# Oligosaccharide and Peptidoglycan of *Ganoderma lucidum* Activate the Immune Response in Human Mononuclear Cells

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Ⓢ *Supporting Information*

**ABSTRACT:** The acid-hydrolyzed fragments of *Ganoderma lucidum* polysaccharides (GLPS) obtained by Smith degradation were separated by size-exclusion chromatography into two major water-soluble fractions: peptidoglycans (GLPS-SF1) and oligosaccharides (GLPS-SF2). Both fractions induced CD69 in human peripheral blood mononuclear cells (hPB-MNCs), and they displayed distinct immunomodulating properties. GLPS-SF1, with a molecular weight of around 20 kDa, were heterogeneous peptidoglycans composed of glucose/mannose (4:1) that exhibited biological activities with Th1 cytokines IL-12, IL-2, TNF-$\alpha$, and IFN-$\gamma$ in hPB-MNCs and stimulated macrophage cytokine expression via Toll-like receptor 4 (TLR4) signaling. For GLPS-SF2, with a molecular weight of around several kilodaltons, its sugar sequence was elucidated by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy as $[-\alpha\text{-}1,4\text{-Glc}-(\beta\text{-}1,4\text{-GlcA})_3-]_n$. This oligosaccharide displayed specific immune property with low monocyte induction, greatly stimulated cell activation and proliferation of NK and T cells. This oligosaccharide isolated from *G. lucidum* polysaccharides with internal glucuronic acids/ glucose repeat unit in a 3:1 ratio may be responsible for the active stimulation of NK and T cells.

**KEYWORDS:** *Ganoderma lucidum, peptidoglycan, oligosaccharide, glucuronic acid, human mononuclear cells*

## ■ INTRODUCTION

*Ganoderma lucidum* (Ling-zhi in Chinese and Reishi in Japanese) is a medicinal mushroom commonly used in traditional medicine in China and other Asian countries. *G. lucidum* is believed to promote health and longevity and is used to prevent and treat various diseases, especially tumors.[1−3] The bioactive and functional natural products and macromolecules from *G. lucidum* as well as their possible clinical applications have been studied.[4−7] Among these compounds, triterpenoids and polysaccharides are known as the most important pharmacologically active constituents of *G. lucidum*. These triterpenoids were reported to have hepatoprotective, anti-hypertensive, hypocholesterolemic, and antihistaminic effects and are effective as anti-angiogenic and anticancer compounds.[3] *G. lucidum* polysaccharides (GLPS) can modulate a wide range of immune functions, both *in vitro* and *in vivo*.[4,8,9] Previous studies have demonstrated that GLPS increase the phagocytosis of macrophages, stimulate CD80, CD86, CD83, CD40, CD54, and the human leukocyte antigen (HLA) DR, stimulate the secretion of various cytokines, including IL-1, IL-6, IL-10, IL-12, IFN-$\beta$, TNF-$\alpha$, p70, p40, and GM-CSF,[8,10−12] increase the number of CD14+CD26+ monocytes and macrophages, CD83+CD1a+ dendritic cells, and CD16+CD56+ natural killer (NK) cells in human cord blood mononuclear cells,[13] and protect cells against free radicals and reduce cell damage caused by mutagens.[5,14,15] Despite these multiple functions of GLPS, little is known about their active structure of sugar sequence

and the mechanisms of action underlying their effect on the human innate immune function. Here, we attempted to analyze the lower molecular mass of the oligosaccharide, which responded to the active immune property.

The polysaccharides stimulate the immune system, which results in the production of cytokines and the activation of anticancer activities of immune cells. GLPS are macromolecules with a molecular mass of above 500 kDa. The sugar elements of GLPS from different species have been reported to contain a glucan backbone with 1,3-, 1,6-, and 1,3,6-linkages, a mannan backbone with 1,4-linkages, and heteroglycan side chains of undefined structures.[3,16−19] The binding epitopes of carbohydrate responsible for the diverse immunomodulating properties and their receptors have not been well-identified.[20] Therefore, a mild acid condition of Smith degradation[21] was chosen as a suitable method, which was applied to degrade GLPS into smaller oligosaccharides. The consequent oligosaccharide was confirmed to have similar immunomodulating activities in the human peripheral blood mononuclear cells (hPB-MNCs) as the native GLPS.

## ■ MATERIALS AND METHODS

**Materials.** Artificially cultivated *G. lucidum* fruit bodies were provided by Pharmanex (San Diego, CA). Biogel P10 was purchased from BioRad. Human peripheral blood was provided by the Taipei Blood Center following the guidelines of the Institutional Review Board (IRB) issued by Academia Sinica, Taiwan. Monosaccharide standards, including D-glucose (Glc), D-mannose (Man), D-galactose (Gal), L-fucose (Fuc), and glucuronic acid (GlcA), phenol, sulfuric acid, trifluoroacetic acid (TFA), cell culture medium RPMI-1640, and chemicals were purchased from Sigma-Aldrich. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Bender Medsystem. The monocyte isolation kit II, NK cell isolation kit II, and Pan T cell isolation kit II were purchased from Miltenyibiotec. Fluorochrome-conjugated monoclonal antibodies were obtained from BD Biosciences. The WST-1 reagent was purchased from Roche. HeNC2 and GG2EE cells were kindly provided by Dr. Danuta Radzioch, McGill University, Montreal, Quebec, Canada.

