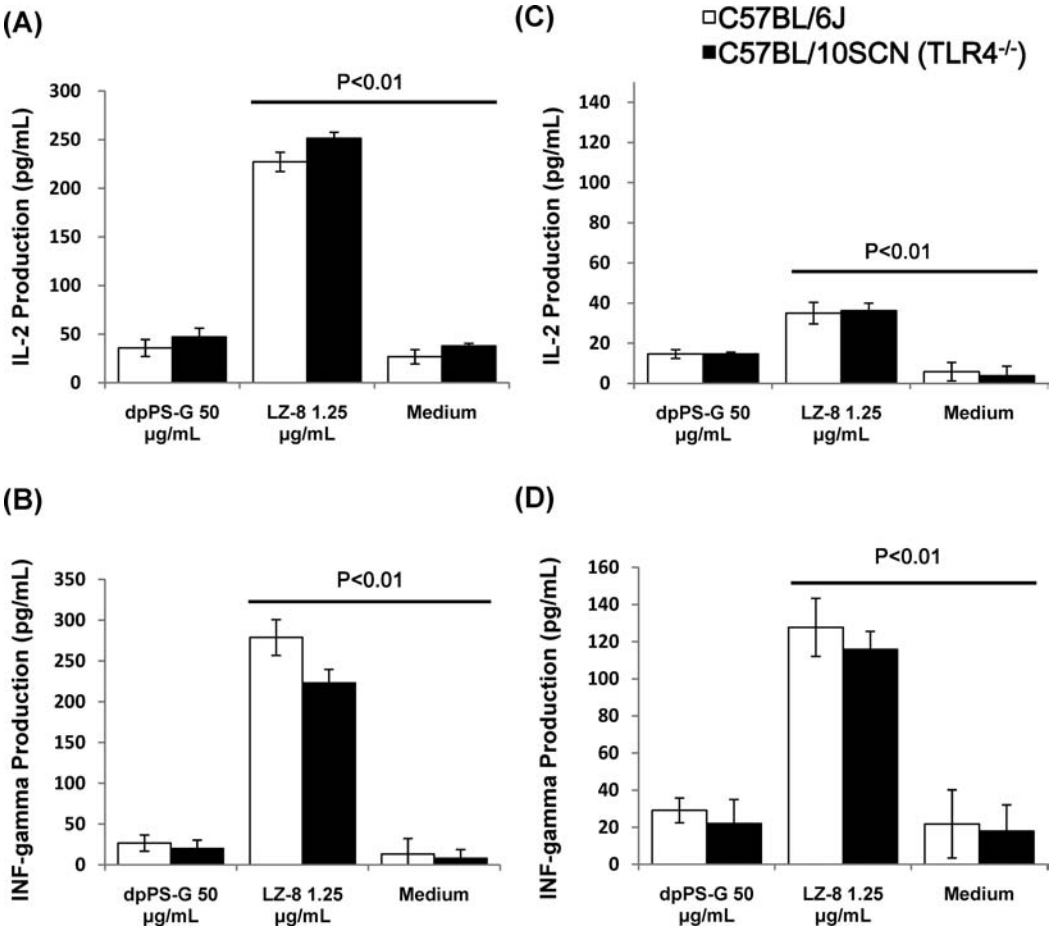

Figure 5. Enhancement of T cell activation by rLZ-8-treated mouse peritoneal macrophages. Mouse peritoneal macrophages were stimulated with dpPS-G, rLZ-8 and medium control for 24 h. After 3 days of coculture with pretreated macrophages, allogeneic (**A**, **B**) $CD4^+$ and (**C**, **D**) $CD8^+$ T cell culture supernatant were analyzed for (**A**, **C**) IL-2 and (**B**, **D**) INF-γ using ELISA assay.

test (*20*). In agreement with these findings, PS-G in our study was determined to have a carbohydrate/protein ratio of 3.1 to 1. The Western blotting also has suggested that PS-G polysaccharide fraction could be detected by anti-nLZ-8 monoclonal antibodies. This indicated that LZ-8 contamination might influence the immune-regulating function of PS-G.

