

Activation of Macrophages by Polysaccharide–protein Complex from *Lycium barbarum* L.

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Macrophages play crucial roles in innate immunity. This paper reports that a polysaccharide–protein complex isolated from *Lycium barbarum* (LBP) is able to activate macrophages. LBP was isolated from *Lycium barbarum* fruit and separated to five homogenous fractions, designated LBPF1, LBPF2, LBPF3, LBPF4 and LBPF5. It was found that LBP (50 mg/kg, i.p.) markedly upregulated the expressions of CD40, CD80, CD86 and MHC class II molecules on peritoneal macrophages. *In vitro* studies showed that LBP and LBPF1–5 activated transcription factors NF- κ B and AP-1 by RAW264.7 macrophage cells, induced TNF- α , IL-1 β , IL-12p40 mRNA expression, and enhanced TNF- α production in a dose-dependent manner. Furthermore, LBP (50 mg/kg, i.p.) significantly enhanced macrophage endocytic and phagocytic capacities *in vivo*. These results indicate that LBP enhances innate immunity by activating macrophages. The mechanism may be through activation of transcription factors NF- κ B and AP-1 to induce TNF- α production and upregulation of MHC class II costimulatory molecules. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: *Lycium barbarum* polysaccharide–protein complex; macrophage; NF- κ B; AP-1; TNF- α ; endocytosis; phagocytosis.

INTRODUCTION

Biological response modifiers (BRMs) are substances that stimulate the body's immune response to infection and disease. BRMs can be cytokines produced endogenously by mammalian immune cells or derivatives of microorganisms, fungi, algae, lichens and photosynthetic plants (Leung *et al.*, 2006; Schepetkin and Quinn, 2006). In recent decades, polysaccharides isolated from botanical sources have extraordinarily attracted a great deal of attention in the biomedical area because of their broad spectrum of therapeutic properties and relatively low toxicity (Schepetkin and Quinn, 2006; Wasser, 2002; Tzianabos, 2000). One of the pharmacological mechanisms of these botanical polysaccharides is thought to influence the innate immunity through activating macrophages (Omarsdottir *et al.*, 2005; Schepetkin *et al.*, 2005).

Lycium barbarum (*L. barbarum*) is an important Chinese medicine used to treat and prevent diseases such as insomnia, liver dysfunction, diabetes, visual degeneration and cancer. It has been recognized that the bioactive components of *L. barbarum* are polysaccharide–protein complex (LBP), which is a type of β -glycan possessing a core backbone of (1 \rightarrow 6)- β -galactosyl residues, about half of which are substituted

at C-3 by galactosyl or arabinosyl groups (Peng and Tian, 2001, Fig. 1). The carbohydrate is linked O-glycosidically to serine/threonine residues of the protein part (Qin *et al.*, 2001). LBP generally consists of six monosaccharides (galactose, glucose, rhamnose, arabinose, mannose and xylose) and 18 amino acids (Huang *et al.*, 1998; Gan *et al.*, 2003, 2004). In structure–function attributes, β -glycan structure is thought to contribute to the biological function (Tzianabos, 2000). Previous studies have shown that LBP can enhance the immune function (Gan *et al.*, 2003, 2004), protect liver damage (Ha *et al.*, 2005), lower the blood glucose level (Luo *et al.*, 2004), reduce the side effects of chemotherapy and radiotherapy (Gong *et al.*, 2004, 2005) and act against cancer (Gan *et al.*, 2004; Zhang *et al.*, 2005; Chao *et al.*, 2006). It is speculated that one of the mechanisms by which LBP enhances immunity may be through induction of the innate immune response by activating macrophages.