**Preparation of Peptidoglycans from GLPS (GLPS-SF1) and Glc/GlcA Mixture Oligosaccharides from GLPS (GLPS-SF2) by Smith Degradation.** Extracts of *G. lucidum* (5 g) were acid-hydrolyzed by Smith degradation according to an established protocol (http://www.organ.su.se/gw/doku.php?id=sop:index). The protocol involved three steps, including oxidation with periodate, reduction with borohydride, and hydrolysis with dilute acid under mild conditions. The yield of water-soluble sugar-containing products was around 22.4% (1.12 g). The product (500 mg in 1 mL double-distilled $H_2O$) prepared by Smith degradation was further separated by size-exclusion chromatography on Biogel P10 (2.6 cm diameter × 70 cm height) with double-distilled $H_2O$ as the eluent (containing 0.02% $NaN_3$, at a flow rate of 1 mL/min). Dextrans were used as size standards (5, 10, and 20 kDa). GLPS-SF1 was collected from the 150−186 mL of total elution volumes of 370 mL, and GLPS-SF2 was
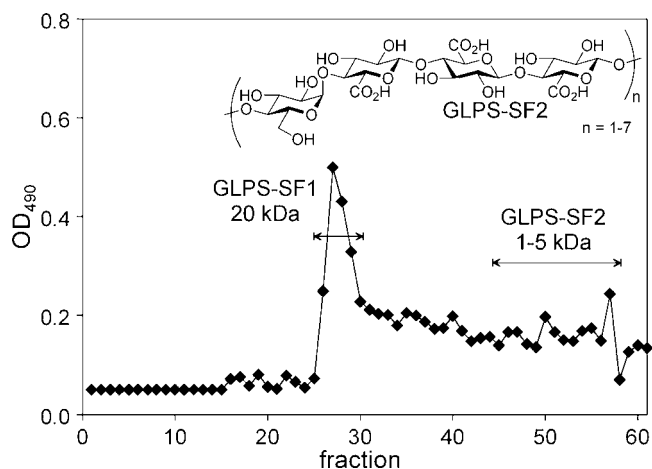


**Figure 1.** Chromatography of GLPS-SF1 and GLPS-SF2 by a Biogel-P10 size-exclusion column and detected by phenol−sulfuric acid at 490 nm.

collected from 270 to 348 mL. Every 6 mL is in each tube (Figure 1). The sugar content of the collected fractions was determined using the phenol−sulfuric acid method and measuring the absorbance at 490 nm.[22] The protein concentration was determined using Bradford's method.[23] The peptide part of the peptidoglycan of *G. lucidum* was removed by protease K; the peptidoglycan (10 mg) was treated with protease K (100 μL, 1 mg/mL, >30 unit/mg, Bioshop) in 5 mL of 50 mM Tris-HCl at pH 8.0 and 1 mM $CaCl_2$ at 37 °C for 1 h, repeated twice, then denatured by 100 °C for 10 min, and centrifuged. The supernatant was dialyzed against water (molecular weight cutoff of 1000 Da) and then lyophilized. The sugar part of the peptidoglycan is collected. Amino acid compositions were detected on the basis of the retention time established for the individual amino acid under defined experimental conditions in high-performance liquid chromatography (HPLC) after acid hydrolysis by heating in 6 N HCl at 110 °C for 24 h and further making derivatives. For the N terminus of peptidoglycans, the procedures of Edman degradation were followed by instruction.

**Carbohydrate Composition/Linkage Analysis by Gas Chromatography−Mass Spectrometry (GC−MS).** The sugar composition of GLPS was determined by acidic hydrolysis with 0.5 M methanolic HCl at 80 °C for 16 h, N-acetylation with methanol/pyridine/acetic anhydride, and trimethylsilylation with Sylon HTP trimethylsilylation reagent (Supelco). The final trimethylsilylated products were kept in *n*-hexane for GC−MS analysis. To analyze the sugar linkages, the trimethylsilylated products were permethylated with $CH_3I$/dimethyl sulfoxide (DMSO) under alkaline conditions, hydrolyzed by 2 M TFA at 120 °C for 2 h, and reduced in $NH_4OH$/$NaBD_4$/ethanol for 2 h at room temperature. The reaction was stopped by adding 100% acetic acid. The derivatives were crystallized using 10% acetic acid in methanol, acetylated, and analyzed by GC−MS. The glucuronic acid linkage was determined by converting glucuronic acid by reduction to the corresponding neutral sugar with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate and sodium borohydride.[24]

GC−MS was carried out on an Agilent gas chromatograph 6890N connected to an Agilent 5975B mass selective detector. A fused silica capillary column (30 m × 0.25 mm inner diameter) at 60 °C was used. The trimethylsilylated products were analyzed at 60 °C for 1 min, increasing to 140 °C at 25 °C/min, increasing to 200 °C at 5 °C/min, and finally, increasing to 300 °C at 10 °C/min. Partial methylated aditol acetate derivatives were analyzed at 60 °C for 1 min, increasing to 290 °C at 8 °C/min, and finally, increasing to 300 °C at 10 °C/min. Peaks were identified by GC−MS and comparing the retention times to those of authentic standards (arabinose, rhamnose, fucose, xylose, galactose, glucose, mannose, N-acetylglucosamine, and N-acetylgalactosamine) using HP EnviroQuant ChemStation (G1701AA, version A.3.00).

**Determination of the Structure of GLPS with Nuclear Magnetic Resonance (NMR) Spectroscopy.** The sugar fragments of GLPS in $D_2O$ (0.5 mg in 0.6 mL) were analyzed by NMR using an AVANCE-500 spectrometer at 300 or 330 K. Chemical shifts are referenced to the residual signal for $D_2O$ at $\delta$ 4.80 ppm (¹H). One-dimensional (1D)/two-dimensional (2D) NMR were carried out with standard pulse sequences provided by Bruker. Selective 1D total correlation spectroscopy (TOCSY) experiments were recorded with mixing times of 60, 90, or 120 ms. Two-dimensional correlation spectroscopy (COSY) spectra were used to assign the proton chemical shifts of H1−H5 of the glucuronic acid and H1−H6 of the glucose. The heteronuclear multiple-quantum coherence (HMQC) spectra were recorded with PL = 120 dB to observe the coupling constants of anomeric positions.