Several methods have been reported to isolate polysaccharides from contaminating proteins. Polysaccharide from *Streptococcus salivarius* was rapidly separated and purified using coupled anion exchange and gel-permeation high-performance liquid chromatography (*29*). Ninety percent of the contaminated proteins in the alkali-extracted polysaccharide from *Mucor rouxii* were eliminated by adjusting the pH of the solution to reach precipitation point (*30*). The Sevag method had high deproteinization efficiency (87.9%) in gellan gum produced by *Sphingomonas paucimobilis*, but it showed several fatal drawbacks such as low recovery, waste disposal problem, complex operation procedure, and ineffectiveness (*31*). Moreover, 10−25% (w/v) TCA provided a very efficient and selective precipitation for almost all the protein content (>90%) without affecting the amount of polysaccharides (*32*). To identify the diverse immunomodulatory role of PS-G and LZ-8, PS-G was subjected to a deproteinization treatment with 15% TCA to remove LZ-8 residue under native conditions. The detection of anti-nLZ-8 monoclonal antibodies showed a remarkable protein reduction (89.7%) of the dpPS-G (from 6% of total PS-G weight to 0.6%). These results suggested that the LZ-8 protein coprecipitated within PS-G could be denatured and precipitated by TCA deproteinization process.

TLRs in vertebrates are able to sense diverse, microbial, and other ligands. Also, they are able to transmit signals via adaptor molecules selectively, but depend on other surface molecules (such as CD14/MD2 and C-type lectin-like receptors) for proximal ligand binding and recognition (*33*). Antigen recognition by TLRs is critical for eliciting the activation of innate immune system and leads to the secretion of many immune mediators such as TNF-α, IL-1β, and several chemokines. Therefore, TLRs are likely to play a considerable role in the initiation and perpetuation of the immune response. The investigation of Reishi-F3 polysaccharide on murine and human macrophages has showed that Reishi-F3 mediated production of inflammatory cytokine was through the receptor TLR4 followed by a cascade of protein kinase C and p38 MAPK signaling (*10*). However, the addition of anti-TLR4 mAb to human dendritic cells has blocked 31% and 39% 1 μg/mL PS-G-induced production of IL-12 p40 and IL-10, respectively (*9*). In our present study, PS-G stimulated $TLR4^{-/-}$ macrophages have identically reduced 47%, 43% and 70% production of TNF-α, IL-1β, and IL-12p70, respectively, toward WT macrophages, while deproteinization process totally abolished the cytokine secretion on $TLR4^{-/-}$ macrophages. In this study, TCA deproteinization greatly decreased the production of TNF-α, IL-1β, and IL-12p70 (**Figure 3**). This suggested that TLR4 is a putative receptor of dpPS-G, mediating the consequent immunomodulating events associated with inflammatory cytokine expression.

Treatment of DC with rLZ-8 enhanced the production of IL-12 p40, IL-10, and IL-23. In addition, treatment of DCs with rLZ-8

enhanced the naive T cell-stimulatory capacity and increased the naive T cell secretion of IFN-γ and IL-10 (*18*). Our results demonstrated that 1.25−5 μg/mL of rLZ-8 was capable of enhancing cytokine secretion (IL-1β and IL-12p70) on WT and TLR4$^{-/-}$ macrophages (**Figure 3**). Matured antigen presenting cells show a high surface expression of MHC class II and CD80/86 (B7.1/7.2) costimulatory molecules. PS-G and rLZ-8-treated DCs can induce MHC class II, CD80, CD86, and CD83 expression, as well as the enhanced production of IL-12 (*18*, *20*). In contrast with DCs, our results suggested only CD86 and MHC class II molecules surface expression on primary macrophages by rLZ-8, PS-G and dpPS-G treatments, while rLZ-8 also activates CD80 and MHC class I moderately (**Figure 4**). However, neutralization with antibodies against TLR4 has been shown to inhibit the rLZ-8-induced production of IL-12 p40 and IL-10 in DCs, and rLZ-8 could stimulate TLR4 or TLR4/MD2-transfected HEK293 cells to produce IL-8 (*18*). TLR4 was well-known as the receptor to recognize the polysaccharides of microorganisms (e.g., LPS) but not proteins. Based on the rLZ-8-induced activation within TLR4$^{-/-}$ CD4+ T cells (**Figure 5A**,**B**) and TLR4$^{-/-}$ CD4+ T cells (**Figure 5C**,**D**), our results were not consistent with Lin et al. (*18*). To further clarify the interaction between TLR4 and rLZ-8, we have performed in vitro binding assay. It was observed that the FITC-conjugated rLZ-8 could bind to the cell surface of TLR4$^{-/-}$ macrophages as demonstrated by a clear shift in fluorescence intensity compared to the nonconjugated rLZ-8 control (**Figure 6**). According to these viewpoints, rLZ-8 might be recognized by macrophages in an obscure TLR4-independent route.

