Lycium barbarum polysaccharide LBPF4-OL may be a new Toll-like receptor 4/MD2-MAPK signaling pathway activator and inducer


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Recognition of the utility of the traditional Chinese medicine Lycium barbarum L. has been gradually increasing in Europe and the Americas. Many immunoregulation and antitumor effects of L. barbarum polysaccharides (LBP) have been reported, but its molecular mechanism is not yet clear. In this study, we reported that the activity of the polysaccharide LBPF4-OL, which was purified from LBP, is closely associated with the TLR4–MAPK signaling pathway. We found that LBPF4-OL can significantly induce TNF-α and IL-1β production in peritoneal macrophages isolated from wild-type (C3H/HeN) but not TLR4-deficient mice (C3H/HeJ). We also determined that the proliferation of LBPF4-OL-stimulated lymphocytes from C3H/HeN mice is significantly weaker than that of lymphocytes from C3H/HeJ mice. Furthermore, through a bio-layer interferometry assay, we found that LPS but not LBPF4-OL can directly associate with the TLR4/MD2 molecular complex. Flow cytometry analysis indicated that LBPF4-OL markedly upregulates TLR4/MD2 expression in both peritoneal macrophages and RAW264.7 cells. As its mechanism of action, LBPF4-OL increases the phosphorylation of p38-MAPK and inhibits the phosphorylation of JNK and ERK1/2, as was observed through Western blot analysis. These data suggest that the L. barbarum polysaccharide LBPF4-OL is a new Toll-like receptor 4/MD2-MAPK signaling pathway activator and inducer.

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1. Introduction

Lycium barbarum is an important traditional Chinese herbal medicine for the promotion of health and longevity and as a food supplement [1,2]. In recent years, the health care functions of L. barbarum are gradually gaining the interest of the US and Europe [3]. Modern medicine found that the polysaccharide of L. barbarum (LBP) is the main active ingredient of L. barbarum. Many beneficial health effects of LBP, such as immune regulation, anti-stress, anti-colon cancer, neuroprotective, and anti-diabetic effects, have been reported [4–12]. Several studies also found that LBP exhibit myocardial protection, eye protection, anti-radiation damage, and anti-aging effects [4–12]. However, the molecular mechanism of LBP has not yet been elucidated. LBP (100 mg/mL) can increase the co-expression of I-A/I-E and CD11c and the secretion of IL-12 p40 in bone marrow-derived dendritic cells (BMDCs), which suggests that LBP are capable of promoting both the phenotypic and functional maturation of murine BMDCs in vitro [13]. LBP can also up-regulate the DC expression of CD40, CD80, CD86, and MHC class H molecules, down-regulate the uptake of Ag by DCs, enhance the co-stimulator activity of DCs, and induce IL-12p40 and p70 production. Furthermore, LBP induce the development of an enhanced Th1 response, and LBP-treated DCs exhibited enhanced Th1 and Th2 responses in vitro and in vivo. Several studies have provided a rationale for the use of LBP under various clinical conditions to enhance host immunity and suggest the use of LBP as a potent adjuvant for the design of DC-based vaccines [14]. We previously found that macrophages, rather than T and B cells, are the principal immunostimulatory target cells of the L. barbarum polysaccharide LBPF4-OL. All of these results suggest that macrophages and dendritic cells are the primary target cells of LBP [15].

At present, TLR4/2 is generally believed to be the main receptor of polysaccharides. A Japanese scholar first reported that safflower polysaccharides activate the transcription factor NF-κB via Toll-like receptor 4 and induce cytokine production [16]. More recently, South Korean and Chinese scholars began to report that the Toll-like receptor-mediated activation of B cells and macrophages by polysaccharides isolated from the cell culture of Acanthopanax senticosus and polysaccharides from Dioscorea batatas induce tumor necrosis factor-alpha secretion via Toll-like receptor 4-mediated protein kinase signaling pathways [17,18]. Anti-TLR4 monoclonal antibody blocking and the use of cells from TLR4-defective mice are two mainstream methods for revealing the molecular mechanism of active polysaccharides, but these methods do not directly indicate whether the polysaccharide is a ligand of TLR4. A
large number of studies suggest that several polysaccharides can significantly activate the TLR4–MAPK signaling pathway and induce TNF-α, IL-1β, and IFN-α/β production. However, it is currently unclear whether the LBP activity is related to the TLR4–MAPK signaling pathway and whether LBP can be directly combined with TLR4. In this study, we investigated the molecular mechanism of the polysaccharide LBPF4-OL, which was extracted from LBP, in peritoneal macrophages and murine RAW264.7 macrophage cells.