Macrophages play a major role in host defense against infection. Macrophages express a broad range of pattern recognition receptors (PRRs) to bind the conserved structures of pathogens, ingest and kill microbes into vesicles, and produce reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (mainly nitric oxide) to destroy microbes (Aderem and Underhill, 1999; Taylor *et al.*, 2005). Activated macrophages also secrete cytokines TNF and IL-1, and chemokines to induce inflammatory reactions to microbes (Pylkkänen *et al.*, 2004). In addition, macrophages can present antigen to T cells and produce IL-12 to coordinate innate and adaptive immune responses (Watford *et al.*, 2003). Furthermore, macrophages are involved in tissue remodeling after infections and injury, clearance of apoptotic cells and hematopoiesis (Tsirogiani *et al.*, 2006; Krysko

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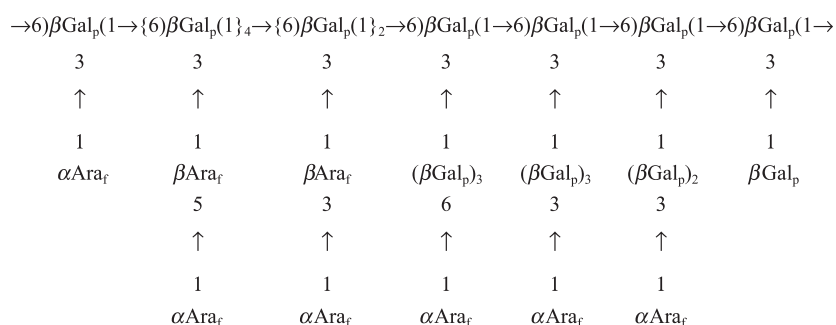


Figure 1. Structure of *L. barbarum* polysaccharide–protein complex LbGp2 (Peng and Tian, 2001).

et al., 2006). The present study investigated the mechanism of macrophage activation induced by LBP and compared the immunostimulatory activities of its different fractions.

MATERIALS AND METHODS

Animal and cell line. Female BALB/c mice, 6 weeks old, were obtained from the Singapore Laboratory Animal Centre. All procedures were approved by the Institutional Animal Care and Use Committee, National University of Singapore. The RAW264.7 murine macrophage cell line was ordered from the American Type Culture Collection (ATCC, TIB-71). The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37 °C in a 5% CO₂ humidified incubator.

Reagents. Ovalbumin (OVA), lipopolysaccharide (LPS, L3755, *Escherichia coli* serotype 026:B6) E-TOXATE[®] kit, FITC-dextran and FITC-*Staphylococcus aureus* were purchased from Sigma (St Louis, MO). The protein assay kit was from Bio-Rad (Hercules, CA). The mouse B cell isolation kit, lipofectamine transfection reagents and SuperScrip[™] first-strand synthesis kit were purchased from Invitrogen (Carlsbad, CA). The RNA extraction kit was ordered from Qiagen (Hilden, Germany). PCR primers and fluorogenic probes for the target gene (TNF- α , IL-1 β and IL-12p40) and endogenous control (β -actin) were purchased as TaqMan[®] Gene Expression Assays from Applied Biosystems (Foster City, CA). Mouse TNF- α , IL-1 β , IL-12p40, IL-12p70 OptiEIA[™] sets, NFAT-luc, AP-1-luc and NF- κ B-luc plasmids, and the following antibodies, including anti-mouse CD11b (Rat IgG2b, APC, M1/70), anti-mouse CD40 (rat IgG2a, FITC, 3/23), anti-mouse CD80 (American hamster IgG2, FITC, 16-10A1), anti-mouse CD86 (rat IgG2a, FITC, GL1), anti-mouse I-A/I-E (rat IgG2a, FITC, 2G9), isotype controls American hamster IgG1 (APC, G235-2356), American hamster IgG2 (FITC, B81-3) and rat IgG2a (FITC, R35-95) were from BD Biosciences (San Diego, CA). Luciferase assay reagents were from Promega (Madison, WI). ³H-thymidine was from GE Healthcare (Buckinghamshire, UK).

LBP preparation and treatment. *L. barbarum* fruit was purchased from Eu Yan Sang Chinese medicine store (Singapore). The fruit was imported from Zhongning

