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Biochemical and Biophysical Research Communications 323 (2004) 133–141
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Immune receptors for polysaccharides from *Ganoderma lucidum*

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Received 22 June 2004
Available online 27 August 2004

Abstract

This study was designed to identify and characterize the immune receptors for polysaccharides from *Ganoderma lucidum*, a Chinese medicinal fungus that exhibits anti-tumor activities via enhancing host immunity. We herein demonstrate that *G. lucidum* polysaccharides (GLPS) activated BALB/c mouse B cells and macrophages, but not T cells, in vitro. However, GLPS was unable to activate splenic B cells from C3H/HeJ mice that have a mutated TLR4 molecule (incapable of signal transduction) in proliferation assays. Rat anti-mouse TLR4 monoclonal antibody (Ab) inhibited the proliferation of BALB/c mouse B cells under GLPS stimulation. Combination of Abs against mouse TLR4 and immunoglobulin (Ig) achieved almost complete inhibition of GLPS-induced B cell proliferation, implying that both membrane Ig and TLR4 are required for GLPS-mediated B cell activation. In addition, GLPS significantly inhibited the binding of mouse peritoneal macrophages with polysaccharides from *Astragalus membranaceus*, which is known to bind directly with TLR4 on macrophage surface. Moreover, GLPS induced IL-1β production by peritoneal macrophages from BALB/c, but not C3H/HeJ, mice, suggesting that TLR4 is also involved in GLPS-mediated macrophage activation. We Further identified a unique 31 kDa serum protein and two intracellular proteins (ribosomal protein S7 and a transcriptional coactivator) capable of binding with GLPS in co-precipitation experiments. Our results may have important implications for our understanding on the molecular mechanisms of immunopotentiating polysaccharides from traditional Chinese medicine.

Keywords: *Ganoderma lucidum*; Polysaccharide; Receptor; TLR4; Immunomodulation

Fungi are an important source of materials in traditional Chinese medicine. Polysaccharide extracts from many species of fungi exhibit immunostimulating and/or anti-tumor activities [1–14]. *Ganoderma Lucidum* (Lingzhi) is a member of the fungus family (lamellae basidiomycete of the family Polyporaceae) that naturally grows on fallen trees and logs of other broad leaf trees. The use of Lingzhi as a longevity- and vigor-promoting "magic herb" dates back more than 2000 years in China. Scientific investigations have repeatedly confirmed Lingzhi's beneficial effect on health in general, it is now frequently promoted as an effective agent against cancers in the Pacific Rim areas, such as China, Japan, Korea, and other Asian countries. Recent advances demonstrate that polysaccharides extracted from the fruit bodies, mycelium, and spores of *G. lucidum* can promote the function of macrophages, B cells, T cells as well as dendritic cells [6–14]. However, molecular mechanisms for the immunobiological function of *G. lucidum* polysaccharides (GLPS) are far from clear. This study was designed to identify and characterize GLPS-binding receptors that are required for its immunobiological function.

Materials and methods

Animals and antibodies. Female BALB/c mice (8–10 weeks old) were purchased from the Experimental Animal Division of Peking University Health Sciences Center, Beijing, China. Female C3H/HeJ mice (8–10 weeks old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Science, China. All animals were

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doi:10.1016/j.bbrc.2004.08.069

maintained in the animal facility of the Department of Immunology. Monoclonal rat anti-mouse TLR4 antibody (Ab) was provided by Invivogen (San Diego, USA). IgG from un-immunized rats or rabbits and polyclonal rabbit IgG Abs against mouse immunoglobulin (Ig) were prepared in this laboratory.

Polysaccharides. GLPS was isolated from boiling water extract of *G. lucidum*, followed by ethanol precipitation, dialysis, and protein depletion using the Sevag method. The product was hazel powder with a molecular weight of 584,900 and contained 16 different amino acid residues. The ratio of polysaccharides to peptides is 93.5:6.5%. The polysaccharides consisted of D-rhamnose, D-xylose, D-fructose, D-galactose, D-mannose, and D-glucose with molar ratio of 0.793:0.964:2.944:0.167:0.389:7.94 and linked together by β-glycosidic linkages.

Fluorescence labeling and characterization of polysaccharides from *Astragalus membranaceus* (APS) was as described previously [15]. Dextran (Mr 70,000) was obtained from Sigma (USA). GLPS, APS, and dextran were dissolved in RPMI 1640 medium (Gibco-BRL), filtered through a 0.22 mm filter, and stored at −20 °C for future use.

Preparation of mouse splenocytes, splenic T, B cells, and peritoneal macrophages. Spleens from BALB/c or C3H/HeJ mice were gently smashed by pressing with the flat surface of a syringe plunger against stainless steel sieve (200 mesh). Red blood cells were lysed by brief treatment with distilled water. The splenocytes were washed twice and then resuspended in R10:RPMI-1640 medium supplemented with 10% (v/v) FCS (Hyclone, USA), penicillin/streptomycin (100 U/ml), L-glutamine (2 mM), and 2-ME (5×10^{-5} M).