CD154 (CD40L) is a protein marker that is primarily expressed on activated T cells. CD154 interactions can influence T cell priming and T cell-mediated effector functions, and upregulate costimulatory molecules to activate APCs for polarization factor secretion (*34*). CD44 is also required in T cell development and maturation of T cell function (*35*). In our present study splenic CD3$^+$ T cell populations exhibited remarkable increases of CD154 and CD44 expression upon the rLZ-8 stimulation, however only slight or no activation was noted with the PS-G and dpPS-G treatment (**Figure 7**). The CD4$^+$ T cells play an important role in establishing and supporting the capabilities of an

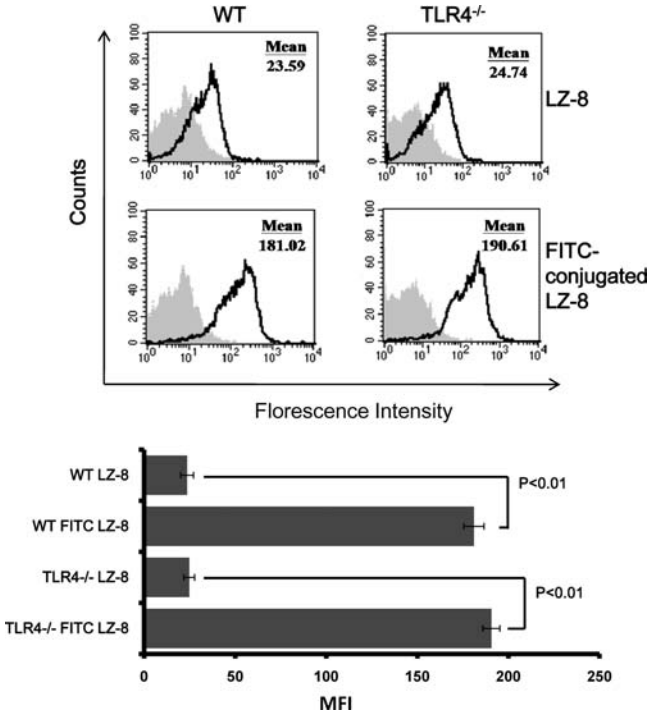

Figure 6. Irrelevance of TLR4 and rLZ-8 in mouse peritoneal macrophages. Peritoneal macrophages from WT, and TLR4$^{-/-}$ mice were unstained (shaded area) or stained with FITC-labeled rLZ-8 and nonlabeled rLZ-8(solid line) for 30 min and then analyzed by flow cytometry. The histograms were quantified and represented as mean fluorescence intensity (MFI).

