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A study on the immune receptors for polysaccharides from the roots of *Astragalus membranaceus*, a Chinese medicinal herb

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Abstract

The immunopotentiating effect of the roots of *Astragalus membranaceus*, a medicinal herb, has been associated with its polysaccharide fractions (*Astragalus* polysaccharides, APS). We herein demonstrate that APS activates mouse B cells and macrophages, but not T cells, in terms of proliferation or cytokine production. Fluorescence-labeled APS (fl-APS) was able to selectively stain murine B cells, macrophages and a also human tumor cell line, THP-1, as determined in flow cytometric analysis and confocal laser scanning microscopy. The specific binding of APS to B cells and macrophages was competitively inhibited by bacterial lipopolysaccharides. Rabbit-anti-mouse immunoglobulin (Ig) antibody was able to inhibit APS-induced proliferation of, and APS binding to, mouse B cells. Additionally, APS effectively stimulated the proliferation of splenic B cells from C3H/HeJ mice that have a mutated TLR4 molecule incapable of signal transduction. These results indicate that APS activates B cells via membrane Ig in a TLR4-independent manner. Interestingly, macrophages from C3H/HeJ mice were unable to respond to APS stimulation, suggesting a positive involvement of the TLR4 molecule in APS-mediated macrophage activation. Monoclonal Ab against mouse TLR4 partially inhibited APS binding with macrophages, implying direct interaction between APS and TLR4 on cell surface. These results may have important implications for our understanding on the molecular mechanisms of immunopotentiating polysaccharides from medicinal herbs.

Keywords: *Astragalus membranaceus*; Polysaccharides; Receptor; TLR4; Immune cells

Polysaccharides from natural sources are a class of macromolecules that can profoundly affect the immune system and therefore have the potential as immunomodulators with wide clinical applications [1]. For example, polysaccharides purified from certain mushrooms have anti-tumor activities via macrophage activation [2,3]. β-Glucans (glucose polymers) from the cell walls of plants, fungi, and bacteria exhibit anti-tumor and anti-infection activities [4–6]. Polysaccharides from various traditional medicinal herbs have been shown to be immunopotentiating both in vivo as well as in vitro [7–19]. The roots of *Astragalus membranaceus* (Huangqi) are amongst the most popular health-promoting herbs in China, their use dates back more than 2000 years, and were recorded in *Shen Nong's Materia Medica* written in the Han dynasty. Scientific investigation in the last two decades has revealed much insight into the pharmacological functions of different components of Huangqi, especially its polysaccharide fractions [18–21]. However, molecular mechanisms for the immunobiological function of *Astragalus* polysaccharides (APS) are far from clear. This study was designed to identify and characterize APS-binding cellular receptors expressed by immune cells.

Materials and methods

Animals and antibodies. Female BALB/c mice were purchased from the Experimental Animal Division of Peking University Health Sciences Center, Beijing, China. Female C3H/HeJ mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of

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Science, China. All animals were maintained in animal facility of the Department of Immunology. Monoclonal rat Abs (IgG) against mouse TLR4 or RP105 were provided by Invivogen (San Diego, USA). Mouse anti-human CD14 mAb was from R&D System (USA). Polyclonal rabbit IgG Abs against mouse Ig and IgG from un-immunized rabbits or rats were prepared in this laboratory.

Preparation of APS. Sliced roots of *A. membranaceus* were extracted three times with boiling water. The supernatant was applied to a DEAE–Sephacel (2.6×100 cm) column and bound materials were eluted with a linear gradient of NaCl (0–2 mol/L NaCl). Carbohydrate concentration in the fractions was determined using the phenol-sulfuric acid method. The fractions containing carbohydrate were pooled and precipitated three times with ethanol. The resultant polysaccharide extract was dialyzed against several changes of water and then lyophilized. Carbohydrate content of the final product was 97%, protein and nuclear acid contamination in the extract was negligible (absorbance at 280 and 260 nm wavelengths close to zero). The molecular weight of the extract was about $3500 \sim 1.58 \times 10^6$ as determined by gel filtration method. Sugar compositional analysis by TLC and gas chromatography showed that it consisted of Rha, Xyl, Glc, Gal, Man, and Fru in the molar ratios of Rha:Xyl:Glc:Gal:Man:Fru = 4.9:4.7:8.3:122.2:2.2:3.1.

Cell culture medium. All cells were cultured in R10: RPMI-1640 (Hyclone, USA) supplemented with 10% (v/v) FCS (Hyclone, USA), penicillin/streptomycin (100 U/ml), L-glutamine (2 mM), and 2-ME (5×10^{-5} M). Tumor cell lines Raji, THP-1, and CCRF-CEM were from ATCC, USA, and maintained in this laboratory.