Freshly prepared BALB/c mouse splenocytes, resuspended in R10 at 10^7 cells/ml, were plated into 90 mm tissue culture dishes and incubated for 4 h to remove adherent cells at 37 °C in a CO_2 incubator. The non-adherent cells were collected, washed twice in PBS, and then incubated with magnetic microbeads coated with rat anti-mouse CD19 mAb (Myltenyi Biotec., Italy) at a density of 10 μl Ab solution/10^7 splenocytes for 30 min at 4 °C. The labeled cells were washed twice with PBS and then applied to a MACS separation column. The effluent was collected as non-B cells. After further washes, the column was removed from the magnet separator and the B cells were flushed out of the column using a plunger. To increase the purity of T cells in the non-B cells, the non-B cell fraction was reapplied to a separation column and the effluent was collected as T cells. B cells were reacted with FITC-anti-mouse Ig Ab (IgG) and T cells were reacted with FITC-anti-mouse CD3 Ab for 30 min at 4 °C and then analyzed by flow cytometry. The purities of B cells and T cells were greater than 95%.

In order to obtain mouse peritoneal macrophages, BALB/c, and C3H/HeJ mice were treated intra-peritoneally with 3% thioglycollate three days before they were sacrificed by cervical amputation. Peritoneal macrophages were harvested by peritoneal lavage using ice-cold Ca^{2+} and Mg^{2+}-free PBS. Cells were collected by centrifugation and resuspended in R10.

Proliferation assay. Freshly prepared mouse splenocytes (4×10^5), T cells or B cells (2×10^5) were cultured in flat-bottomed 96-well plates (Nunc, Denmark) in a volume of 200 μl per well in the presence, or absence, of different concentrations of GLPS, dextran, LPS or ConA. The cultures were incubated at 37 °C and 5% CO_2 for three days. In the last 8 h of incubation, [^{3}H]thymidine ([^{3}H]TdR, 0.2 μCi/well) was added into each well. The cells were harvested, using a 96-well plate harvester (Tomtec, USA), onto fiberglass filters and radioactivity on the filter matt was counted in a MicroBeta Trilux LSC counter (EG & G Wallac, USA).

Ig and cytokine assays. BALB/c mouse splenocytes (4×10^5/well) or purified B cells (2×10^5/well) were incubated with, or without, GLPS (100 μg/ml), dextran (100 μg/ml) or LPS (5 μg/ml) for four days in 24-well Costar plates. Concentration of IgG in the culture supernatant was determined by ELISA using plates coated with polyclonal rabbit anti-mouse Ig Abs. HRP-conjugated goat anti-mouse IgG (Sigma, USA) was used as detecting Ab.

BALB/c or C3H/HeJ mouse peritoneal macrophages were cultured with dextran, GLPS (100 μg/ml), LPS (5 μg/ml) or IFN-γ (6.25 ng/ml) for 48 h. Concentration of IL-1β in the supernatant was determined using an ELISA kit (Sigma, USA) following manufacturer's instruction.

RT-PCR detection of IL-2 mRNA expression by T cells. BALB/c mouse splenocytes were cultured in 24-well plate in the presence or absence of dextran, GLPS (100 μg/ml) or ConA (5 μg/ml) for 24 h. The cells were harvested and total cellular RNA was extracted using Trizol Reagent (Invitrogen, USA). Equal amounts of RNA were reverse-transcribed into cDNA using oligo$(dT)_{16}$ primers. For PCR, samples were heated to 94 °C for 5 min and cycled 40 times at 94 °C for 30 s, 51 °C for 1 min, and 72 °C for 2 min, after which an additional extension step at 72 °C for 10 min was included. PCR products were electrophoresed in 1% agarose gel followed by staining with ethidium bromide. The sequences used were as follows: IL-2, sense: 5′ CTT GCC CAA GCA GGC CAC AG 3′; antisense: 5′ GAG CCT TAT GTG TTG TAA GC 3′. β-actin, sense: 5′ GTG GGG CGC CCC AGG CAC CA 3′; antisense: 5′ CTT CCT TAA TGT CAC GCA CGA T TTC 3′. The IL-2 and β-actin primers produce amplified products at 307 and 540 bp, respectively.

Determination of endotoxin contamination. Endotoxin concentrations in GLPS and LPS samples (30 μg/ml of GLPS and 0.01 μg/ml of LPS) were measured using LAL chromogenic assay kit (Yihua Biotech., Shanghai, China) according to the manufacturer's instruction.

Competitive binding assay. Staining of cells using fluorescence-labeled APS (fl-APS) was as described previously [15]. For GLPS competition assays, freshly prepared BALB/c mouse peritoneal macrophages (1×10^6/tube) were treated with GLPS or dextran at 5 mg/ml for 40 min at 4 °C and then fl-APS (0.3 mg/ml) for another hour at 4 °C. After washes, the cells were resuspended in 1 ml PBS for flow cytometric analysis.