2. Material and methods

2.1. Animals

Six-week-old female BALB/c mice and C3H/HeJ mice were purchased from Vital River Experimental Animal Center (Beijing, China) and were housed in a controlled environment (12-h light/12-h dark photoperiod, 22 ± 2 °C, 45–55% relative humidity). Female C3H/HeJ mice were obtained from The Jackson Laboratory. All of the mice were used at the age of 8 to 10 weeks. All of the experiments were conducted according to the National Guide for the Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Beijing Institute of Pharmacology and Toxicology.

2.2. Reagents

Lipopolysaccharide (LPS from Escherichia coli, serotype055:BS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediamine tetraacetic acid disodium salt (EDTA-Na₂) was purchased from Sinopharm Chemical Reagent (Shanghai, China). TNF-α and IL-1β ELISA kits were purchased from Dakewe Biotech (Beijing, China). Anti-mouse TLR4/MD2, anti-CR3, and anti-TLR2 were purchased from BioLegend. Extracellular signal-regulated kinase (ERK), C-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) were purchased from Cell Signaling Technology (Beverly, MA, USA). RPMI (Roswell Park Memorial Institute) 1640 medium was purchased from Gibco BRL (Life Technologies, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, and ³H-thymidine was obtained from GE Healthcare (Buckinghamshire, UK).

2.3. Preparation of LBPF4-OL

LBPF4-OL was isolated from L. barbarum fruit as described previously [6]. First, crude LBP was isolated from L. barbarum fruit. Briefly, dried L. barbarum fruit was extracted with water, and the water extract was precipitated with ethanol. The free proteins were removed using the Sevag reagent (CHCl₃:n-BuOH = 4:1). The crude LBP was obtained through dialysis and lyophilization. Second, the polysaccharide complex LBPF4 was isolated from LBP. Briefly, LBP was separated through DEAE-cellulose ion exchange chromatography (successively eluted with water, followed by 0.05 M, 0.1 M, 0.25 M, and 0.5 M NaHCO₃, and five homogenous fractions, designated LBPF1, LBPF2, LBPF3, LBPF4, and LBPF5, were obtained. LBPF4 was then purified mainly through gel filtration chromatography using a Sephadex G-100 column with 0.1 M sodium chloride–water solution as the eluent, and LBPF4 was obtained. Third, LBPF4-OL was released from LBPF4. Briefly, 80 mg of LBPF4 was dissolved in 3.5 mL of 0.1 N Tris–hydrochloric acid buffer (pH 8.0) containing 0.1% calcium chloride. Then, 1% (g/g) Pronase E was added to the buffer, and the sample was allowed to digest for 24 h at 37 °C and then an additional 48 h with a supplement of 0.5% (g/g) Pronase E. Then, 100 μL of methylbenzene was used to inhibit the growth of the bacterium. LBPF4-OL was finally purified using a Sephadex G-100 column, and the polysaccharide LBPF4-OL released from LBPF4 was obtained. The molecular weight of LBPF4-OL was 181 kDa, as determined by SDS-PAGE. The protein content was found to be ~0%, as determined through the Bradford method using a protein assay kit according to the manufacturer’s protocol. LBPF4-OL was dissolved in PBS or normal saline and filtered through a 0.22-µm filter.

2.4. Isolation of peritoneal macrophages

Mice were injected i.p. with 1 mL of 3% thioglycolate (Sigma-Aldrich). After three days, the peritoneum was washed with 10 mL of ice-cold PBS. The cells were incubated on 24-well plates with DMEM (Invitrogen Life Technologies) containing 10% FCS (HyClone Laboratories), 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and nonessential amino acids for 3 h at 37 °C in 5% CO₂. The nonadherent cells were removed by washing.

2.5. ELISA

The resident cells from the peritoneal cavity of mice were collected, and the macrophage population was enriched according to the method described by Shalev et al. [19]. The levels of TNF-α and IL-1β in the supernatants were measured with precoated TNF-α and IL-1β ELISA kits according to the manufacturer’s instructions. The absorbance was measured at 450 nm with a reference wavelength of 570 nm using a spectrophotometer (BMG POLARStar Galaxy v 4.0, Germany).

2.6. Cell line

RAW 264.7 is a tumor macrophage cell line derived from BALB/c mice (20) that retains phagocytic and Ag-presenting capabilities (21). RAW 264.7 cells were obtained from American Type Culture Collection (ATCC) and were raised in full medium at 37 °C in a 5% CO₂ atmosphere.