**MS Spectroscopy.** Mass spectra were recorded as described previously in detail.[25] Briefly, a Prince CE system (Prince Technologies, The Netherlands) was coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Canada). A sheath solution [2:1 isopropanol/methanol (vol/vol)] was delivered at a flow rate of 1.0 μL/min and separated on a 90 cm bare fused-silica capillary using 15 mM ammonium acetate in deionized water at pH 9.0. The positive-ion detection mode was at an electrospray ionization voltage of 5.2 kV. A high orifice voltage (+350 V) was used to promote in-source collision-induced dissociation (pseudo-tandem mass spectrometry), and the tandem mass spectra were acquired in the enhanced product ion (EPI) scan mode at a scan rate of 400 Da/s. The samples were dissolved in deionized water.

**Isolation and Stimulation of hPB-MNCs.** hPB-MNCs were separated from buffy coats by Ficoll-Hypaque centrifugation. The mononuclear cells were washed with 2 mM ethylenediaminetetraacetic acid (EDTA) containing phosphate-buffered saline (PBS) and adjusted to a cell density of $2 \times 10^6$ cells/mL in complete medium [RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 4.5 g/L glucose, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 unit/mL penicillin,

and 100 unit/mL streptomycin]. Cells were treated with sugar fragments and incubated at 37 °C and 5% $CO_2$ for 24 h. *Escherichia coli* lipopolysaccharide (LPS) as a positive control was treated at 1 μg/mL.

**Detection of the Cluster of Differentiation (CD) Marker Expression in hPB-MNCs Using Flow Cytometry.** Harvested cells were washed with PBS and suspended at a concentration of $1 \times 10^6$ cells/100 μL of PBS. Fluorochrome-conjugated monoclonal antibodies (10 μL) were added to each 100 μL cell suspension, and the mixture was incubated at 4 °C for 30 min. In double-staining experiments, two types of monoclonal antibodies conjugated with different fluorochromes were added to each cell suspension. As negative controls, aliquots of cell suspensions were mixed with the same fluorochrome-conjugated irrelevant isotype-matched monoclonal antibody. The cells were washed twice with PBS and suspended in 4% paraformaldehyde for 10 min to fix the cells, which were then stored at 4 °C for flow cytometry. Cells were cytofluorometrically analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA); in each sample, $2 \times 10^4$ cells were analyzed using WinMDI, version 2.8 (Windows Multiple Document Interface for Flow Cytometry). Statistical markers were set using the irrelevant isotype-matched controls as reference.

**Isolation of Monocytes, T Lymphocytes, and NK Cells from hPB-MNCs.** NK cells and T lymphocytes were isolated by depletion of nonmonocytes and non-NK cells and non-T lymphocytes, respectively (negative selection) using human monocytes isolation kit II, NK cell isolation kit II, and Pan T cell isolation kit II (Miltenyibiotec). Total hPB-MNCs ($1 \times 10^7$ cells) were suspended in MACS cell separation buffer and incubated with 10 μL of biotin-conjugated monoclonal antibodies (primary labeling reagent) at 4 °C for 10 min. The antibiotin monoclonal antibodies conjugated with magnetic microbeads (20 μL; secondary labeling reagent) were then added and incubated at 4 °C for 15 min. No washing was required between the two labeling steps. After labeling, the cells were washed with MACS cell separation buffer and applied to separation columns. Before loading the samples, the columns were placed in the magnetic field of a MACS separator and rinsed with MACS cell separation buffer. The unlabeled cells that passed through the columns were collected and cultured in complete medium to a density of $2 \times 10^6$ cells/mL. The purities of isolated monocytes, NK cells, and T lymphocytes cells were evaluated by staining with the anti-CD14 fluorescein isothiocyanate (FITC), anti-CD56 FITC, and anti-CD3 FITC, respectively, and analyzed by flow cytometry. The purity of each cell population used in this study was >90%.

**Determination of Th1 Cytokine Concentrations in Medium by ELISA.** At the end of the incubation, the samples were centrifuged and the supernatant was collected; this conditioned medium was used for cytokine quantification using human IL-12, TNF-α, IL-2, and IFN-γ ELISA kits (Bender Medsystem). Conditioned medium (100 μL) was added to sample wells, followed by 50 μL of biotin-conjugated solution. After 2 h of incubation, sample wells were washed with 0.1% Tween-20 PBS buffer 3 times. To each sample well, 100 μL of diluted streptavidin–horseradish peroxidase (HRP) was added, and the mixtures were incubated for 1 h. After washing, tetramethylbenzidine (TMB) substrate solution (100 μL) was added to each sample well, and the reaction was stopped by adding 100 μL of 2 N $H_2SO_4$. The cytokine concentration was measured according to the color intensity using a microplate reader (MaxII DYNEX, HP DJ610) at 450 nm.

**Detection of Cell Proliferation Using the WST-1 Assay.** Primary T lymphocytes and NK cells were isolated from hPB-MNCs by MACS. Cells ($1 \times 10^4$) were in 100 μL with or without GLPS-SF2 (200 μg/mL) and incubated for 3 and 7 days. WST-1 reagent (10 μL) was then added to each well, and the mixtures were gently mixed and then incubated for 4 h. The formazan formed was quantified using an ELISA plate reader at 450 nm.