Precipitation of polysaccharide-binding proteins. GPS, APS, and dextran were immobilized onto epoxy-activated Sepharose 6B (Pharmacia, USA) gel following the manufacturer's instruction. BALB/c mouse splenocytes or THP-1 cells were lysed in lysis buffer (50 mmol/L Tris–CL, 1 mM EDTA, 150 mM NaCl, 100 μg/ml PMSF, 1% NP40, and 0.05% SDS) at 4 °C for 1 h. The lysate was then pre-cleared by incubating with Sepharose 6B at 4 °C for 1 h followed by Sepharose 6B coupled with GLPS, APS or dextran for another hour. After three washes, the precipitates were loaded onto SDS–PAGE 12% gels for electrophoresis followed by Coomassie or silver staining. In some experiments, the unique GLPS-binding protein band was excised from the Coomassie blue-stained gel and in-gel digested with trypsin. The digested peptides were separated by HPLC and subsequently amino acid sequence was analyzed using an electrospray tandem mass spectrometer (LCQ EDCA XP, Finnigeanm, USA) by Huada, Beijing, China. The MS/MS data were searched against a protein sequence database using Mascot software.

Statistical analysis. All experiments described here have been repeated at least three times. Results are presented as means ± standard error of the mean (SEM). Comparison of the data were performed using Student's *t* test. Significance was defined as a P value of <0.05%.

Results

Selective activation of murine B cells by GLPS in vitro

As illustrated in Fig. 1A, GLPS was able to elicit vigorous proliferation of BALB/c mouse splenocytes. Subsequent proliferation assays using purified mouse splenic B and T cells showed that the former were the main GLPS responders in the splenocyte population (Figs. 1B and C). Furthermore, increased Ig production was

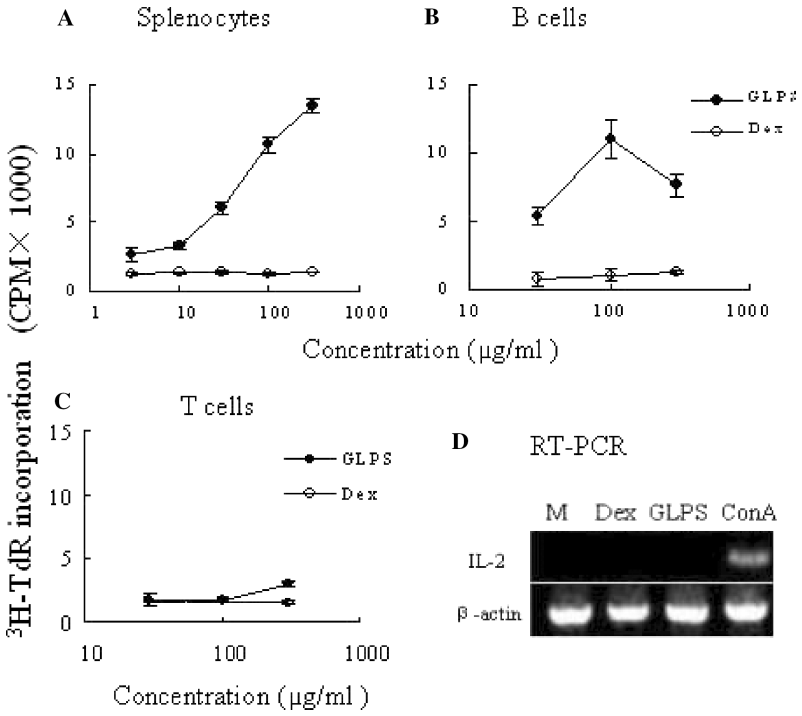

Fig. 1. Selective activation of murine B cells by GLPS. BALB/c mouse splenocytes (A) or fractionated splenic B (B) and T (C) cells were stimulated with GLPS or dextran (Dex) at indicated concentrations for 72 h. [^{3}H]Thymidine was added to the cultures for the last 8 h of incubation and the results are expressed as [^{3}H]thymidine incorporation (CPM). Agarose gel analysis of RT-PCR products for detection of IL-2 mRNA expression by splenocytes after stimulation with or without (M), dextran (Dex), GLPS or ConA is shown in (D).

observed in the culture supernatant of BALB/c mouse splenocytes and purified B cells stimulated with GLPS (Fig. 2). Interestingly, the splenocytes produced significantly more Ig compared with purified B cells, implying a possibility that T cells (or other cells of the splenocyte population) were also involved in GLPS-mediated activation of B cells. However, GLPS stimulation of splenocytes did not induce any detectable expression of IL-2 mRNA (Fig. 1D), therefore excluding the possibility for GLPS to directly activate T cells.