Preparation of fluorescence-labeled APS and dextran. This was essentially the same as described by Glabe et al. [22]. Briefly, APS or dextran (MW 70,000, Sigma, USA) solution (20 mg/ml in 1 ml water) was mixed with 0.2 ml CNBr (50 mg/ml in H_2O), under magnetic stirring, and maintained at pH 11 for 15 min by addition of 0.2 M NaOH. After dialysis against sodium borate buffer at pH 8.0 for 20 h, the CNBr-activated APS or dextran was mixed with 2 mg fluoresceinamine (Sigma, USA) for 10 h in a volume less than 4 ml. The resultant fluorescence-labeled polysaccharide was separated from the free fluoresceinamine by gel filtration on a Sephadex G-50 (Pharmacia, USA) column (2×40 cm). Elution fractions (3 ml/tube) were collected and concentration of fluoresceinamine was determined by absorbance at 440 nm wavelength. A standard curve was prepared using serial diluted fluoresceinamine in PBS (from 2 to 200 μM). The standard curve was constructed using serial diluted dextran.

Preparation of mouse splenocytes, splenic T, B, adherent 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 complete R10.

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 at 37 °C in a CO_2 incubator. The non-adherent cells were collected, washed twice in PBS, and then incubated with MACS magnetic microbeads coated with rat anti-mouse CD19 mAb (Myltenyi Biotec, Italy) at a density of 10 μl antibody 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 antibody (IgG) and T cells were reacted with FITC-anti-mouse CD3 antibody for 30 min at 4 °C. The purities of B cells and T cells in two parts were greater than 95% by flow cytometry analysis. Adherent cells (mostly macrophages) were washed twice with PBS and collected by pipeting up and down.

In order to obtain mouse peritoneal macrophages, BALB/c and C3H/HeJ mice were treated 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 ice-cold PBS.

Proliferation assays. Freshly prepared 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/well in the presence or absence of different concentrations of LPS, APS, fl-APS or dextran. The cultures were incubated at 37 °C and 5% CO_2 for 3 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 then 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).

RT-PCR detection of IL-2 mRNA expression by T cells. BALB/c mouse spleen cells were cultured in 24-well plate in the presence or absence of dextran, APS (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) and then 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 1 min, and 72 °C 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 amplified products at 307 and 540 bp, respectively.

Cytokine production by peritoneal macrophages. Mouse peritoneal macrophages were cultured with dextran, APS (100 μg/ml) or LPS (5 μg/ml) for 40 h. Concentrations of IL-1β and TNF-α in the supernatant were determined using ELISA kits provided by Pharmingen (USA) and Sigma (USA), respectively.

Fluorescence staining, FACS analysis, and confocal laser scanning microscopy. Cell suspensions prepared as above were centrifuged and the cell pellets (1×10^6/tube) were incubated, after resuspension, with 50 μl fl-dextran or fl-APS (150 μg/ml) for 1 h at 4 °C The cells were washed three times in PBS and resuspended in 1 ml staining buffer (PBS supplemented with 0.1% NaN_3 and 0.2% BSA) for flow cytometric analysis on a FACS Caliver (Becton–Dickinson). For confocal laser scanning microscopy, the stained THP-1 cells were spread on a glass slide, fixed and observed using a Leica TCS SP2 microscope (Germany). In some experiments, the mouse splenic adherent cells, purified B lymphocytes, THP-1 cell line, and peritoneal macrophages were treated with 5 μg/ml trypsin (Gibco, USA) in 1 ml PBS for 20 min at 37 °C before staining.

Laminarin, a low molecular weight neutral β-glucan polymer, was purchased from Sigma (USA). Oligomannose was provided by Dr. D.X. Fu, Proceeding and Engineering Institute of Chinese Academy of Sciences (Beijing, China). To perform inhibition assays, the cells (1×10^6/tube) were treated with dextran, laminarin, oligomannose or LPS (Sigma, USA) at 1 mg/ml or Abs at 400 μg/ml, for 1 h 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.

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

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 was performed using Student's t test. Significance was defined as a P value of $<0.05\%$.

Results

Fluorescence labeling of APS and dextran

In order to be able to identify cells expressing specific receptor(s) for APS, APS and dextran were conjugated with fluoresceinamine using the CNBr-activation method as described in Materials and methods. The resultant fluorescence-labeled APS (fl-APS) and dextran (fl-dextran) were separated from free fluoresceinamine in the labeling mixture by filtration on a Sephadex G-50 column. All fractions were monitored for carbohydrate and fluoresceinamine concentrations. As shown in Fig. 1, a small amount of fluoresceinamine co-eluted with polysaccharides, while free fluoresceinamine eluted much more slowly. Fluorescence-labeled polysaccharide fractions were mixed, adjusted to 500 μg/ml, and stored at −20 °C for future use. The concentration of fluoresceinamine in the fl-APS and fl-dextran preparations was approximately 5–10 μg/ml.