## ■ RESULTS

**Hydrolysis of *G. lucidum* Polysaccharides by Smith Degradation.** To determine which GLPS moiety played an immunological role, GLPS was partially hydrolyzed by Smith degradation. The products were separated by Biogel-P10 size-exclusion chromatography into two groups, GLPS-SF1 and GLPS-SF2, according to their molecular masses, as shown in Figure 1. The yields of GLPS-SF1 and GLPS-SF2 were 34.4 and 8.6%, respectively. In comparison to the standard dextran, the molecular weight of GLPS-SF1 was about 20 and 1−5 kDa for GLPS-SF2. Meanwhile, GLPS-SF1 was detected as a peptide-containing polysaccharide, and GLPS-SF2 was determined to be an oligosaccharide.

**Fragment Properties of GLPS-SF1.** GLPS-SF1 was a peptidoglycan with a molecular weight more than 20 kDa. Owing to the high molecular weight, the resolution of proton NMR spectroscopy could not be obtained with better resolution. Here, we reported only the carbohydrate and amino acid composition of GLPS-SF1. From GC−MS, the molar ratio of glucose/mannose was about 4:1, and 1,6-glucosidic linkage and 1,4-mannosidic linkage were determined as the sugar backbone. Otherwise, GLPS-SF1 contained the following amino acids: Asp/Asn (14.1%), Glu/Gln (11.7%), Gly (13.5%), Ala (11.8%), Cys (13%), Ser (8.3%), Val (7.5%), Leu (6%), Phe (4.65%), Ile (4.44%), Lys (2%), Arg (2%), Tyr (1.2%), and Try/Pro (not quantified). GLPS-SF1 consisted of heterogeneous peptidoglycans because there were no consistent N-terminal sequences from N-terminal analysis (data not shown).

**Carbohydrate Sequence of GLPS-SF2 Oligosaccharide.** From GC−MS, the carbohydrate composition and linkages of GLPS-SF2 were obtained. Glucose and glucuronic acid residues were analyzed with glycosidic 1,4-linkage. The intergradient indicated that the ratio was 1:3 for Glc/GlcA in the $^1H$ NMR spectrum (Figure 2a). According to 1D TOCSY
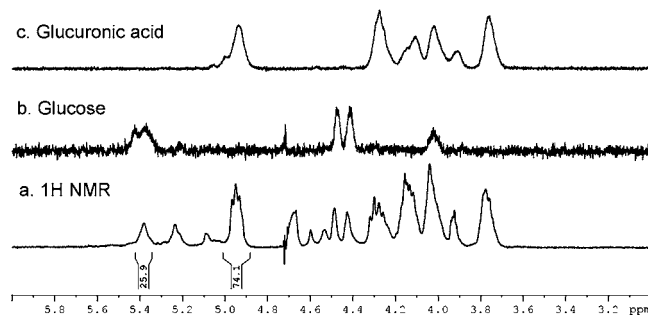