2.7. Splenocyte proliferation assay

C3H/HeN and C3H/HeJ mice were sacrificed. Their spleen was aseptically removed and minced through a 40-µm nylon cell strainer to achieve a single-cell suspension. The red blood cells were depleted with Tris–NH₄Cl lysis buffer (0.144 M NH₄Cl, 0.017 M Tris–HCl). A total of 5 × 10⁵ spleen cells were cultured with LPS at serial concentrations, including 5, 10, 50, 100, and 200 µg/mL. The spleen cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C in a 5% CO₂ humidified incubator for 72 h and were pulsed with ³H-thymidine (1 µCi/well) during the last 18 h of incubation. The cells were harvested on glass fiber filters using a Filtermate cell harvester (Packard). The amount of ³H-thymidine incorporated into the cells was measured using a β-scintillation counter (Beckman LS6500). The results are expressed as cpm (counters per minute) of the stimulated cells and cpm of the unstimulated cells.

2.8. Flow cytometry

The cells were washed with cold PBS containing 0.1% NaCl and 1% FBS. A total of 10⁶ cells were incubated with PE-conjugated anti-mouse TLR4/MD2 or isotype control IgG (BD Biosciences) for 20 min at room temperature. The cells were washed in PBS containing 0.1% FBS and analyzed by flow cytometry (BD Calibur™).

2.9. Western blot

The cells were washed in 1 × PBS and lysed in lysis buffer [10 mM Tris–HCl (pH 7.5), 10 mM NaH₂PO₄/NaHPO₄ (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaPi, 1 mM phenylmethylsulphonyl fluoride, and 2 µg/mL pepstatin A] for 30 min on ice. The lysates were then centrifuged at 30,000 rpm and 4 °C for 30 min. The supernatant was collected, and the protein content was then measured using a Bio-Rad protein assay kit before its analysis. The cytoplasmic or nuclear protein samples
were loaded at a concentration of 10 μg of protein/lane, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10–15% gel, and transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF membrane, 0.2 μm; Bio-Rad). The membranes were blocked with 5% nonfat powdered milk in 1 × Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 h and then incubated with primary antibodies overnight at 4 °C. The membranes were then treated with horse-radish peroxidase-coupled secondary antibodies for 1 h at 4 °C and washed with T-TBS after each antibody binding reaction. The detection of each protein was performed using an enhanced chemiluminescence (ECL) kit (Millipore Co., USA).

2.10. Bio-layer interferometry (BLI)

The kinetic estimates were obtained using the ForteBio Octet RED system (ForteBio). The octet analysis was performed at 30 °C in HBS-EP + 0.1 mg/mL BSA running buffer. Briefly, the anti-human IgG Fc capture (AHC) sensors from ForteBio were used to capture the TLR4/MD2-Fc onto the kinetic surface of the sensor. TLR4/MD2-Fc was loaded to saturation onto the anti-human Fc biosensors, washed in buffer (30 s), and placed for 3 min in the wells containing LBPF4-OL, LPS, and FBS. The one-shot KD was calculated based on the 1:1 model, and the average KD was extracted from the binding results obtained for five different concentrations of the analyses.

2.11. Statistical analysis

Unless specifically mentioned, all of the data are expressed as the means ± S.D. The ANOVA procedure was used to assess the significance of the differences between groups. For each significant treatment effect, Dunnett-type multiple comparisons were used to compare the means of multiple groups. All of the statistical analyses were conducted using the SPSS 12.0 software package. A value of P < 0.05 was used as the threshold for significance.

3. Results

3.1. Anti-TLR4/2 treatment inhibits LBPF4-OL-induced TNF-α and IL-1β production

Our previous research study found that LBPF4-OL induces the production of multiple cytokines, including TNF-α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, and GM-CSF, in splenocytes in a dose-dependent manner [15]. Of all of these cytokines, TNF-α and IL-1β were the most significantly up-regulated. To determine whether the reported polysaccharide-related receptors, including TLR4/2 and CR3, are related to the activities of L. barbarum polysaccharides, macropages were treated with anti-TLR4/2 antibody, and the concentrations of TNF-α and IL-1β in the cell culture supernatants were examined. Treatment with LBPF4-OL for 24 h stimulated TNF-α and IL-1β production compared with the medium control (Fig. 1A). Anti-TLR4 (0.31, 0.62, 1.25, and 2.5 μg/mL) treatment inhibited the LBPF4-OL (100 μg/mL)-induced secretion of TNF-α and IL-1β in a concentration-dependent manner. Anti-TLR4 (0.62 μg) also significantly inhibited the LPS (5 μg/mL)-induced secretion of TNF-α and IL-1β (Fig. 1B). We further found that anti-TLR2 (0.31, 0.62, 1.25, and 2.5 μg/mL) treatment partially inhibited the LBPF4-OL (100 μg)-induced TNF-α and IL-1β secretion, and anti-TLR2 (0.62 μg/mL) has no apparent effect on the LPS (5 μg/mL)-induced TNF-α and IL-1β secretion (Fig. 1C). Neither anti-CD11b nor anti-CD18/anti-CD11b combined with anti-CD18 had an effect on the LBPF4-OL- and LPS-induced TNF-α and IL-1β production (Fig. 1D). These results suggest that the LBPF4-OL-induced TNF-α and IL-1β production is TLR4/2-related and not CR3-related.