No endotoxin contamination in GLPS

Gram-negative bacterial LPS (i.e., endotoxin) is a typical thymus-independent (TI) Ag capable of activating B cells without T cell help [16,17]. In order to

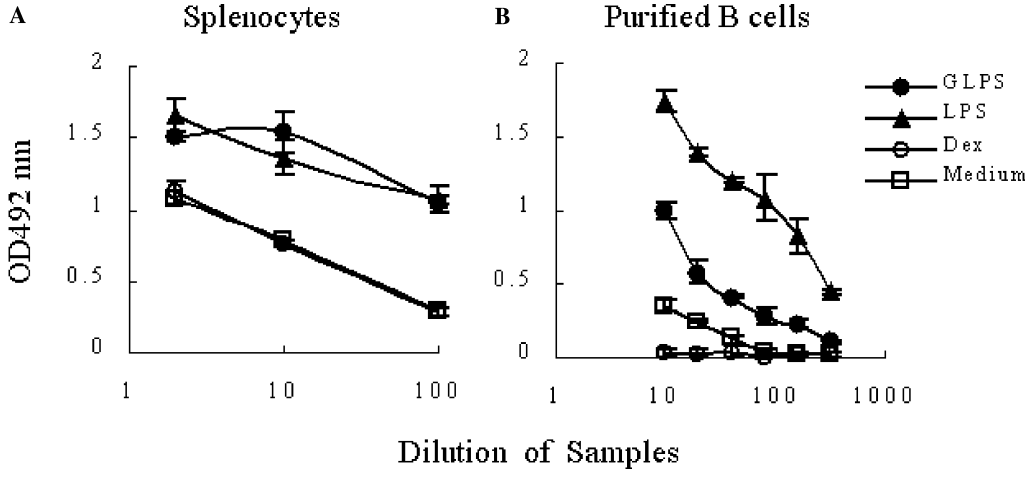

Fig. 2. GLPS-induced Ig production by splenic B cells. BALB/c mouse splenocytes (A) or purified B cells (B) were stimulated with GLPS (100 μg/ml), dextran (100 μg/ml) or LPS (5 μg/ml) for 4 days. Ig concentration in the culture supernatants (serial diluted) was determined using ELISA. The results are expressed as absorbance at 492 nm wavelengths.

Table 1
Quantitation of endotoxin activity in LPS and GLPS[a]

	Concentration (μg/ml)	Endotoxin[b] (Eu/ml)	Eu/C[c]
GLPS	30	0.335	1.12×10^{-2}
LPS	0.01	0.313	31.3

[a] LAL chromogenic assay was performed to determine endotoxin units (Eu) in samples of LPS and GLPS.
[b] A standard curve was prepared for each experiment using standard endotoxin for calculation of Eu in the samples.
[c] Eu/C = Eu (U/ml)/sample concentration (μg/ml).

exclude the possibility that GLPS preparations used in our study had been contaminated with LPS, endotoxin activity in GLPS was measured by LAL chromogenic method and the results are summarized in Table 1. Endotoxin activity in the GLPS preparation was approximately 0.035%, indicating that LPS contamination in GLPS was negligible.

TLR4 and membrane Ig as functional B cell receptors for GLPS

Polysaccharides from various medicinal herbs are able to activate B cells and/or macrophages via TLR4-mediated signaling [15,18–21]. C3H/HeJ mice have a point mutation in their TLR-4 molecule (amino acid 712, localized in the cytosolic domain) that leads to unresponsiveness of their macrophages and B cells to LPS stimulation [22]. In the experiment shown in Fig. 3, splenocytes from BALB/c and C3H/HeJ mice were compared for ability to respond to GLPS stimulation in vitro. Like LPS, GLPS induced vigorous proliferation of splenocytes (B cells) from BALB/c, but not C3H/HeJ, mice, indicating that TLR4 signaling is required for GLPS-mediated B cell activation. This was also supported by the experiment where anti-mouse TLR4 mAb partially inhibited GLPS-induced proliferation of BALB/c mouse splenocytes (Table 2).

In the experiments summarized in Table 2, rabbit anti-mouse Ig Abs (IgG) effectively inhibited GLPS-induced proliferation of BALB/c mouse splenocytes, suggesting that mIg of B cells was also a functional receptor for GLPS. When Abs against TLR4 and mIg were used together, almost complete inhibition of GLPS-induced proliferation by B cells was achieved (Table 2).

Role of TLR4 in GLPS-mediated activation of macrophages

TLR4 is one of the main pattern recognition receptors expressed by macrophages [17,23]. In a previous study, we showed that fluorescence-labeled APS (polysaccharides from *Astragalus membranaceus*) positively stained human and mouse macrophages in a TLR4-dependent manner [15]. Interestingly, GLPS was able to competitively inhibit the binding of fl-APS with mouse peritoneal macrophages as determined by flow cytometric analysis (Fig. 4), implying that GLPS is able to bind directly with TLR4 on macrophage surface. Furthermore, GLPS induced significant IL-1β production by peritoneal macrophages from BALB/c but not C3H/HeJ mice (Fig. 5), also suggesting that GLPS activated macrophages via TLR4.