**Figure 2.** $^1H$ NMR and selective 1D TOCSY spectra for GLPS-SF2 at 330 K.

and COSY experiments (spectra b and c of Figure 2 and panels a and b of Figure 3), each proton signal could be assigned, in which the anomeric proton resonance of Glc was at δ 5.40 and 4.79 ppm for GlcA, respectively (Table 1). The chemical shift of the anomeric proton of the glucose unit was more downfield and is responsible for the α configuration, and the β configuration of the glucuronic acid residue was determined by the $^1J_{C-H}$ coupling constant (160 Hz) (Figure 3d). The HMBC experiment can be applied to analyze the long-range $^1H-^{13}C$ $^2J$ and $^3J$ coupling constants and explain the glycosidic linkage (Figure 3c), and the cross-peaks $H1_{Glc}/C4_{GlcA}$ and $H1_{GlcA}/C4_{GlcA'}$ indicated the α-1,4-linkage and β-1,4-linkage, respectively. The carbohydrate sequence $-α$-1,4-Glc-(β-1,4-GlcA)$_3-$ of GLPS-SF2 could be further examined by electrospray ionization−mass spectrometry (ESI−MS). The ESI−tandem mass spectrometry (MS/MS) spectrum indicated that GLPS-SF2 is Glc$^{Smith\ degradation}-$(GlcA)$_3$, as shown in Figure
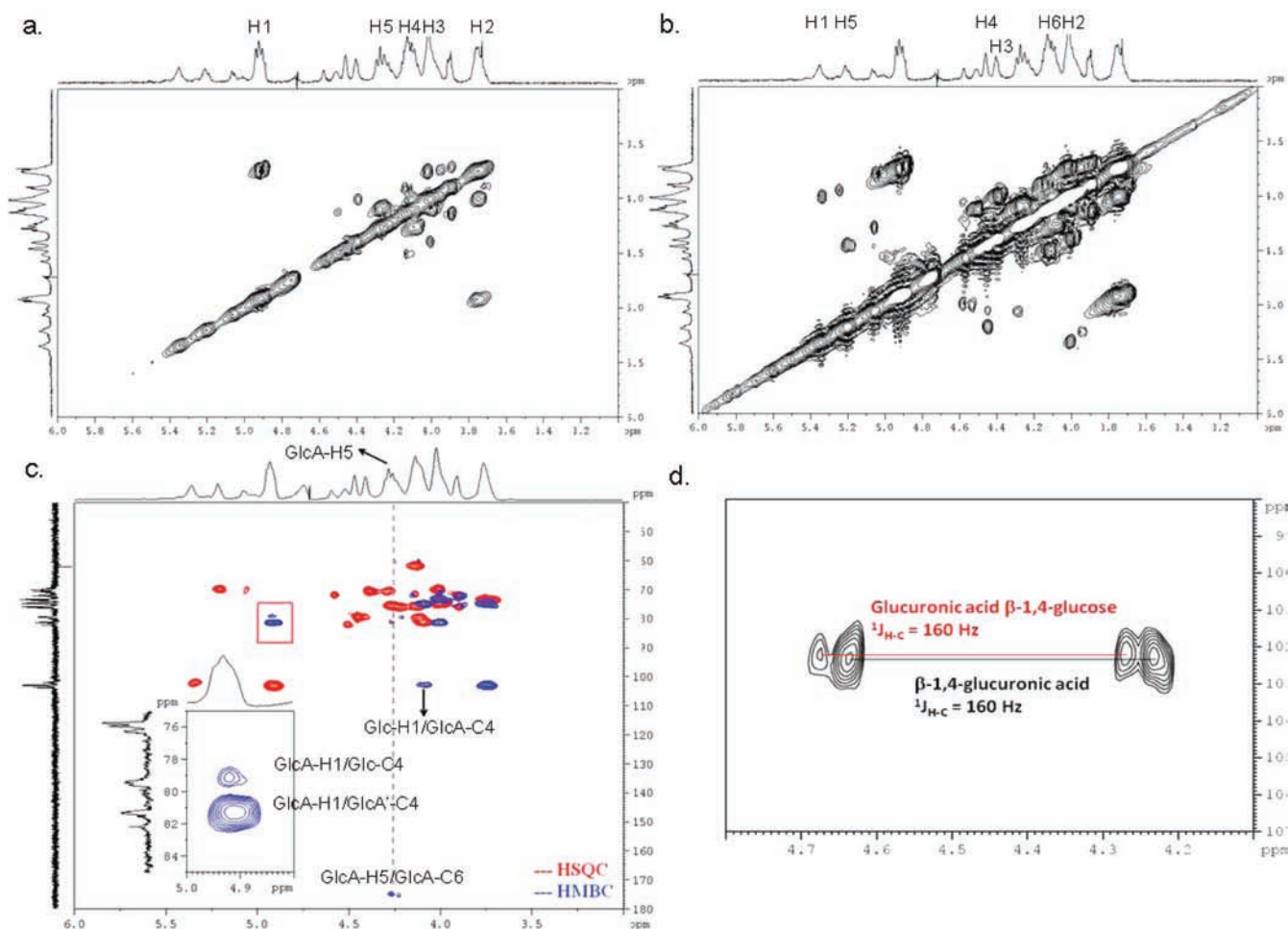
**Figure 3.** Proton chemical-shift assignment for GLPS-SF2 at 330 K. (a) $^1H-^1H$ COSY assignment of the glucuronic acid moiety. (b) $^1H-^1H$ COSY spectrum of the glucose moiety. (c) $^1H-^{13}C$ HSQC and HMBC spectra of GLPS-SF2. (d) Two-dimensional $^1H-^{13}C$ HMQC spectra for $^1J$ coupling constants of anomeric positions.

**Table 1. $^1$H and $^{13}$C NMR Chemical Shifts for GLPS-SF2 at 330 K$^a$**

| residue | | position | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| →4)-β-D-GlcA−(1→) | $^1$H | 4.97 | 3.79 | 4.06 | 4.14 | 4.30 | |
| | $^{13}$C | 102.9 | 73.4 | 74.7 | 81.3 | 75.8 | 174.9 |
| →4)-α-D-Glc−(1→) | $^1$H | 5.40 | 4.16 | 4.44 | 4.50 | 5.23 | 4.17 |
| | $^{13}$C | 101.9 | 70.1 | 70.6 | 79.5 | 70.0 | 62.2 |

$^a$Sample in $D_2O$, with the chemical shift in parts per million (ppm).

4. The fragments $m/z$ 687 and 136 of GLPS-SF2 indicated a tetrasaccharide sequence of $(GlcA)_3$ and a Glc$^{Smith\ degradation}$ residue, a reduced derivative of glucose after Smith degradation, respectively. From size-exclusion chromatography, the molecular weight of GLPS-SF2 was distributed from 1 to 5 kDa. This result indicated that the repeat unit number ($n$) of oligosaccharide $[−α-1,4-Glc−(β-1,4-GlcA)_3−]_n$ could range from 1 to 7.

**GLPS-SF1 and GLPS-SF2 Fractions Retain the Immuno-activating Properties.** When the immune activation properties of the GLPS-SF1 and GLPS-SF2 fractions are compared to that of undegraded GLPS, it was revealed that both fractions retained CD69 inducing activity, similar to undegraded GLPS, by staining with anti-CD69-PE antibody in hPB-MNCs (Figure 5a). Furthermore, when the cells were

stained with individual antibody that specifically recognized a cell surface marker, we observed that GLPS and the fractions induced CD69 expression in monocytes (marker CD14), T lymphocytes (marker CD3), and NK cells (marker CD56) in hPB-MNCs similarly (see Figure S5b of the Supporting Information). Besides, the degraded products GLPS-SF1 and GLPS-SF2 also induced the Th1 cytokines IL-12, IL-2, TNF-α, and IFN-γ in hPB-MNCs (Figure 5b).