Activation of B lymphocytes and macrophages by APS in vitro

As illustrated in Fig. 2A, APS induced significant proliferation of BALB/c mouse splenocytes in vitro. In addition, fl-APS was equally effective in stimulating splenocytes in the same experiments, suggesting that conjugation of APS with fluoresceinamine did not significantly alter its immunobiological activity.

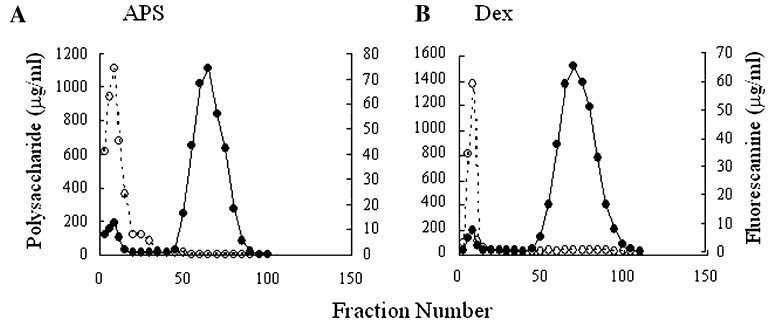

Fig. 1. Polysaccharide labeling with fluoresceinamine. CNBr-activated APS (A) and dextran (B) were reacted with fluoresceinamine for 10 h at room temperature. The mixtures were fractionated on a Sephadex-G 50 column, concentrations (μg/ml) of fluoresceinamine (●) and carbohydrate (○) in each fraction were determined.

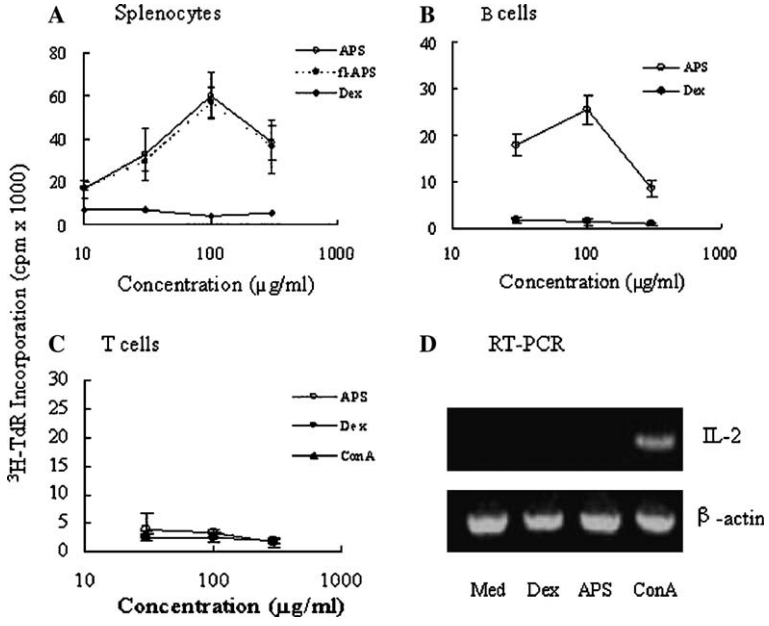

Fig. 2. APS-induced proliferation of mouse splenic cells. BALB/c mouse splenocytes (A) or fractionated splenic B (B) and T (C) cells were stimulated with APS, fl-APS or dextran at indicated concentrations for 72 h. [^{3}H]Thymidine was added to the cultures for the last 8 h of incubation and the results are expressed as [^{3}H]thymidine incorporation (CPM). IL-2 and β-actin mRNA expression in splenocytes after stimulation with APS, dextran, ConA or medium alone (Med) was detected by RT-PCR. The PCR products were analyzed by agarose gel electrophoresis (D).

Table 1
Cytokine production by peritoneal macrophage stimulated with APS[a]

Cytokine	APS	LPS	Dex	Medium
TNF-α	0.15 ± 0.015	0.80 ± 0.049	0.03 ± 0.005	0.04 ± 0.005
IL-1β	0.18 ± 0.003	0.30 ± 0.004	0.08 ± 0.003	0.10 ± 0.006

[a] Mouse peritoneal macrophages were treated with APS (100 μg/ml), LPS (5 μg/ml), and dextran (100 μg/ml) for 40 h. The culture supernatant was then collected and concentrations of IL-1β and TNF-α were determined by ELISA. The results are expressed as absorbance at $OD_{492\,nm}$ wavelength.

Subsequent assays using purified mouse splenic B and T cells confirmed that the former were the main responders to APS stimulation in vitro (Figs. 2B and C). The fact that APS was unable to induce IL-2 mRNA expression by splenocytes also excluded the possibility for APS to directly activate T cells (Fig. 2D).

When peritoneal macrophages of BALB/c mice were stimulated with APS or dextran for 40 h, significantly more IL-1β and TNF-α were found in the culture supernatant of the APS group (Table 1), suggesting that APS was also able to activate murine macrophages.