Identification of GLPS-binding serum proteins

GLPS-, APS-, and dextran-conjugated Sepharose 6B beads were prepared and used to precipitate GLPS-binding proteins in fetal calf serum. Compared with dextran, both APS and GLPS precipitated specific protein bands of approximately 75, 43, and 38 kDa (Fig. 6). In addition, a unique protein band of 31 kDa was present in the GLPS group (Fig. 6), although human and mouse sera did not contain such a GLPS-binding protein (data not shown). To verify if the GLPS-binding serum proteins play any significant role in GLPS-mediated B cell activation, fetal

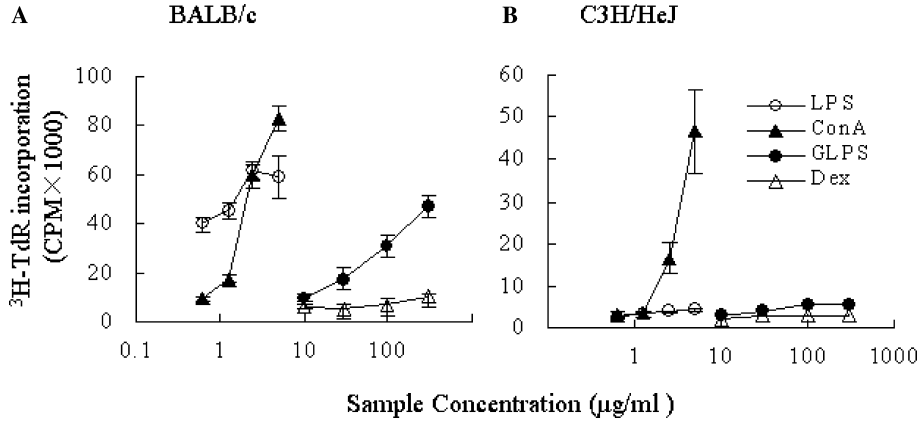

Fig. 3. Unresponsiveness of C3H/HeJ mouse B cells to GLPS stimulation in vitro. Splenocytes from BALB/c (A) and C3H/HeJ (B) mice were stimulated with dextran (Dex), GLPS, LPS or ConA in standard proliferation assays. [^{3}H]Thymidine was added to the cultures for the last 8 h of incubation and the results are expressed as [^{3}H]thymidine incorporation (CPM).

Table 2
Ab inhibition of GLPS-stimulated proliferation of splenocytes[a]

Inhibiting Abs	Stimulators			
	Dex (100 μg/ml)	GLPS (100 μg/ml)	LPS (5 μg/ml)	ConA (5 μg/ml)
No Ab control	17.3 ± 2.5	94.9 ± 9.5	100.6 ± 8.3	144.5 ± 0.5
Rabbit anti-mouse Ig	ND[b]	38.1 ± 7.4 (73.2%)[c]	79.8 ± 13.4 (24.9%)[c]	137.7 ± 5.5 (5.3%)[c]
Rat anti-mouse TLR4	ND	69.2 ± 6.1 (33.0%)	63.2 ± 6.2 (44.9%)	146.0 ± 12.7 (0%)
Rabbit anti-mouse Ig + rat anti-mouse TLR4	ND	22.8 ± 4.0 (92.9%)	66.0 ± 8.0 (41.5%)	139.8 ± 9.1 (3.7%)
Rabbit IgG	ND	92.6 ± 13.2 (2.4%)	100.9 ± 18.9 (0%)	140.1 ± 3.7 (3.5%)
Rat IgG	ND	96.8 ± 2.8 (3.0%)	101.8 ± 19.3 (0%)	149.6 ± 2.4 (0%)
Rabbit IgG + rat IgG	ND	94.7 ± 5.1 (4.0%)	106.0 ± 14.6 (0%)	144.6 ± 5.0 (0%)

[a] BALB/c mouse splenocytes were stimulated with Dex, GLPS, LPS or ConA in the presence, or absence, of rabbit anti-mouse Ig polyclonal Abs and/or rat anti-mouse TLR4 mAb for 72 h. Rabbit IgG and rat IgG were included as controls. [^{3}H]Thymidine was added to the cultures for the last 8 h of incubation and the results are expressed as [^{3}H]thymidine incorporation (CPM × 1000) ± standard deviation. The reading of medium alone group was 18.6 ± 1.51 (CPM × 1000).

[b] ND, not determined.

[c] Percent inhibition, shown in brackets, was calculated as: $(CPM_{without\ Ab} - CPM_{with\ Ab})/(CPM_{without\ Ab})$.