3.2. Anti-TLR4/MD2 treatment inhibits LBPF4-OL-induced lymphocyte proliferation

The immunoenhancement activity of LBP is mainly reported to stimulate mouse splenocyte proliferation [15,20,21] and to induce the phenotypic and functional maturation of DCs with strong immunogenicity [14]. A recent study found that the water-soluble polysaccharide fraction LBP-IV isolated from L. barbarum leaves significantly enhanced the proliferation of splenocytes stimulated by Con A or LPS [22]. To investigate whether TLR4 and CR3 are related to the immunological enhancement activity of LBPF4-OL, we further observed the effect of LBPF4-OL on lymphocyte proliferation through antibody pretreatment. BALB/c splenocytes were prepared, and lymphocyte separation medium was used to obtain the lymphocytes. The lymphocytes were pre-treated with anti-TLR4 and anti-CR3 antibody for 1 h in vitro, and the 3H-TdR incorporation method was then used to analyze the LBPF4-OL- and LPS-induced lymphocyte proliferation. Fig. 2A shows that LBPF4-OL induces lymphocyte proliferation in a concentration-dependent manner, but the stimulation intensity is significantly weaker than that obtained with LPS. Fig. 2B and C shows that anti-TLR4 can completely inhibit the LBPF4-OL-induced lymphocyte proliferation, but anti-CR3 has no effect on the LBPF4-OL-induced lymphocyte proliferation. These results indicate that the LBPF4-OL-induced lymphocyte proliferation is TLR4-dependent and not CR3-dependent.

3.3. LBPF4-OL cannot induce TNF-α and IL-1β production in peritoneal macrophages from C3H/HeJ mice

The C3H/HeJ mouse is TLR4-defective [23]. To further clarify whether TLR4 is a LBPF4-OL activity-related site and to determine whether the production of inflammatory cytokines induced by LBPF4-OL is associated with TLR4, the supernatant TNF-α and IL-1β concentrations of peritoneal macrophages from C3H/HeJ mice were analyzed. As shown in Fig. 3, LBPF4-OL (50, 100 μg/mL) significantly increased the TNF-α and IL-1β production by peritoneal macrophages from C3H/HeN mice but had no effect on the TNF-α and IL-1β production by macrophages from C3H/HeJ mice. In addition, we found that the TNF-α and IL-1β production by peritoneal macrophages from C3H/HeJ mice was obviously weaker than that obtained from C3H/HeN macrophages. The results show that TLR4 is in fact an activity-association receptor of LBPF4-OL for the induction of TNF-α and IL-1β production, but it is unclear whether TLR4 is a LBPF4-OL binding site.

3.4. LBPF4-OL-stimulated proliferation of lymphocytes from C3H/HeJ mice is weaker than that of lymphocytes from C3H/HeN mice

To determine whether the lymphocyte proliferation induced by LBPF4-OL is associated with TLR4, the in vitro proliferation rates of the lymphocytes from C3H/HeJ and C3H/HeN mice were compared through

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**Fig. 1.** Anti-TLR4/2 treatment inhibits LBPF4-OL-induced TNF-α and IL-1β production. BALB/c mice were sacrificed, and their spleen cells were prepared. The spleen cells (5 × 107) were stimulated with LPS (5 μg/mL), Dextran (100 μg/mL), anti-TLR4 (0.31–2.5 μg/mL), anti-TLR2 (0.31–2.5 μg/mL), anti-CR3 (0.31–1.25 μg/mL), and LBPF4-OL (10–100 μg/mL) for 24 h. (A) LBPF4-OL induced TNF-α and IL-1β production in peritoneal macrophages in vitro. **P < 0.01 compared with the medium control; †P < 0.01 compared with LPS; ‡P < 0.01 compared with LBPF4-OL. (B) Anti-TLR4 treatment inhibited LBPF4-OL (100 μg/mL)-induced TNF-α and IL-1β production. **P < 0.01 compared with the medium control; †P < 0.01 compared with LPS; ‡P < 0.01 compared with LBPF4-OL. (C) Anti-TLR2 treatment inhibits LBPF4-OL (100 μg/mL)-induced TNF-α and IL-1β production. **P < 0.01 compared with the medium control. (D) Anti-CR3 treatment had no effect on LBPF4-OL (100 μg/mL)-induced TNF-α and IL-1β production. **P < 0.01 compared with the medium control. The production of TNF-α and IL-1β was measured by ELISA. The data represent the means ± SD of four replicates.
the $^3$H-TdR incorporation method. As shown in Fig. 4, the effect of LBPF4-OL on the proliferation of C3H/HeN mouse splenocytes is obviously weaker than that on the proliferation of C3H/HeJ mouse splenocytes, and this effect is dependent on the concentration (5 to 200 $\mu$g/mL) of LBPF4-OL. However, the proliferation of lymphocytes from C3H/HeJ mice induced by LBPF4-OL is significantly higher than that observed in the control group. The results show that TLR4 is in fact an activity association receptor of LBPF4-OL for the induction of lymphocyte proliferation. The results also indicate that other receptors in addition to TLR4 may participate in the LBPF4-OL-induced lymphocyte proliferation.