Selective binding of APS to murine macrophages, B cells, and human THP-1 cell line

To demonstrate the specific binding of APS to target cells, freshly prepared BALB/c mouse macrophages, splenic T cells and B cells were stained with fl-APS or fl-dextran (for 1 h at 4 °C) and then subjected to flow cytometric analysis. Consistence with the functional study results, mouse splenocytes, peritoneal macrophages, splenic adherent cells (mostly macrophages), and B cells, but not T cells, were positively stained by fl-APS (Figs. 3A–E). Pre-treatment with trypsin significantly decreased the binding of APS to B cells and macrophages (Figs. 3F–H). When fl-APS-stained peritoneal macrophages were incubated at 4 or 37 °C for up to 6 h, their fluorescence intensity remained almost unchanged (Fig. 4), suggesting that the binding between APS and cell surface receptors was quite stable.

We next examined human tumor cell lines of T, B cell and monocytic origins for ability to bind with fl-APS. THP-1 (monocyte/macrophage line), but not Raji (B lymphoma line) and CCRF-CEM (T leukemia line), cells were positively stained by fl-APS (Figs. 5A, B, and D). Pretreatment of THP-1 cells with trypsin also abolished their ability to bind with fl-APS (Fig. 5C). Specific binding of fl-APS to THP-1 cells was also observed under a confocal laser-scanning microscope (Fig. 5E).

Competitive inhibition of fl-APS binding to cells by LPS

The immunostimulating activity of APS was similar (albeit much less potent) to that of Gram-negative bacterial lipopolysaccharide (LPS), which is a typical thymus-independent (TI) Ag capable of inducing B cell activation without T cell help and also the production of

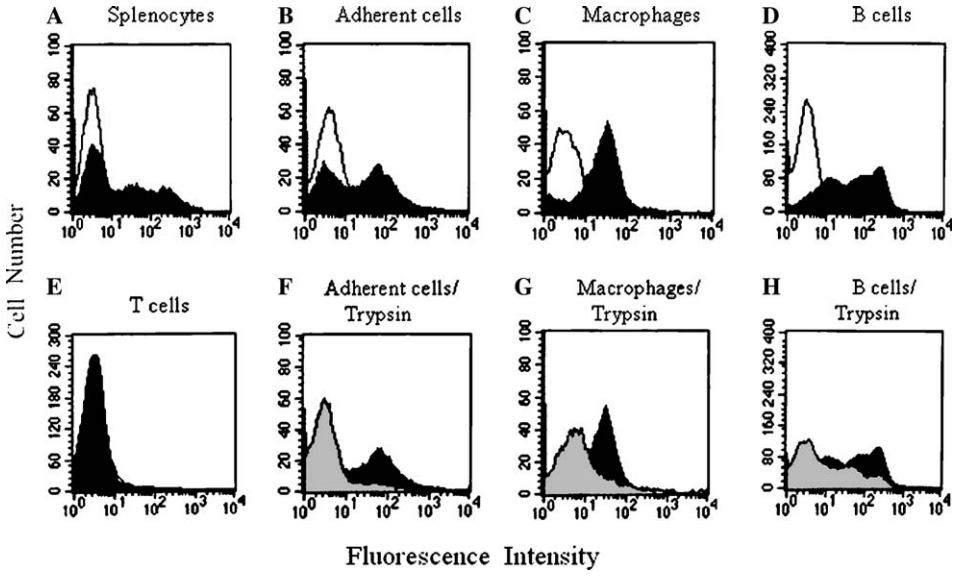

Fig. 3. Selective binding of fl-APS to mouse B cells and macrophages. BALB/c mouse splenocytes (A), splenic adherent cells (B), peritoneal macrophages (C), splenic B (D), and T cells (E) were stained with fl-dextran (unshaded histograms) or fl-APS (dark histograms) for 1 hr and then subjected to flow cytometric analysis. Pretreatment of splenic adherent cells (F), peritoneal macrophages (G), and B cells (H) with trypsin (gray histograms) significantly decreased their ability to bind to fl-APS.

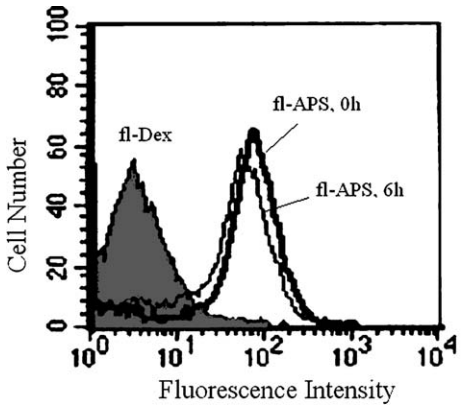

Fig. 4. Stable binding of APS with mouse macrophages. BALB/c mouse peritoneal macrophages were treated with fl-dextran or fl-APS for 1 h followed by thorough washes. The cells were then incubated in a CO_2 incubator at 37 °C for 0 or 6 h before flow cytometric analysis. There was no significant decrease in the fluorescence intensity of fl-APS-stained macrophages after 6 h incubation.

cytokines (e.g., IL-1β and TNF-α) by macrophages [23,24]. It was therefore of importance to exclude the possibility that APS preparations used in our study had been contaminated with LPS. As shown in Table 2, endotoxin activity in APS was approximately 0.15‰ of that in LPS, indicating that LPS contamination in APS, if any, was negligible.