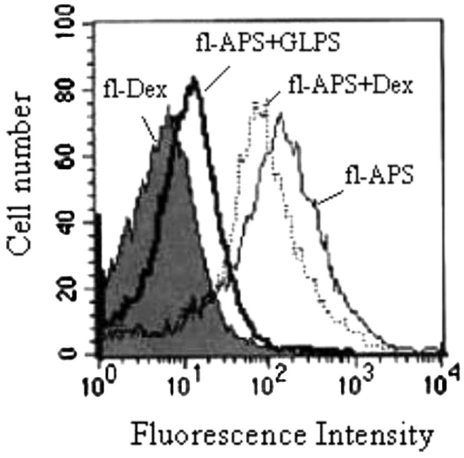

Fig. 4. GLPS inhibition of fl-APS binding to macrophages. BALB/c mouse peritoneal macrophages were stained with fl-APS in the presence, or absence, of GLPS or dextran (Dex) for 1 h at 4 °C and then subjected to flow cytometric analysis.

calf serum was pretreated with GLPS–Sepharose 6B or dextran–Sepharose 6B and then compared for ability to support the proliferation of splenic cells stimulated with GLPS in vitro. As shown in Fig. 7, depletion of GLPS-binding proteins in fetal serum resulted in a moderate reduction in GLPS-mediated proliferative responses of mouse splenocytes.

Other potential cellular receptors for GLPS

In order to further identify cellular proteins capable of specific binding with GLPS, lysate of BALB/c mouse splenocytes or THP-1 cells was precipitated using Sepharose 6B coupled with GLPS, APS or dextran. As illustrated in Figs. 8A and B, a unique protein band of 23 kDa was present in the GLPS groups in silver-stained SDS–PAGE gels (indicated by arrow). Presence of soluble GLPS in THP-1 cell lysate effectively inhibited the precipitation of the 23 kDa molecule by GLPS–Sepharose 6B (Fig. 8C), confirming the specificity of the binding between GLPS and the target protein(s). The 23 kDa protein band was cut out and analyzed for amino acid sequence. As shown in Table 3, the 23 kDa band contained two protein molecules, ribosomal protein S7 and a transcription coactivator, basic region-leucine zipper (bZIP) enhancing factor [24].

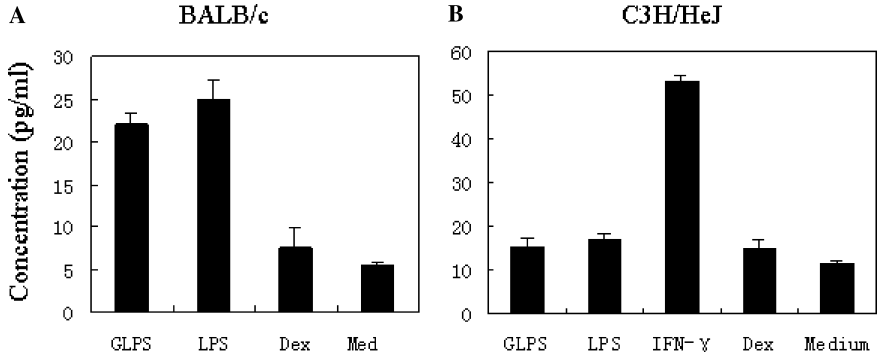

Fig. 5. GLPS-induced IL-1β production by mouse macrophages. BALB/c and C3H/HeJ mouse peritoneal macrophages were treated with GLPS (100 μg/ml), LPS (5 μg/ml), dextran (Dex, 100 μg/ml) or IFN-γ for 48 h. IL-1β concentration (pg/ml) in the culture supernatant was determined by ELISA.

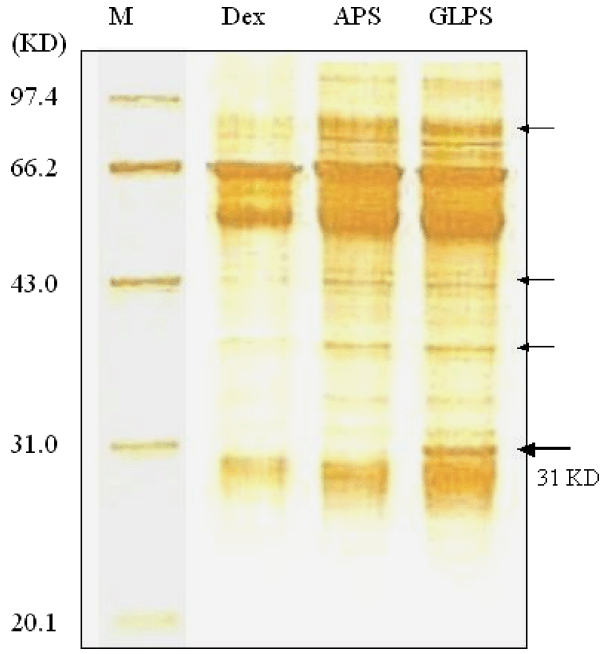

Fig. 6. Identification of GLPS-binding serum proteins. Fetal calf serum was incubated with the Sepharose 6B conjugated with GLPS, APS or dextran (Dex) at 4 °C for 1 h followed by thorough washes. The precipitates were then subjected to SDS–PAGE 12% gel analysis (silver staining). Molecular weight markers (M) were loaded in the left lane.