county, Ningxia province China. Voucher herbarium specimens are deposited at the WHO Immunology Centre, National University of Singapore. LBP was isolated from *L. barbarum* fruit as described previously (Chen *et al.*, 2008). Briefly, *L. barbarum* dried fruit was extracted with water and the water extract was precipitated with ethanol. Free proteins were removed with Sevag reagent (CHCl₃:*n*-BuOH = 4:1). Crude LBP was obtained by dialysis and lyophilization. The LBP was then separated by DEAE-cellulose ion exchange chromatography (successively eluted with water, followed by 0.05 M, 0.1 M, 0.2 M and 0.5 M NaCl) and further purified by size exclusion chromatography (eluted with water) using FPLC. Five homogenous fractions, designated as LBPF1, LBPF2, LBPF3, LBPF4 and LBPF5 were obtained (Chen *et al.*, 2008). The molecular weights of LBPF1, LBPF2, LBPF3 and LBPF4 were 150 kDa, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Chen *et al.*, 2008). The molecular weight of LBPF5 was 293 kDa, as determined by size exclusion chromatography (Chen *et al.*, 2008). The carbohydrate contents were 48.2%, 30.5%, 34.5%, 20.3% and 23.5%, respectively, as determined by phenol–sulfuric acid assay (Chen *et al.*, 2008). The protein contents were 1.2%, 4.8%, 4.1%, 13.7% and 17.3%, respectively, as measured by the Bradford method using a protein assay kit (Chen *et al.*, 2008). The LBP and LBPF1–5 were dissolved in PBS or normal saline (for *in vivo* experiment), filtered through a 0.22 µm filter and stored at 4 °C. The RAW264.7 cells were stimulated with LBP or LBPF1–5 at various concentrations and time points at 37 °C in a 5% CO₂ humidified incubator for *in vitro* assays. The LBP was administered *i.p.* to mice daily for 7 days at 50 mg/kg for *in vivo* studies.

Flow cytometric analysis. Cells were washed with cold wash buffer (PBS/0.1% NaN₃/1.0% FBS). 10⁶ cells were stained with 0.5 µg of APC-conjugated anti-mouse CD11b and 0.5 µg of FITC-conjugated anti-mouse CD40, CD80, CD86, I-A/I-E, or isotype control in 100 µL for 40 min at 4 °C. The cells were washed, resuspended in PBS/2% paraformaldehyde and analysed by flow cytometry (Dako).

Luciferase assay. The RAW264.7 cells were transiently transfected with NFAT-luc, AP-1-luc or NF- κ B plasmid DNA using lipofectamine transfection reagent according to the manufacturer's protocol. The transfected cells were incubated for 48 h. 1 × 10⁵ cells were stimulated with LBP or LBPF1–5 100 µg/mL for 6 h. PMA (20 ng/mL) plus ionomycin (0.5 µg/mL) was a positive control.

At the end of stimulation, the cells were harvested and lysed with 20 μ L of lysis buffer (25 mM Tris-phosphate, 8 mM MgCl₂, 2 mM DTT, 1% Triton X-100, and 10% glycerol, 2 mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid). The lysate was mixed with 100 μ L of luciferase assay reagent. Luciferase activity was measured by a luminometer (Biotrace). The background obtained with the lysis buffer was subtracted in each experimental value, and the specific trans-activation was expressed as the fold induction over untreated cells.

Real-time RT-PCR. Total RNA was extracted from LBP- or LBPF1-5-treated RAW264.7 cells using Qiagen RNeasy mini kit and reverse transcribed to cDNA using the Invitrogen SuperScrip™ first-strand synthesis system. The PCR primers and fluorogenic probes for the target genes (TNF- α , IL-1 β and IL-12p40) and endogenous control (β -actin) were purchased as TaqMan® Gene Expression Assays. The PCR volume was 20 μ L, composed of 1 μ L of 20 \times mixed primers and probe, 10 μ L of 2 \times TaqMan® Fast Universal PCR Master Mix and 9 μ L of cDNA template (500 ng diluted in RNase-free water). PCR was performed in an optical 96-well reaction plate on the ABI 7500 Fast Real-time PCR System. Each sample was run in triplicate. The thermal cycle conditions were 20 s hold at 95 °C, followed by 50 cycles of 1 s at 95 °C (denature) and 20 s at 60 °C (annealing/extension). The relative quantification of the target gene expression was calculated by comparative C_T ($\Delta\Delta C_T$) method using the SDS 1.3.1 software. Values reported have a 95% confidence interval (CI) as determined by the software.

ELISA. TNF- α , IL-1 β and IL-12p40 and p70 were quantified by sandwich ELISA using BD Biosciences OptiEIA™ set according to the manufacturer's instructions. The absorbance was measured at 450 nm with a reference wavelength of 570 nm using a spectrometer (Tecan Sunrise).