To address the question whether APS and LPS shared the same cellular receptor(s), LPS was used to inhibit the binding of fl-APS to target cells in flow cytometric analysis. Fig. 6 shows that LPS effectively, although not completely, inhibited fl-APS binding to THP-1 cells and mouse splenic B cells. In contrast, dextran, oligomannose, and laminarin did not exhibit any significant inhibitory effect in similar experiments (data not shown).

Membrane Ig of B lymphocytes as functional receptors for APS

In the experiment shown in Fig. 7, rabbit anti-mouse Ig antibody (IgG) significantly inhibited APS-induced proliferation of BALB/c mouse splenocytes, while the same Abs did not affect ConA-induced T cell proliferation. Furthermore, rabbit anti-mouse Ig antibody partially blocked APS binding to splenic B cells but not macrophages (Fig. 8). These results provided concrete evidence for mIg as an APS-binding receptor in B cells.

TLR4 is not involved in APS-induced B cell activation

C3H/HeJ mice have a point mutation in their TLR-4 molecule (amino acid 712, localized in the cytosolic domain) that leads to the unresponsiveness of their macrophages and B cells to LPS stimulation [25].

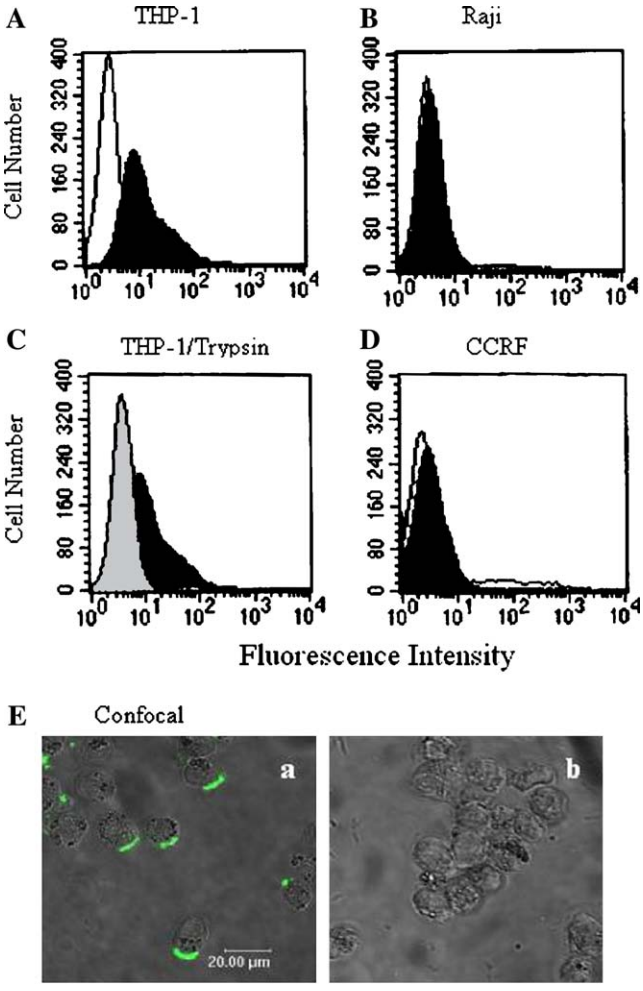

Fig. 5. Selective binding of fl-APS to THP-1 cells. THP-1 (A), Raji (B), and CCRF-CEM (D) cells were stained with fl-dextran (unshaded histograms) or fl-APS (dark histograms) for 1 h for flow cytometric analysis. When THP-1 cells were pretreated with trypsin (gray histograms), their binding ability to fl-APS was abolished (C). THP-1 cells stained with fl-APS (a) or fl-dextran (b) were also observed using a confocal laser-scanning microscope. Micrographs in (E) represent merged images obtained through the field light and green fluorescence channels.

Splenocytes from BALB/c and C3H/HeJ mice were tested for ability to respond to APS stimulation in proliferation assays. As expected, LPS induced vigorous proliferation of splenocytes (B cells) from BALB/c, but

Table 2
Quantitation of endotoxin activity in LPS and APS[a]

	Concentration (μg/ml)	Endotoxin (Eu/ml)	Eu/C
APS	30	0.014	4.67×10^{-4}
LPS	0.01	0.313	31.3

[a] LAL chromogenic assay was performed to determine LPS (endotoxin) activity in samples of LPS (0.01 μg/ml) and APS (30 μg/ml). A standard curve was prepared for each experiment using the standard endotoxin. Thus, LPS contamination in APS is 0.015‰.