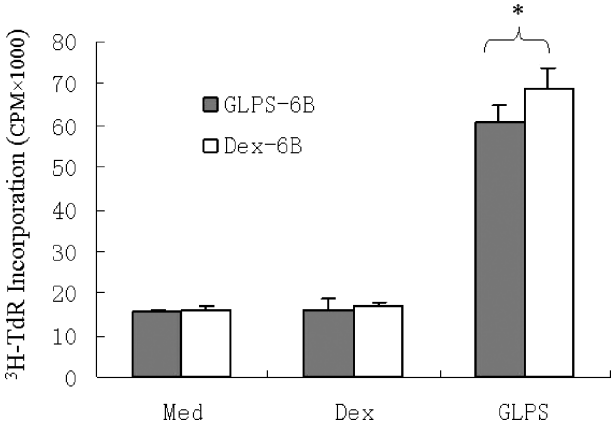

Fig. 7. Effect of depletion of GLPS-binding serum protein in FCS. BALB/c mouse splenocytes were stimulated with, or without (Med), dextran (Dex) or GLPS (GLPS) in RPMI-1640 medium supplemented with fetal calf serum that had been pre-cleared with dextran- (Dex-6B) or GLPS-conjugated Sepharose 6B (GLPS-6B). [^{3}H]Thymidine was added for the last 8 h of the three day culture and the results are expressed as [^{3}H]thymidine incorporation (CPM). *Statistically significant difference between the two groups ($P < 0.05$).

Discussion

Polysaccharides from medicinal materials exhibit diverse immunopotentiating activities [8,10–14,18–21,25–28]. We have shown that APS [15] and GLPS (in this paper) activate mouse B cells and macrophages but not T cells. Polysaccharides from *Phellinus linteus* can stimulate B cells, T cells, and macrophages [8], while lentinan and β(1 → 3)-glucan isolated from *Lentinus edodes* is a stimulator of T cells and macrophages, but not B cells [28]. The relationship between polysaccharide origin, structure, and their immunomodulation activity remains to be further characterized [10,11].

Characteristics of GLPS and APS are compared in Table 4. Both mIg and TLR4 are required for GLPS activation of mouse B lymphocytes (Table 2), whilst APS activates B cells independent of TLR4 [15]. Polysaccharides from several other medicinal herbs are also able to interact with TLR4 [18–22]. For instance, Ando et al. [21] reported that safflower polysaccharides activated NF-κB signaling pathway via TLR4 in macrophages. Polysaccharides from the roots of *Acanthopanax koreanum* and *Platyoden grandiflorum* stimulate B cells through TLR4, CD19, and CD79b [19,25].

In this study, we identified several bovine serum proteins able to bind with GLPS with high affinity, including a 31 kDa protein that binds with GLPS but not APS (Fig. 6). Depletion of GLPS-binding proteins in fetal calf serum resulted in moderate reduction in its ability to support the proliferation of mouse splenocytes stimulated with GLPS (Fig. 7), suggesting that GLPS-binding serum proteins could play an important role in GLPS activation of B lymphocytes, although its mechanism is still unclear. Serum proteins capable of binding with LPS play important roles in stimulating innate immunity in the host. For example, LPS-binding-protein catalyzes LPS monomers from LPS aggregates to other LPS receptor molecule [29]. CD14, RP105, MD-1, and MD2 are also LPS-binding proteins that can function as adaptor molecules mediating interaction between LPS and TLR4 [17,30].

Our coprecipitation experiments revealed ribosomal protein S7 and bZIP enhancing factor (BEF) as GLPS-binding intracellular proteins (Fig. 8, Table 3). It has to be noted, however, that TLR4 was not identified in these experiments. This may be explained by the fact that the S7 and bZIP enhancer are significantly more abundant than TLR4 in cells and are therefore easily identifiable in silver-stained SDS–PAGE gels. More sensitive detection methods (such as [^{35}S]methionine labeling and radioautography) are needed for identification of membrane molecules such as TLR4. It is also possible that the binding affinity between GLPS and these two proteins is much higher than with TLR4. Third, in the lysis buffer, TLR4 might not be able maintain a proper structure for effective binding with GLPS. Nonetheless, the significance of these GLPS-binding cellular proteins for GLPS-mediated immunoregulation merits further investigation. It is possible that, following direct binding with TLR4 on cell surface, GLPS could get internalized by macrophages thereby exerting regulatory function intracellularly. LPS from