**GLPS-SF1 and GLPS-SF2 Fractions Revealed Distinct Immunomodulating Properties on Different Cell Populations.** After Smith degradation, GLPS-SF1 and GLPS-SF2 fractions were each able to activate Th1 cytokines in hPB-MNCs. However, the specific structural signature of each fraction may affect the affinity and specificity of the targeted cell populations. To further evaluate the difference in the
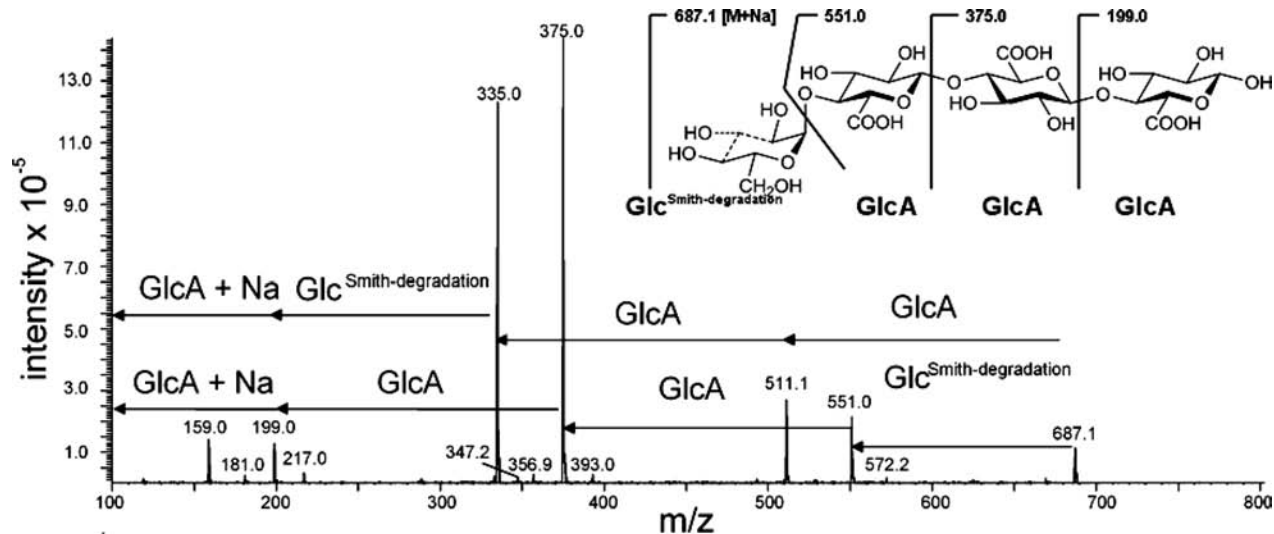
**Figure 4.** MS/MS spectra for GLPS-SF2. The glucose residue was modified after Smith degradation.
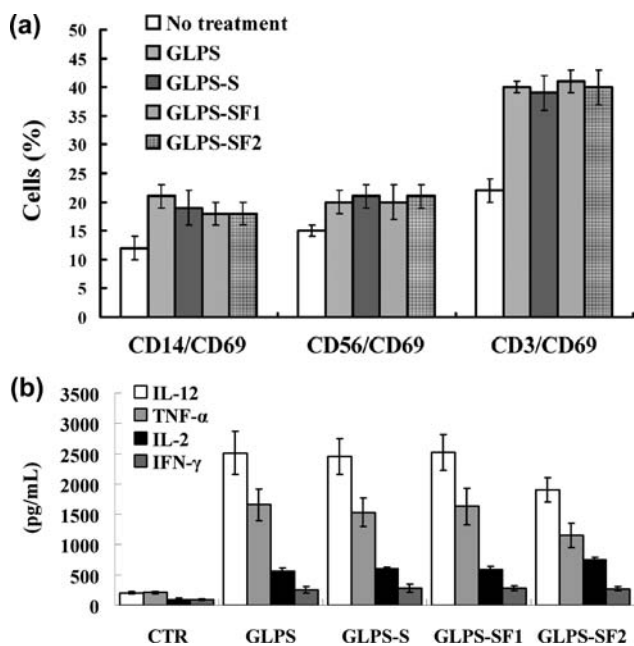


**Figure 5.** Monocytes, T lymphocytes, and NK cells were activated by GLPS and its products by Smith degradation. (a) hPB-MNCs (4 × $10^6$) cultured in 2 mL complete medium were treated with the indicated fractions (100 μg/mL) for 24 h and then double-stained with antibodies against CD69 and with antibodies against either CD14, CD3, or CD56. The percentages of CD14+/CD69+, CD3+/CD69+, and CD56+/CD69+ cells were calculated and normalized to the value of the control. (b) Conditioned medium was harvested at 24 h and assayed for the cytokine concentrations by ELISA. Results are expressed as the mean ± standard error of the mean (SEM) from three independent experiments. GLPS-S = GLPS hydrolyzed products of Smith degradation.

structural–functional relationships between GLPS-SF1 and GLPS-SF2, the effect of each fraction on the purified cell population sorted by the immunomagnetic separation was examined. In purified primary CD14+ monocytes, flow cytometry data showed that GLPS-SF1 significantly induced the expression of the co-stimulatory molecules CD80/CD86 and the Th1 cytokines IL-12/TNF-α more than that of GLPS-SF2 by approximately 10%. In contrast, GLPS-SF2 only slightly

induced the expression of CD80/CD86 and IL-12/TNF-α in monocytes (see Figures S1 and S2 of the Supporting Information). Moreover, in purified T lymphocytes and NK cells, although both fractions induced the expression of CD69 and IFN-γ in T lymphocytes and NK cells to similar levels, GLPS-SF2 induced IL-2 secretion to higher levels. GLPS-SF2 also induced IL-2 secretion in the human T cell line Jurkat (see Figures S3–S6 of the Supporting Information).