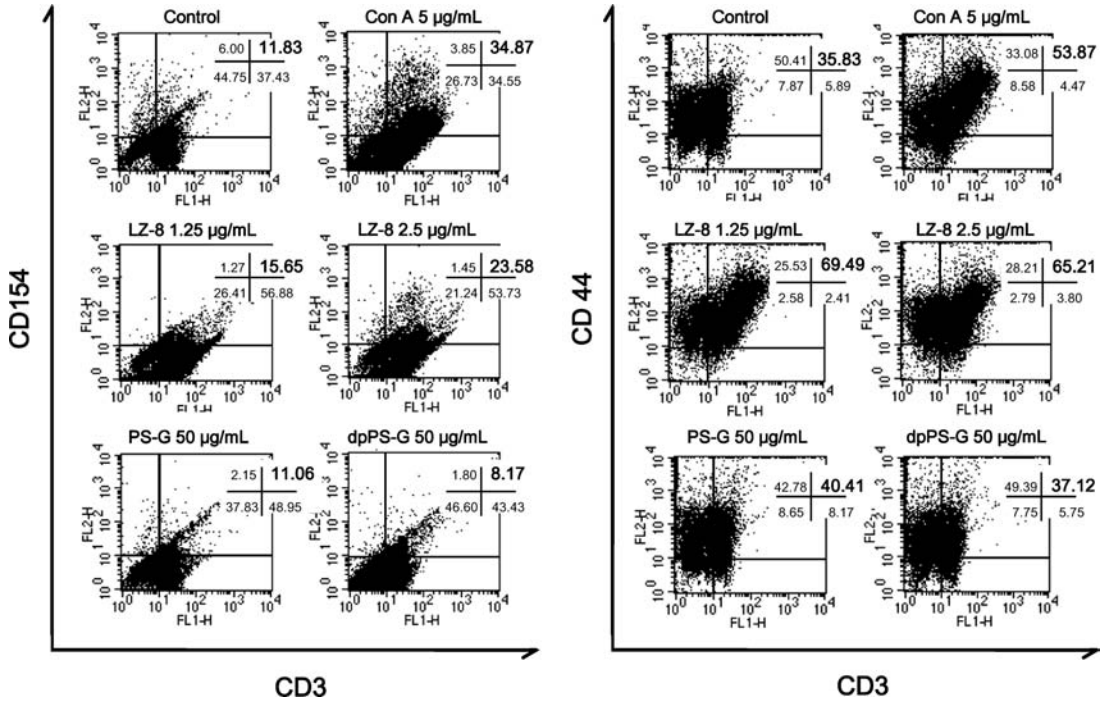

Figure 7. LZ-8 unitarily activates CD154 and CD44 marker expression on CD3$^+$ T cell. Splenocytes from C57BL/6J mice were isolated and stimulated with rLZ-8 (1.25 and 2.5 μg/mL), PS-G (50 μg/mL), dpPS-G 50 (μg/mL), or LPS (5 μg/mL) for 48 h. The cells were harvested and stained with FITC-conjugated anti-CD3, and PE conjugated anti-CD154/CD44. The flow cytometry data are representatives of two independent sets of analysis.