Endocytosis and phagocytosis assay. Mice were injected i.p. with 1 mL of FITC-dextran (1 mg/mL, endocytosis assay) or 1 mL of FITC-*Staphylococcus aureus* (1 mg/mL, phagocytosis assay) 30 min prior to killing. Peritoneal cells were harvested, washed and resuspended in DMEM medium supplemented with 10% FBS. The cells were applied to 24-well tissue culture plates with a microscope glass cover slip in the bottom at 37 °C in a 5% CO₂ humidified incubator for 6 h. The cover slip with adherent cells was picked up and mounted with DABCO-glycerol media. The cells were observed and photographed under a fluorescent microscope (Olympus BX-60, magnification, $\times 40$).

Test of LPS contamination. LPS contamination was tested by *Limulus* ameobocytes lysate (LAL) assay and B cell proliferation assay. The LAL assay was performed using an E-TOXATE® kit according to the manufacturer's instruction. In brief, 100 μ L of samples (10 mg/mL), standards or endotoxin-free water (negative control) was mixed with 100 μ L of LAL for 1 h at 37 °C and observed for gelation. B cell proliferation was performed using negatively selected B cells from BALB/c mouse spleen. 2 $\times 10^5$ B cells were stimulated with LPS (1 μ g/mL), or LBP (100 μ g/mL), or LBPF1-5

(100 μ g/mL) at 37 °C in a 5% CO₂ humidified incubator for 72 h. The cells were pulsed with ³H-thymidine (0.5 μ Ci/well) for the last 18 h. The amount of ³H-thymidine incorporated into the cells was measured using a β -scintillation counter (Packard TopCount).

Statistical analysis. Data are presented as mean \pm SD, except for the relative quantification of cytokine mRNA, which was presented as 95% of CI. Each experiment was repeated at least three times. Differences were analysed for significance using the Student's unpaired, two-tailed *t*-test by the SPSS 13.0 software. A value of *p* < 0.05 was used as the threshold for significance.

RESULTS

Effects of LBP on the expression of CD40, CD80, CD86 and MHC class II molecules on macrophages

Macrophages are types of antigen presenting cells which enhance their antigen presenting ability by upregulating the expression of MHC class II molecules and costimulators such as CD40, CD80 and CD86 (Hancock *et al.*, 1996). To investigate whether LBP upregulates the expression of such molecules on macrophages, LBP i.p. was injected i.p. into mice and the peritoneal macrophages were harvested 7 days later. Expression of such molecules was analysed by flow cytometry. As shown in Fig. 2, 3.9% of peritoneal cells in the mice injected with saline expressed CD40. In contrast, the expression was increased to 74.1% in the LBP-treated mice. Similarly, the expression of CD80, CD86 and MHC class II after LBP treatment were upregulated from 18.3%, 14.5% and 64.5% to 94.1%, 90.4% and 96.9%, respectively.

Effects of LBP and LBPF1-5 on activation of transcription factors

Transcription factors are critical for macrophage activation. Transcriptional signaling is necessary for inducible expression of a suite of genes required to initiate inflammation and eliminate pathogens (Guha and Mackman, 2001). Therefore, the study investigated whether LBP activates three key transcription factors, including NFAT, AP-1 and NF- κ B. The RAW264.7 cells were transfected with the three corresponding plasmids containing the luciferase reporter gene, then the transfected cells were stimulated with LBP and LBPF1-5, and the luciferase activity was measured by luciferase assay. As shown in Fig. 3, LBP and LBPF1-5 significantly activated AP-1 and NF- κ B (*p* < 0.01–0.05, compared with medium). The luciferase activities were increased 1.5- to 2.5-fold by LBP or LBPF1-5 stimulation. In contrast, NAFT was completely suppressed.