3.5. LBPF4-OL cannot directly bind to TLR4 in vitro

Many of the bioactivities of botanical polysaccharides are closely associated with TLR4; however, it has not yet been reported whether these polysaccharides can be directly combined with TLR4. LPS is a glycolipid located in the outer membrane of Gram-negative bacteria and is composed of an amphipathic lipid A component, hydrophilic polysaccharides in the core, and O-antigen. Lipid A represents the conserved molecular pattern of LPS and is the main inducer of the immunological responses to LPS [24]. TLR4 in association with MD-2 is responsible for the physiological recognition of LPS [25]. In this study, a new method based on bio-layer interferometry (BLI) was used to determine the binding and dissociation of LBPF4-OL and LPS with TLR4 in vitro [26]. As shown in Fig. 5A, the AHC sensor combined effectively with the FC fragment of human rat recombinant TLR4 molecules, and the maximum response value was found to be 0.8 nm. Fig. 5B shows that LPS directly binds to TLR4 and rapidly reached saturation within 10 s in a concentration-dependent manner. Because we cannot acquire the molecular weight of LPS, it is impossible to quantify the interactions through equilibrium binding analyses. This result shows that LPS can bind directly to TLR4 in vitro. As a comparison, the association of LBPF4-OL with TLR4 was tested under the same conditions, and we found that LBPF4-OL at different concentrations (31.25–500 $\mu$g/mL) cannot bind to TLR4. These results suggest that LBPF4-OL cannot directly associate with TLR4.

3.6. LBPF4-OL increases TLR4 expression on the membranes of peritoneal macrophages and RAW264.7 cells

It has been reported that one of the molecular mechanisms of the immune-enhancing effect of polysaccharides is the induction of the expression of TLR4 on the cytomebrane. Therefore, we further observed the effect of LBPF4-OL on the expression of TLR4 on peritoneal macrophages and RAW264.7 cells by flow cytometry. As indicated in Fig. 6A, peritoneal macrophages stimulated with LBPF4-OL or LPS for 24 h cannot induce the expression of TLR4 on the cytomebrane, and a high expression of TLR4 was observed in these cells after stimulation for 48 h with both LPS and LBPF4-OL. A partial recovery of the TLR4 expression was observed in cells stimulated with LBPF4-OL for

Fig. 2. Anti-TLR4/MD2 treatment inhibits LBPF4-OL-induced lymphocyte proliferation. BALB/c mice were sacrificed, and their spleen cells were prepared. The spleen cells (5 × 10^5) were pretreated with anti-CR3 (0.62 $\mu$g/mL) and anti-TLR4/MD2 (0.62 $\mu$g/mL) and then stimulated with LPS (5 $\mu$g/mL) and LBPF4-OL (100 $\mu$g/mL) for 72 h. (A) Anti-TLR4/MD2 treatment inhibits the LBPF4-OL-induced proliferation of the spleen cells. *P < 0.05 and **P < 0.01 compared with the medium control; #P < 0.05 and ##P < 0.01 compared with LPS treatment. (B) Anti-CR3 treatment has no effect on the LBPF4-OL-induced proliferation of spleen cells; *P < 0.05 and **P < 0.01 compared with the medium control. The cell proliferation was measured by $^3$H-thymidine incorporation assay. The data represented as mean the means ± SD of four replicates.
In contrast, the LPS-induced TLR4 expression was completely recovered after 72 h. In addition, we observed the effect of LBPF4-OL on TLR4 expression in the macrophage cell line RAW264.7. In contrast to its effect on peritoneal macrophages, the expression of TLR4 on the RAW264.7 membrane was significantly increased by both LBPF4-OL and LPS after 24 h.

Fig. 3. LBPF4-OL cannot induce TNF-α and IL-1β production by peritoneal macrophages from C3H/HeJ mice. Both C3H/HeN and C3H/HeJ mice were sacrificed, and their spleen cells were prepared. The spleen cells (5 × 10⁵) were stimulated with LPS (5 μg/mL), galactan (100 μg/mL), and LBPF4-OL (10–100 μg/mL) for 24 h. (A) LBPF4-OL could not induce TNF-α production by the peritoneal macrophages from C3H/HeJ mice. (B) LBPF4-OL could not induce IL-1β production by the peritoneal macrophages from C3H/HeJ mice. *P < 0.01 compared with the LPS-stimulated spleen cells from C3H/HeN mice; #P < 0.05 and ##P < 0.01 compared with the LBPF4-OL-stimulated spleen cells from C3H/HeN mice. The TNF-α and IL-1β production levels were measured by ELISA. The data represent the means ± SD of four replicates.