**GLPS-SF1 Stimulates Cytokine Expression of the Macrophage via the Toll-like Receptor 4 (TLR4) Receptor.** The results suggested that monocyte-/macrophage-activating properties of GLPS were mainly contributed by the moiety of GLPS-SF1. GLPS and GLPS-SF1 induced the production of TNF-α in normal mouse macrophages (HeNC2) expressing TLR4, but GLPS-SF2 could not (Figure 6). When
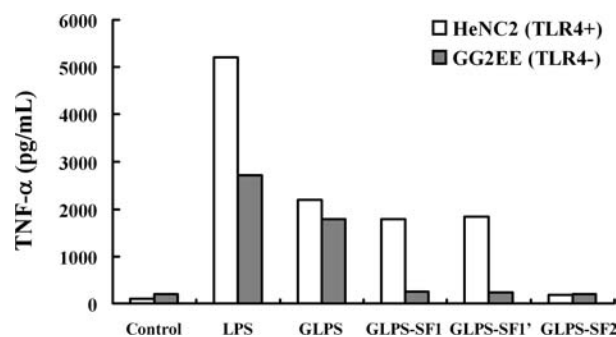


**Figure 6.** Impaired cytokine production in TLR4-deficient mouse macrophages stimulated by GLPS. HeNC2 (TLR4+) and GG2EE (TLR4−) cells (5 × $10^5$ /mL) were incubated with *E. coli* LPS (1 μg/mL), GLPS (100 μg/mL), GLPS-SF1 (100 μg/mL), GLPS-SF1′ (removed peptides of GLPS-SF1, 100 μg/mL), and GLPS-SF2 for 24 h. The TNF-α concentration in the conditioned medium was determined by ELISA.

we removed the peptides using protease K, the carbohydrate moiety of GLPS-SF1 (named GLPS-SF1′) still induced TNF-α activity, which suggested that the polysaccharides of GLPS-SF1 were responsible for the induction of cytokine activity. The TNF-α inducing activity of both GLPS-SF1 and GLPS-SF1′ was impaired in TLR4-deficient macrophages (GG2EE; Figure 6). These results suggested that TLR4 is one of the putative

receptors for GLPS-SF1- and GLPS-SF1′-mediated TNF-$\alpha$ expressions.

**GLPS-SF2 Specifically Stimulated Cell Activation and Proliferation in T Lymphocytes and NK Cells.** GLPS-SF2 had specific immunomodulating properties in T lymphocytes and NK cells but not in monocytes. GLPS-SF2 induced the expression of the activation marker CD69 and the secretion of IFN-r and IL-2 in T lymphocytes and NK cells. The immune activation of T lymphocytes and NK cells was usually associated with cell proliferation. Thus, we further examined whether GLPS-SF2 induced cell proliferation in T lymphocytes and NK cells. T lymphocytes and NK cells were purified from hPB-MNCs and cultured with or without GLPS-SF2 stimulation. Cell proliferation was assayed using the WST-1 live-cell-staining method after 3 and 7 days. As shown in Figure 7,
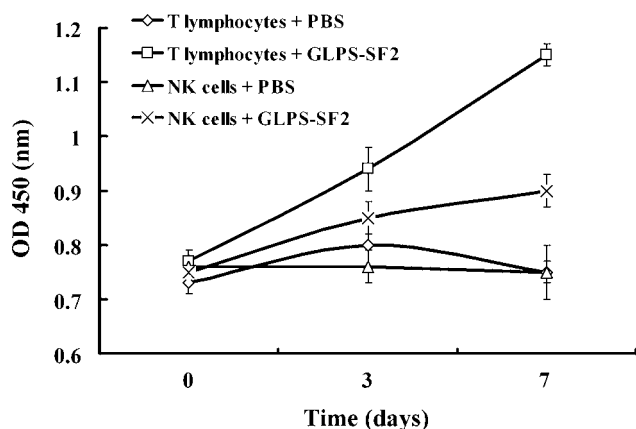


**Figure 7.** Immunomodulating properties of GLPS-SF2 in inducing T lymphocyte and NK cell proliferation. Primary T lymphocytes and NK cells were purified from hPB-MNCs by magnetic negative separation. Primary cells $(1 \times 10^4)$ cultured in 100 $\mu$L of complete medium were treated with GLPS-SF2 (200 $\mu$g/mL) or an equal volume of PBS for 3 and 7 days. Cell proliferation was determined by the WST-1 assay. The optical densities of T lymphocytes and NK cells at 450 nm are shown as diamonds and squares, respectively. Data are expressed as the mean ± SEM of triplicate wells.

GLPS-SF2 significantly enhanced both T lymphocyte and NK cell proliferation. The result indicated that GLPS-SF2 is a potent T lymphocyte and NK cell stimulator.

## ■ DISCUSSION

A number of studies have demonstrated that GLPS extensively modulate immune function both *in vivo* and *in vitro*.[8,26,27] In the current study, we used hPB-MNCs for examining the immunomodulating properties of GLPS. Being a multiple cell type, hPB-MNC allows for a systematic approach for immunoscreening. The major fractions of isolated hPB-MNCs contain both innate and adaptive immune cells. The former contains monocytes/macrophages and NK cells, and and the latter contains T and B lymphocytes.[28] Our data indicated that GLPS directly affected monocytes, NK cells, and T lymphocytes. These effects of GLPS on different types of immune cells may arise from their complex structures of heteroglycans or peptide−glycans.