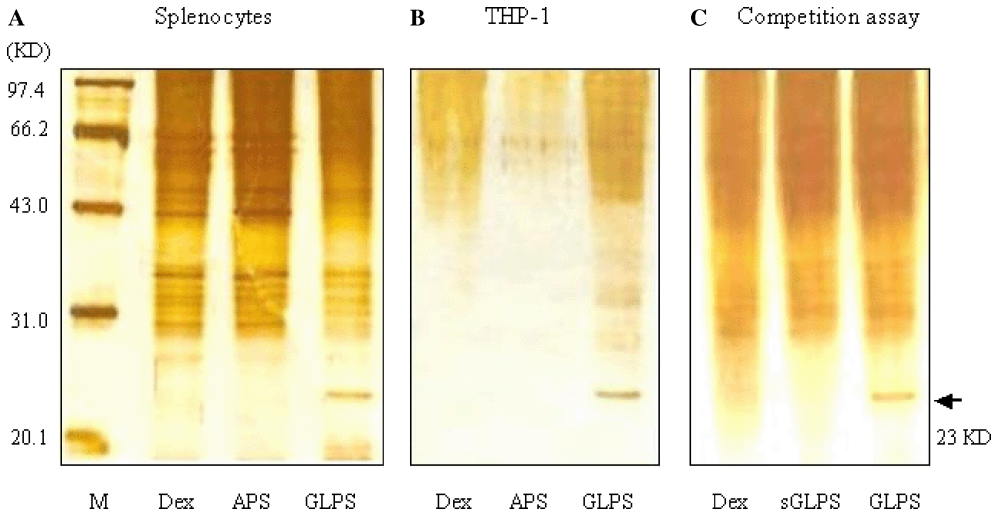

Fig. 8. Identification of GLPS-binding cellular protein(s). Pre-cleared lysates of BALB/c mouse splenocytes (A) and THP-1 cells (B) were incubated with Sepharose 6B coupled with GLPS, APS or Dex at 4 ° for 1 h followed by thorough washes. The precipitates were analyzed using SDS–PAGE 12% gel (silver staining). In (C), soluble GLPS (sGLPS) was added to the lysate of THP-1 cells before precipitation using GLPS–Sepharose 6B (GLPS). Molecular weight markers (M) were loaded in the left lane.

Table 3
Sequence analysis of two GLPS-binding cellular proteins[a]

Partial amino acid sequences of the protein band[b]	Protein molecules identified[c]	GenBank Accession No.	Cellular localization
TLTAVHDAILEDLVFPSEIVGKR	Ribosomal protein S7 (residues 121–143)	AAH02866	Cytoplasm
QQLSAEELDAQLDAYNAR	Transcriptional coactivator (bZIP enhancing factor) (residues 234–251)	NP_005773	Nuclear

[a] The unique GLPS-binding protein band, shown in Fig. 8, was excised and in-gel digested with trypsin for sequence analysis.
[b] Partial sequences (single letter amino acid codes) of the peptides were determined.
[c] Searches of the protein database in GenBank revealed 100% homology between these sequences and the ribosomal protein S7 and bZIP enhancing factor.

Table 4
Comparison of APS and GLPS

	APS[a]	GLPS
Direct binding with TLR4	Yes. Anti-mTLR4 mAb inhibited fl-APS binding to mouse macrophages	Yes. GLPS competitively inhibited fl-APS binding to macrophages (Fig. 4)
Direct binding with mIg of B cells	Yes. Anti-mIg Abs inhibited APS-mediated B cell proliferation and their binding with fl-APS	Yes. Anti-mIg Abs inhibited GLPS-induced B cell proliferation (Table 2)
Other potential cellular receptors	None identified so far (Fig. 8)	7S ribosomal protein and bZIP enhancer (Fig. 8, Table 3)
Unique serum binding protein	75, 43, and 38 kDa proteins in bovine serum (Fig. 6)	A 31 kDa protein in addition to the 75, 43, and 38 kDa proteins in bovine serum (Fig. 6)
B cell activation	Yes. Through mIg, TLR4-independent	Yes. Through both mIg and TLR4 (Table 2)
Macrophage activation	Yes. Induces cytokine production, TLR4-dependent (Fig. 5)	
Dendritic cells	Not determined	Promote maturation and function [14]
T cell activation	Unable (Fig. 1)	

[a] See Ref. [15] for APS function and immune receptors.

Gram-negative bacteria has an intracellular receptor (Nod1) that confers macrophage responsiveness to bacterial infection [31,32]. Ribosome is the site of protein synthesis in all organisms. S7 is one of the two proteins that initiate assembly of the 30S ribosomal protein. It is required for proper folding of 16S ribosomal RNA [33]. BEF functions as a molecular chaperone, promoting the folding of bZIP monomers to dimers and stimulating DNA binding [24]. It is also required for efficient transcriptional activation by bZIP proteins in vivo [24]. It

is thus reasonable to suggest that molecules such as GLPS capable of interacting with S7 and/or BEF have the potential to regulate cell function.

We have demonstrated previously that APS is able to directly bind to mIg and activate B cells in a TLR4-independent manner [15] and also suggested that APS must possess repeating epitopes allowing effective cross-link of mIg on B cell surface. Presumably GLPS shares similar epitopes with APS, as it is also capable of driving the proliferation (Figs. 1 and 3) and Ig production (Fig. 2) by B cells via mIg. In contrast to APS, however, GLPS also requires TLR4 for B cell activation in vitro (Fig. 3, Table 2). It is likely that mIg and TLR4 transduce synergized signals for GLPS-mediated B cell activation, which is also a mechanism used by LPS (and other TI-1 Ags) to activate B lymphocytes [16,17].

Acknowledgments

This work was supported by grants from the National Key Basic Research Program (2001CB510007) and "211" Project of China.

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