Fig. 4. The LBPF4-OL-stimulated proliferation of lymphocytes from C3H/HeJ mice is weaker than that of lymphocytes from C3H/HeN mice. Both C3H/HeN and C3H/HeJ mice were sacrificed, and their spleen cells were prepared. The spleen cells (5 × 10⁵) were stimulated with LBPF4-OL (5–200 μg/mL) for 72 h and pulsed with ³H-thymidine (1 μCi/well) during the last 18 h. The cells were harvested on glass fiber filters using a Filtermate cell harvester. *P < 0.05 and **P < 0.01 compared with the LBPF4-OL-stimulated spleen cells from C3H/HeN mice. The ³H-thymidine incorporation method was used to determine the cell proliferation. The data represent the means ± SD of four replicates.
expression of TLR4 in LBPF4-OL-treated cells was recovered to a normal level, but the TLR4 expression in LPS-treated cells was significantly lower than that observed in the control group after 48 h. These results suggest that LBPF4-OL and LPS trigger different molecular mechanisms to induce TLR4 expression. The above results also suggest that the immune-enhancing effect of LBPF4-OL is due to TLR4 expression.

3.7. LBPF4-OL regulates the phosphorylation of MAPKs

Mitogen-activated protein kinase (MAPK) signal transduction pathways are ubiquitous and highly evolutionarily conserved mechanisms of eukaryotic cell regulation [27]. The multiple MAPK pathways present in all eukaryotic cells enable coordinated and integrated responses to diverse stimuli. It is definite that both LPS and the other botanical
polysaccharide can effectively evoke the MAPK signal pathway through TLR4. However, the effects of L. barbarum polysaccharides on MAPK signal pathway have not yet been reported. In the present study, the effect of LBPF4-OL on p-38, ERK1/2, and SAPK/JNK phosphorylation was observed through the WB method (Fig. 7). We found that phospho-p38 was markedly increased after stimulation with LBPF4-OL at a concentration of 100 μg/mL for 30 min, and the level of phospho-p38 was recovered after 45 min. In contrast, phospho-ERK1/2 was significantly inhibited early during treatment (within 30 min) with LBPF4-OL and returned to the normal level after 45 min. LBPF4-OL has no effect on the level of SAPK over a period of 1 h. We also found that LBPF4-OL markedly inhibits the phospho-JNK level within 1 h. In contrast, we observed that the treatment of cells with LPS for 45 min significantly enhanced the levels of phos-p-38, phos-ERK1/2, and phospho-SAPK/JNK. These results indicate that LBPF4-OL can regulate the p-38, ERK1/2, and JNK MAPK signaling pathways in macrophages.

4. Discussion

Immunomodulation and antitumor effects are one of the main activities of L. barbarum polysaccharide. Previous studies have shown that the polysaccharide–protein complex from L. barbarum (LBP3p) can significantly inhibit the growth of transplantable sarcoma S180 and increase macrophage phagocytosis; in addition, the form of antibody secreted by spleen cells, spleen lymphocyte proliferation, CTL activity, and IL-2 mRNA expression level were increased, and lipid peroxidation was reduced in S180-bearing mice [28]. Moreover, L. barbarum polysaccharide (LBP), which was isolated with boiling water from L. barbarum fruits, can inhibit the proliferation of HeLa cells [29]. The present study provides the first demonstration that L. barbarum polysaccharide stimulates tumor necrosis factor-alpha (TNF-α) and IL-1β generation and promotes lymphocyte proliferation in a TLR4-dependent manner. TNF-α, a pro-inflammatory cytokine that was initially identified and named as a result of its anti-tumoral properties, is involved in the pathogenesis of multiple chronic inflammatory or autoimmune diseases. TNF-α can act on monocytes and macrophages in an autocrine manner to enhance various functional responses and induce the expression of other immunoregulatory and inflammatory mediators. IL-1β is involved in fever during the induction of the acute-phase protein response. In addition, IL-1β cooperates with IFN-γ and IL-12 to induce tumor death by NK cells. It has been reported that, after an initial phase of growth, some murine tumor cells transfected with the TNF-α gene either completely regress or demonstrate decreased tumorigenicity. Consistently, Ganoderma atrum polysaccharide (PSG-1) can activate macrophages and increase TNF-α, IL-1β, and nitric oxide production via TLR4-dependent signaling pathways, improve immunity, and inhibit tumor growth [30]. All of these results strongly suggest that TLR4 plays an important role in the immunomodulation and antitumor effects of L. barbarum polysaccharide LBPF4-OL.