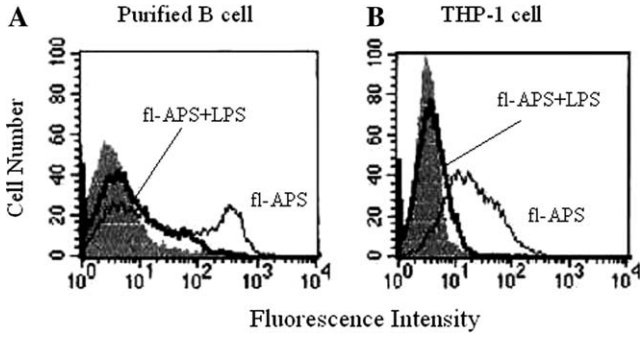

Fig. 6. LPS inhibition of APS binding to cells. Freshly prepared BALB/c mouse B cells (A) and THP-1 cells (B) were stained with fl-APS or fl-dextran (gray histograms) in the presence, or absence, of LPS at 1 mg/ml for 1 h at 4 °C for flow cytometric analysis. LPS significantly inhibited the binding of APS with target cells.

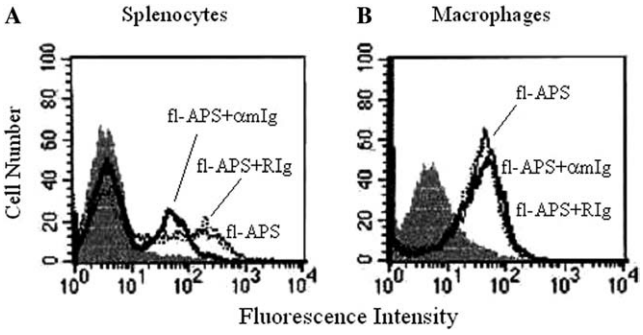

Fig. 8. Inhibition of fl-APS binding to mouse B cells by anti-mouse Ig Ab. BALB/c mouse splenocytes (A) and peritoneal macrophages (B) were treated with fl-dextran (shaded histograms) or fl-APS in the presence, or absence, of rabbit-anti-mouse Ig Ab (αmIg) or control rabbit IgG (RIg).

not C3H/HeJ, mice (Fig. 9). By contrast, splenocytes from both strains of animals responded equally well to APS stimulation (Fig. 9), suggesting that TLR4-mediated signaling pathway was not involved (or at least not essential) in APS activation of murine B cells.

Role of TLR4 in APS-mediated activation of macrophages

In order to verify if TLR4 was required for APS activation of macrophages, peritoneal macrophages from C3H/HeJ mice were incubated with APS, LPS, dextran or IFN-γ for 40 h and then assayed for TNF-α concentration in their culture supernatant. As shown in Fig. 10, C3H/HeJ mouse macrophages (expressing the mutant TLR4) did not respond to APS stimulation in terms of TNF-α production. Given that APS was able to activate macrophages from BALB/c mice (Table 1), it is likely that TLR4 plays an important role in APS-mediated macrophage activation. Furthermore, mAb against mouse TLR4 significantly inhibited binding of fl-APS with peritoneal macrophages of BALB/c mice (Fig. 11), suggesting direct interaction between APS and TLR4 on cell surface.

Discussion

Apparently APS can bind, with intermediate to high affinity, to mIg expressed by a significant proportion of B cell clones in the peripheral B cell repertoire (Figs. 3D and 6A). The broad distribution of fluorescence intensity in fl-APS-stained B cells reflects their polyclonal nature (Figs. 3D and 6A). In 8–10-week-old mice, most of their peripheral B cells are in a resting stage, only a very small proportion (less than 5%) exists as lymphoblasts or plasma cells. It is thus unlikely that the broad distribution of fluorescence intensity in fl-APS-stained B cells depended upon the maturity (activation stages) of B cells. The inability of fl-APS to stain Raji cells (Fig. 5B) can be explained by the fact that Raji cells are monoclonal and their mIg might have low affinity for APS. Our results demonstrate that APS is able to bind with mIg and also induce the proliferation (Fig. 2) and