Although GLPS have such far-reaching effects on the immune system, little is known about their fine structure and the relationship between the structure and biological activity. The degree of substitution on the backbone chain and the

length of the side chains may be important factors in determining their conformation in aqueous solution and thereby the biological activities.[29] Glycopeptides have a wide variety of biological functions, and these functions may be dependent upon either the oligopeptide moiety, the carbohydrate moiety, or both. The elucidation of the structure−function relationship of GLPS is challenging because they are macromolecules and must be degraded into small fragments for structural analyses. A combination of the suitable method and optimized conditions is required to efficiently degrade the polysaccharides into fragments that retain bioactive properties. In our case, Smith degradation was employed, which selectively degrades a polysaccharide to either a polysaccharide with a smaller repeat or an oligosaccharide. The products are usually obtained from a terminal, two-substituted, four-substituted, and six-substituted sugar residue.[21] After Smith degradation and fractionation of GLPS, we successfully obtained two fractions that retained many immunological activities, e.g., their effect on IL-2, IL-12, INF-$\gamma$, and TNF-$\alpha$.

One of the bioactive fractions isolated, GLPS-SF1, might be similar to Glc/Man polysaccharides reported previously that stimulate IL-12 and use TLR4 as a cell receptor.[10−12] The previously described protein-bound polysaccharide PSG consists of 95% polysaccharides and 5% peptides. The polysaccharides of PSG contain mainly a 1,6-D-glucan moiety. PSG is immunologically stimulatory through the NF-$\kappa$B and p38 MAPK pathways in TLR4 receptors.[10] GLPS-SF1 (20 kDa) and its carbohydrate moiety were TLR4-dependent in a mouse macrophage system. However, the second bioactive fraction isolated, GLPS-SF2, differs from the previously reported polysaccharides. GLPS-SF2 was TLR4-independent (Figure 6) and consists of GlcA/Glc. Moreover, we showed that GLPS-SF2 is involved in T lymphocyte and NK cell activation, which is the first report of such a function. In a previous study, an acidic polysaccharide GLA was shown to consist of D-glucose and D-glucuronic acid in a molar ratio of 1:2.4,[30] but its immunological activities were not studied.

Previous studies have shown that some GLPS with specific structural signatures have T-lymphocyte-stimulating activity. For example, $\beta$-1,3-glucan polysaccharides with branches of terminal glucosyl residues substituted at C6 of the glucose residues in the main chain have been shown to induce T cell proliferation.[31] Specifically, two heteroglycans (PL-1 and PL-4) enhance the proliferation of T and B lymphocytes. PL-1 has a backbone consisting of $\alpha$-1,4-glucopyranosyl and $\beta$-1,6-galactopyranosyl residues with branches at O6 of the glucose residues and O2 of the galactose residues; the branches are composed of terminal glucose, 1,6-linked glucosyl residues, and a terminal rhamnose. PL-4 is comprised of 1,3-, 1,4-, and 1,6-linked $\beta$-glucopyranosyl residues and 1,6-linked $\beta$-mannopyranosyl residues.[32] A water-insoluble glucan from *G. lucidum* on oral tumor cells had no cytotoxic effect *in vitro*.[33] When the insoluble $\beta$-1,3-glucan of *G. lucidum* was sulfated or carboxymethylated, it could inhibit the *in vitro* proliferation of S-180 tumor cells through cell-cycle arrest in the $G_2$/M phase, induce apoptosis, and even reduce tumor volumes in female BALB/c mice.[34]

Unlike the isolation procedures used in previous studies, we degraded GLPS by Smith degradation, then separated the fractions by gel filtration, and collected the low-molecular-weight fractions. We identified this acidic polysaccharide component of GLPS, GLPS-SF2, with an −$\alpha$-1,4-Glc−($\beta$-1,4-GlcA)$_3$− sequence. GLPS-SF2 stimulated not only T

lymphocytes but also NK cells. GLPS-SF2 also induced cell proliferation and the secretion of IL-2/IFN-$\gamma$ from both T and NK cells. In addition, we present the first report of GLPS directly stimulating NK cells isolated from hPB-MNC. The $[-\alpha\text{-}1,4\text{-}Glc-(\beta\text{-}1,4\text{-}GlcA)_3-]_n$ structure of GLPS-SF2 contributed to the activation of NK cells.

In this study, we established the relationship between the structural features of GLPS and their immunomodulating mechanisms. GLPS-SF1 and GLPS-SF2 were identified from GLPS after Smith degradation and exhibited distinct bioactivities. GLPS-SF1 consisted of heterogeneous glycopeptides. The sugar part was composed of glucose and mannose in a molar ratio of 4:1, with main sugar 1,6-glucan linkage. GLPS-SF1 showed diverse immune stimulating properties in human monocytes, NK cells, and T lymphocytes as well as induced cytokine expression via TLR4-dependent signaling in mouse macrophages. GLPS-SF2 were acidic oligosaccharides with the $[-\alpha\text{-}1,4\text{-}Glc-(\beta\text{-}1,4\text{-}GlcA)_3-]_n$ structure. This structural feature was studied and described in the current study. GLPS-SF2 was shown to have immunomodulating activities specific on NK cells and T lymphocytes by inducing the IL-2 cytokine expression and cell proliferation.

## ■ ASSOCIATED CONTENT

### ⑤ Supporting Information

Immuno-activating properties of GLPS Smith degradation products in purified monocytes (Figure S1), purified monocytes and monocytic cell lines (Figure S2), purified T lymphocytes and Jurkat cells (Figures S3 and S6), and purified NK cells (Figures S4 and S5). 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.

## ■ ABBREVIATIONS USED

GLPS, *Ganoderma lucidum* polysaccharides; GLPS-SF1, peptidoglycans from GLPS; GLPS-SF2, oligosaccharides from GLPS; hPB-MNC, human peripheral blood mononuclear cell; TLR4, toll-like receptor 4

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