In this study, we found that the underlying mechanisms induced by LBPF4-OL and LPS on the TLR4–MAPK signaling pathway are different. First, we found that the anti-TLR4, anti-TLR2, and anti-CR3 treatments resulted in markedly different effects on the LPS- and LBPF4-OL-induced secretion of inflammatory cytokines. As shown in Fig. 1, both anti-TLR4 and anti-TLR2 treatments significantly inhibited the LBPF4-OL-induced TNF-α and IL-1β secretion. However, anti-TLR4 and not anti-TLR2 inhibited the LPS-induced TNF-α and IL-1β production by macrophages. In addition, as shown in Fig. 3, we found that LBPF4-OL markedly induced the secretion of TNF-α and IL-1β by C3H/HeJ mouse peritoneal macrophages but had no effect on the secretion of cytokines by macrophages from TLR4-defective C3H/HeJ mice. On the contrary, LPS significantly induced the secretion of TNF-α and IL-1β by C3H/HeJ mouse peritoneal macrophages in vitro. These results are consistent with the activity characteristics of LPS. Studies have shown that the LPS–TLR4 interaction requires the participation of many other molecules, such as CD14/LBP and HSP70, and this molecular community is known as the LPS receptor family [31–33]. In addition, these receptors are clustered together and are involved in LPS-induced transmembrane signal transduction. Studies have also found that TLR4 deficiency cannot fully reduce the activity of LPS. These results indicate that, in addition to TLR4, LBPF4-OL can induce the secretion of inflammatory factors by TLR2, and LPS can induce the secretion of inflammatory factors through an unknown receptor. Second, there is a clear difference in the TLR4 expression induced by LPS and LBPF4-OL. Vicky Sender found that LPS can significantly induce TLR4 expression on the cell membrane of alveolar macrophages, and this expression peaks after 30 min.
some studies have indicated that the activation of TLR4 can reduce allergic reactions. A previous study found that Tlr4^{-/-} mice have reduced proportions of CD4^{+}Foxp3^{+} Tregs in the colonic lamina propria and exhibit increased susceptibility to food-induced allergic responses. Moreover, it has also been reported that dermal exposure to the TLR4 ligand lipopolysaccharide (LPS) or TLR2 ligand can directly or indirectly promote diabetes [51].

and then gradually decreases [34]. Our experimental results indicate that the LPS-induced TLR4 expression on peritoneal macrophages continues to be higher than the normal levels after 12 h and recovers to the normal levels after 24 h. The TLR4 expression induced by LBPF4-OL after 12 and 24 h is significantly higher than that obtained with LPS (Fig. 6). These results suggest that the LBPF4-OL-induced TLR4 expression is significantly stronger than the effect induced by LPS. Finally, there are significant differences in the LPS- and LBPF4-OL-induced MAPK signaling pathway. Our results show that LPS significantly promotes the phosphorylation of P-38, ERK1/2, and JNK MAPKs. However, LBPF4-OL only promoted the phosphorylation of P-38 and inhibited the phosphorylation of ERK1/2 and JNK (Fig. 7). These results indicate that LPS plays a facilitating role and LBPF4-OL plays a regulatory role in the MAPK signaling pathway.

Recent studies have shown that Goji berries can cause allergic reactions in both exposed and unexposed food-allergic individuals, especially those who are allergic to lipid-transfer proteins (LTPs) from other foods [35]. However, it seems impossible that L. barbarum polysaccharide LBPF4-OL causes allergic reactions, even though some studies have indicated that the activation of TLR4 can reduce allergic reactions. A previous study found that Tlr4^{-/-} mice have reduced proportions of CD4^{+}Foxp3^{+} Tregs in the colonic lamina propria and exhibit increased susceptibility to food-induced allergic responses. Moreover, it has also been reported that dermal exposure to the TLR4 ligand lipopolysaccharide (LPS) or TLR2 ligand (Pam(3)) Cys suppresses asthmatic responses by reducing airway hyperreactivity, mucus production, Th2-type inflammation in the lungs, and IgE antibodies in the serum in a dose-dependent manner. In addition, LBPF4-OL contains no protein, as was confirmed by the Bradford method in our previous research. In consequence, as a potent immunity-enhancing agent, LBPF4-OL may not cause an allergic reaction; on the contrary, it may attenuate an allergic reaction. Thus, the observation of the anti-allergic effect of LBPF4-OL may be a valuable future research direction.