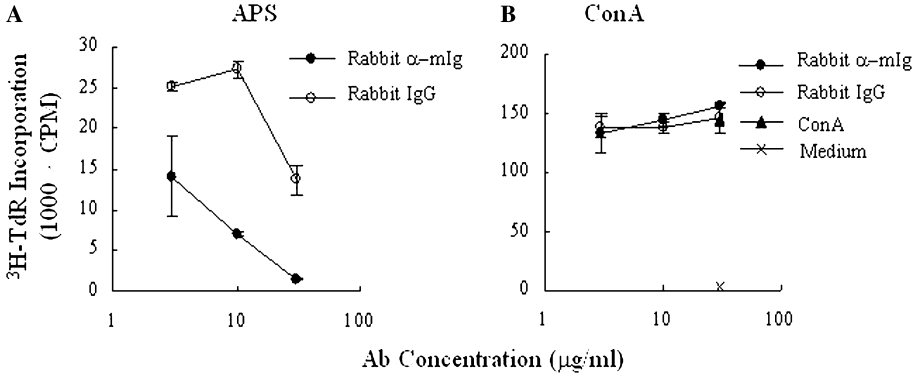

Fig. 7. Anti-mIg Ab-mediated suppression on APS-induced B cell proliferation. BALB/c mouse splenocytes were stimulated with APS (A) or ConA (B) in the presence or absence of rabbit-anti-mouse Ig Ab (IgG) or control rabbit IgG for 72 h. [^{3}H]Thymidine was added to the cultures for the last 8 h of incubation and the results are expressed as [^{3}H]thymidine incorporation (CPM).

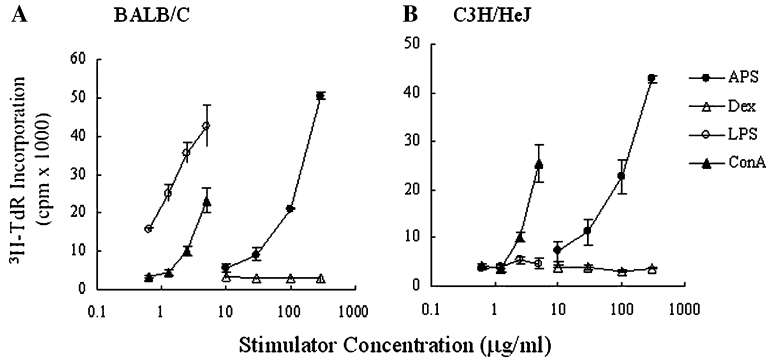

Fig. 9. Differential responses of splenocytes from C3H/HeJ mice to stimulation with APS and LPS in vitro. Splenocytes from BALB/c (A) or C3H/HeJ (B) mice were stimulated with dextran, APS, LPS or ConA in standard proliferation assays. [^{3}H]Thymidine was added to the cultures for the last 8 h of incubation and the results are expressed as [^{3}H]thymidine incorporation (CPM). C3H/HeJ mouse splenocytes did not respond to LPS stimulation but responded well to APS activation.

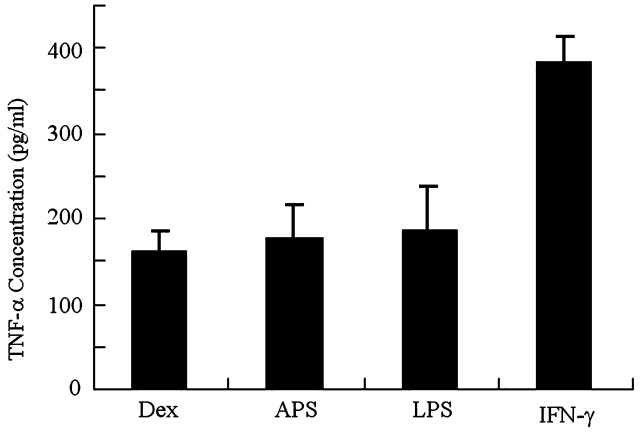

Fig. 10. Hyporesponsiveness of C3H/HeJ mouse macrophages to APS stimulation in vitro. Peritoneal macrophages from C3H/HeJ mice were stimulated with dextran, APS or γ-IFN for 40 h. Concentration of TNF-α in the culture supernatant was determined using ELISA kit. The results are expressed as concentration of TNF-α (pg/ml).

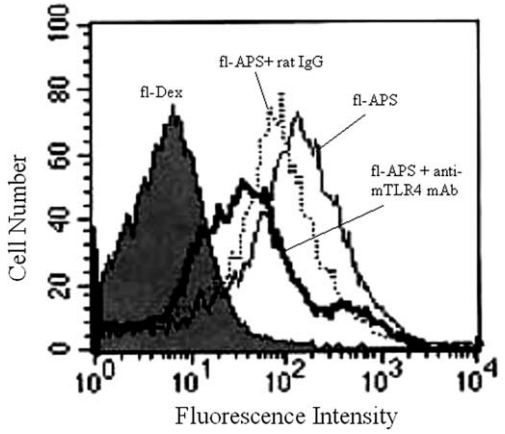

Fig. 11. Inhibition of fl-APS binding to macrophages by anti-TLR4 mAb. BALB/c mouse peritoneal macrophages were treated with rat IgG or rat mAb against mouse TLR4 for 30 min at 4 °C followed by fl-APS staining. Cells treated with fl-dextran alone were included as control.