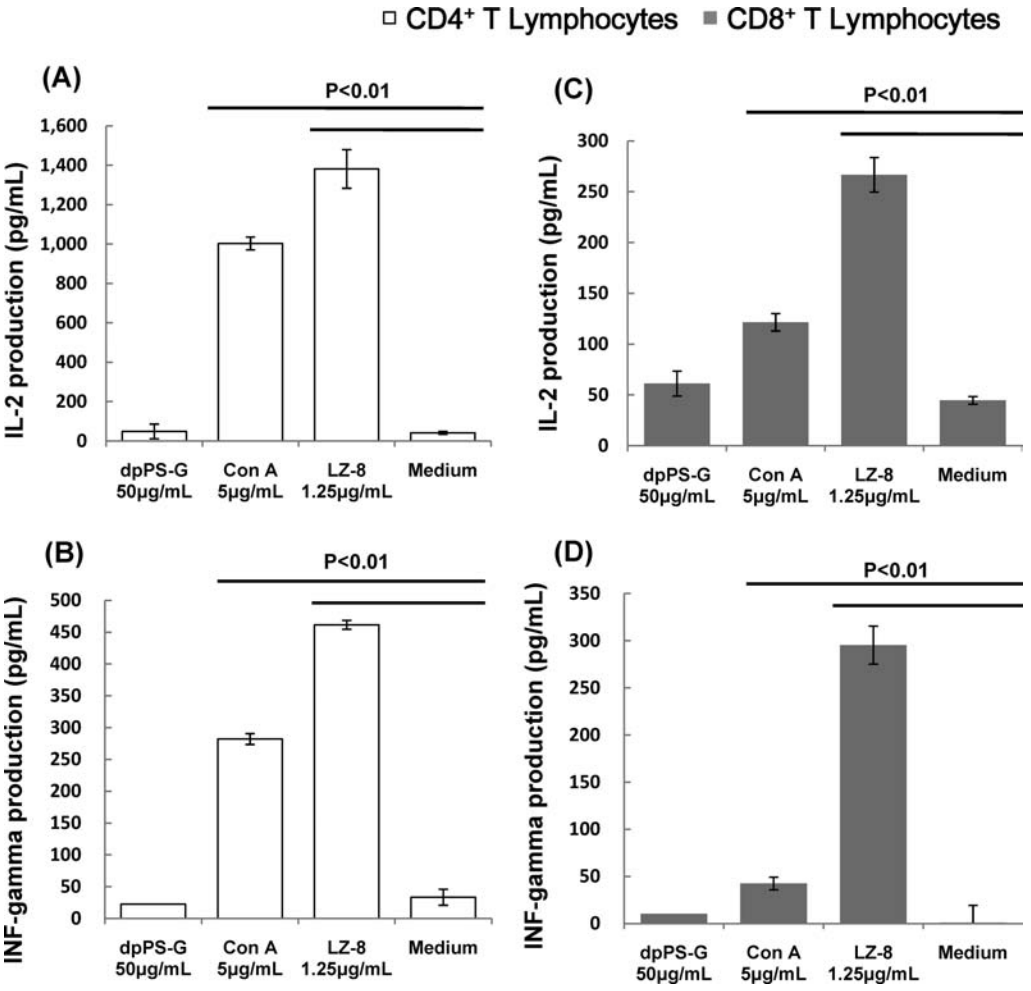

Figure 8. LZ-8 unitarily activates purified $CD4^+$ and $CD8^+$ naive T lymphocyte. $CD4^+$ and $CD8^+$ T cell were directly stimulated with dpPS-G, rLZ-8 and medium control for 3 days. Culture supernatants were harvested and subjected to ELISA assay for (**A**, **C**) IL-2 and (**B**, **D**) INF-γ. Open bars represent the $CD4^+$ T cell, and shaded bars represent $CD8^+$ T cell.

adaptive immune system, while the $CD8^+$ subgroup is capable of inducing the death of pathogen-infected or tumor cells (*36*). PS-G and rLZ-8-induced DC maturation resulted in an enhanced allostimulatory activity, as demonstrated by the enhanced proliferative responses and IFN-γ secretion of naive, allogeneic T cells (*9*, *18*). As evidenced by allogenic mixed leukocyte reaction, rLZ-8 treated-macrophages significantly stimulate the IL-2 and INF-γ production level of naive $CD4^+$ and $CD8^+$ T cells, while dpPS-G-treated macrophages did not induce activation and polarization in allogenic T cells. It has been reported that LZ-8 could stimulate human PBL to secrete IL-2 (*15*). It also induced IL-2 gene expression via the Src-family protein tyrosine kinase, reactive oxygen species (ROS), and differential protein kinase-dependent pathways within human primary T cells and cultured Jurkat T cells (*17*). In agreement with these findings, we found that direct-treatment of rLZ-8 on $CD3^+$, $CD4^+$, and $CD8^+$ T cells exhibited obvious T cell maturation and dramatic mitogenic properties in increasing IL-2 and IFN-γ secretion, while dpPS-G could not prime $CD3^+$, $CD4^+$, and $CD8^+$ T cells unitarily.

In conclusion, the present study not only has demonstrated the existence of LZ-8 protein contamination within PS-G samples but also has clarified the diverse roles of PS-G and LZ-8 in regulating murine macrophage and T lymphocytic cells. The PS-G coprecipitated LZ-8 protein could lead to the direct activation of murine T lymphocytes instead of contributed by Reishi polysaccharide itself. Furthermore, the multiple roles of PS-G and LZ-8 suggested different applications of various Reishi compounds in activating innate and/or adaptive immunity.

ABBREVIATIONS USED

FIP, fungal immunomodulatory protein; FPLC, fast protein liquid chromatography; IL-1β, interleukin-1 beta; IL-2, interleukin-2; INF-γ, interferon-gamma; LAL, *Limulus* amoebocyte lysate; LPS, lipopolysaccharide; LZ-8, Ling Zhi-8; PS-G, polysaccharide from *Ganoderma lucidum*; mAbs, monoclonal antibodies; qPCR, quantitative real-time polymerase chain reaction; TNF-α, tumor necrosis factor-alpha; TLR, Toll-like receptor.

Supporting Information Available: Table of endotoxin contents of LZ-8 and PS-G measured by LAL gel-clot assay and figures depicting ELISA quantitative standard curve of nLZ-8, elution profile of PS-G and dpPS-G from HPLC gel filtration column analysis, and diverse activation effects of dpPS-G, PS-G, and rLZ-8 on mouse peritoneal macrophage inflammatory cytokine mRNA expression. This material is available free of charge via the Internet at http://pubs.acs.org.

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