LPS is currently the most widely studied TLR4 ligand, but the biological activity of LPS may require the participation of more than one protein. These proteins are called a receptor cluster [32,36–38]. Through its interaction with the receptor cluster, LPS activates intracellular signaling by binding with TLR4. In addition, it has been reported that some polysaccharide activities are closely associated with TLR4. The Japanese scholar Izuru Ando first reported that saflower polysaccharides activate the transcription factor NF-kB via Toll-like receptor 4 and induce cytokine production [16]. Later, South Korean and Chinese scholars began to report that the Toll-like receptor-mediated activation of B cells and macrophages by the polysaccharides isolated from the cell culture of A. senticosus and the polysaccharides from D. batatas induce tumor necrosis factor-alpha secretion via Toll-like receptor 4-mediated protein kinase signaling pathways [17,18]. Anti-TLR4/MD2 monoclonal antibody blocking and the use of cells from TLR4-defective mice are two mainstream methods used to reveal the mechanisms of active polysaccharides, but these methods do not directly indicate whether the polysaccharide of interest is a ligand of TLR4. Therefore, in this study, in addition to these two methods, bio-layer interferometry technology (BLI) was also used to observe the binding of LBPF4-OL to TLR4. The experimental results show that LBPF4-OL does not directly bind to TLR4 in vitro. Based on the results, we speculated that LBPF4-OL may act similarly to LPS, which requires a variety of receptor clusters to participate in the activation of TLR4. In addition, the results also indicate that more data are required to determine whether the reported polysaccharides, such as saflower polysaccharide, the activity of which is associated with TLR4, can interact directly with TLR4 in vitro.

Dziarski first reported that LPS induces tyrosine phosphorylation and the activation of MAPKs, such as ERK1/2, p38, and SAPK/JNK, in macrophages [39]. Because the MAPK signal itself has not yet been fully elucidated, particularly the core signaling pathways of the JNK MAPK molecular mechanism [27], it is presently difficult to reveal the intrinsic link between the biological activity of a polysaccharide and the MAPK signaling pathway. The ERK, JNK, and P38 MAPK core pathways and their targets are activated by stresses, such as radiation injury, fatigue, oxidative stress, and inflammatory mediators [40,41]. L. barbarum polysaccharides have significant resistance to radiation damage and alleviate physical fatigue and all types of oxidative stress [42–44]. However, the effect of L. barbarum polysaccharides on the MAPK signal has not been reported. This study found that the L. barbarum polysaccharide LBPF4-OL significantly induces the phosphorylation of p38–MAPK and also significantly inhibits the phosphorylation of ERK and JNK. Many polysaccharides, such as the Ganoderma lucidum (Reishi or Ling-Zhi) polysaccharide EORP [45], the Paecilomyces polysaccharide PCP [46], the acetyl fucoidan from Cladosiphon okamuranus [47], and the polysaccharides from Angelica dahurica, can increase the phosphorylation of P38, JNK, and ERK1/2 MAPK [48]. In contrast, previous studies have reported that P38α leads to feedback control loops that suppress the activities of upstream MAP kinase kinase kinases (MAP3Ks), such as TAK1 and MLKs [49,50], which are associated with the activation of other pro-inflammatory pathways, including those that lead to the activation of JNK. It is possible that this mechanism may explain why the phosphorylation of P38–MAPK was increased and the phosphorylation of JNK was inhibited by LBPF4-OL.

Previous studies have found that the activation of P38 and JNK signaling can directly or indirectly promote diabetes [51]. Strong evidence...
has emerged that JNK, especially JNK1, possibly in conjunction with other MAPK groups, can directly contribute to insulin resistance [52]. These findings are consistent with the anti-diabetic effect and the inhibitory effect of LBP4F-OL on JNK phosphorylation. However, further studies are required to determine whether the anti-diabetic effect of *L. barbarum* polysaccharide is directly linked to JNK.

In conclusion, we found that *L. barbarum* polysaccharide LBP4F-OL promotes TNF-α and IL-1β secretion and lymphocyte proliferation in a THR4/MD2-dependent manner. We also found that LBP4F-OL is stronger than LPS in inducing THR4 expression in macrophages. In addition, LBP4F-OL can significantly enhance the phosphorylation of P38 and inhibit JNK and ERK1/2 MAPK phosphorylation, whereas LPS can induce P38, JNK, and ERK1/2 MAPK phosphorylation. These results indicate than LPS in inducing TLR4 expression in macrophages. In addition, Wang X-y, Bi Z-g, Wang Y-g, Jiang Y, Wang Y-f. Effects of ginsenoside and polysaccharide on vascular endothelial growth factor in the process of acute ocular hypertension. PLoS One 2012;7:e45469-e.

In conclusion, we found that *L. barbarum* polysaccharide is a new Toll-like receptor 4/MD2-MAPK signaling activator and inducer.

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**References**