Ig production of murine B cells (data not shown). LPS relies on both mIg and TLR4 molecules for B cell activation, which may transduce synergized signals for more efficient activation [24,26]. Because APS is able to activate B cells in the absence of TLR4 signaling (Fig. 9), it must be particularly effective in cross-linking mIg on the B cell surface. The ability of LPS to inhibit APS binding to B cells (Fig. 6A) suggests that the polysaccharides of LPS share similar epitopes with APS. Polysaccharides found in several other medicinal herbs such as the roots of *Acanthopanax koreanum*, *Acanthopanax senticosus*, *Platyloden grandiflorum*, and *angelan* are also able to stimulate B cells [12–16]. These polysaccharides activate B cells through TLR2, TLR4, and also CD19 and CD79b [12–16]. The involvement of CD19 and CD79b implies the possibility that these polysaccharides could also bind directly with mIg of B cells.

Potential macrophage receptors for APS are listed in Table 3. The ability of LPS to competitively inhibit the binding of APS to THP-1 cells (Fig. 6B) and macrophages (not shown) suggests that they may share the same receptor(s) expressed by macrophages. Macrophages from BALB/c mice responded to APS stimulation by producing increased amount of IL-1β and TNF-α (Table 1), however macrophages from C3H/HeJ mice did not respond to APS stimulation in vitro (Fig. 10), suggesting TLR4 (or receptor complex associated with TLR4) as one of the candidate APS receptors. The inhibition of fl-APS binding with macrophages by anti-TLR4 mAb (Fig. 11) also supports this notion. Polysaccharides from several other medicinal herbs are able to activate macrophages through TLR-4-dependent pathway [12,16,17]. Ando et al. [17] reported that safflower polysaccharides activated the transcription factor NF-κB signaling pathway via TLR4 in macrophages. We have also found that polysaccharides from *Ganoderma lucidum* (Lingzhi) relied on TLR4 for its macrophage stimulation activity (manuscript in preparation). LPS typically consisted of an essentially invariant lipid

Table 3
Possible receptors for APS and LPS expressed by B cells and macrophages

Receptors	B cells	Macrophages
mIg	High expression [24]	No expression
	Low to high affinity for APS. Anti-mIg Abs blocked APS binding with B cells (Fig. 8A) and inhibited APS-induced proliferation of B cells (Fig. 7A)	
TLR4	Low expression [29]	High expression [29,30]
	High affinity for LPS lipid A [26], low affinity for APS? Anti-TLR4 mAb blocked APS binding macrophages (Fig. 11). LPS competitively inhibited APS binding with target cells (Fig. 6)	
ARXs	No expression?	High expression?
	Intermediate affinity for APS and polysaccharides of LPS	
Mannose receptor	No expression [31]	High expression [31]
	No or low affinity for APS. Mannose and mannan did not inhibit (not shown)	
CD14	No or low expression [32]	High expression [33]
	Low affinity for APS. Anti-CD14 mAb did not inhibit APS binding to THP-1 (not shown)	
RP105	High expression [34]	No expression [34]
	Low affinity for APS. Anti-RP105 mAb did not inhibit APS binding with B cells (not shown)	
β-Glucan receptor	No or low expression [6,35–37]	High expression [6,35–37]
	No or low affinity for APS. Laminarin did not significantly inhibit its binding with THP-1 cells or macrophages (not shown)	

A, a non-repeating "core" oligosaccharide, and a distal polysaccharide (or O-antigen) [24]. It has been demonstrated that the lipid A moiety of LPS is a high affinity ligand for TLR4 [26]. There is also evidence, however, showing that the polysaccharide portion plays an indispensable role in LPS-induced cell activation via TLR4 [27]. Polysaccharides may employ molecules such as CD14, RP105, MD1 or MD2 as a bridge to indirectly interact with TLR4 [28–30,32]. In our experiments, mAbs against CD14, RP105, and MD2 did not significantly inhibit the binding of APS to THP-1 cells and/or B cells (data not shown). Our competitive inhibition assays using various sugars excluded mannose receptor, CR3 (CD11b/CD18), lactosylceramide, dectin-1 as the main APS receptor(s) (data not shown). However, the existence of a TLR4-unrelated APS recognition receptor (ABR-X, Table 3) on macrophage surface is likely, because anti-TLR4 mAb only showed partial inhibition of fl-APS binding (Fig. 11). Ongoing experiments in our laboratory will thoroughly address this question.

Macromolecules capable of activating innate immune system are of great value both in basic research and also therapeutic applications. For more than a century LPS stood as the archetypal agent capable of activating the innate immune system. However, the application of LPS as an immunopotentiating adjuvant has been hampered by its toxicity associated with its lipid A, which is responsible for induction of fever (causes neutrophils to release pyrogen), initiation of both complement and blood coagulation cascades and possibly endotoxic shock. APS exhibits similar (albeit less potent) immunostimulating effect and is essentially nontoxic. When BALB/C mice were injected i.v. with 200 μg LPS or fivefold more of APS, 50% of the LPS group died within 12 h whilst none of the animals in the APS group showed any signs of ailment (data not shown). These qualities of APS make it an ideal candidate as an immunomodulator agent in humans.

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