LBP and LBPF1-5 induce TNF- α , IL-1- β and IL-12p40 mRNA expression

As activation of transcription factors initiates gene transcription, the study next investigated whether LBP induces TNF- α , IL-1 β and IL-12p40 mRNA expression. After RAW264.7 cells were treated with LBP or LBPF1-5

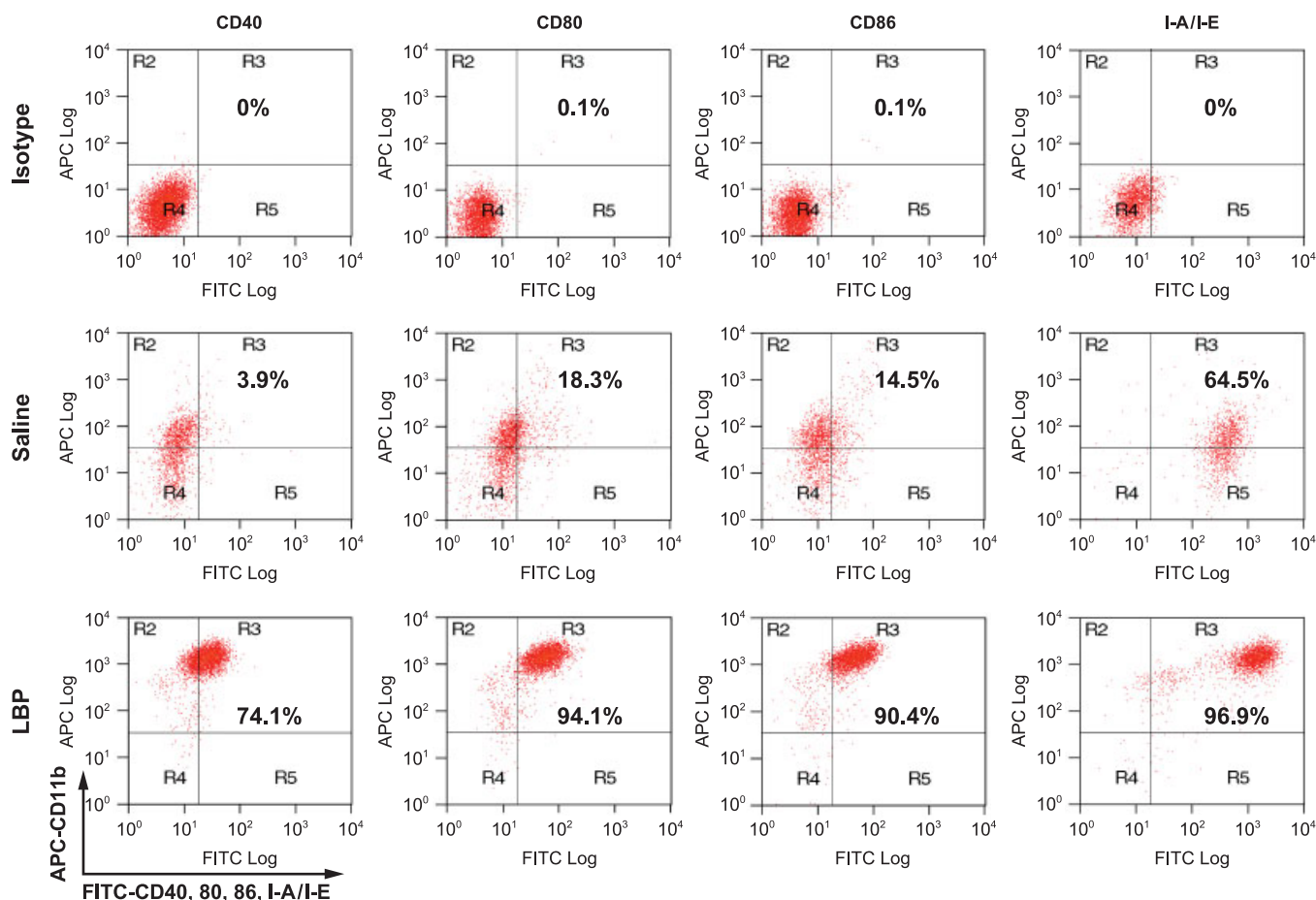


Figure 2. Effects of LBP on the expressions of CD40, CD80, CD86 and MHC class II molecules on macrophages. BALB/c mice were administered i.p. with LBP (50 mg/kg) daily for 7 days. Peritoneal macrophages were harvested, washed and stained with APC-conjugated anti-CD11b and FITC-conjugated anti-CD40, CD80, CD86 or I-A/I-E mAbs. Results are representative of three independent experiments.

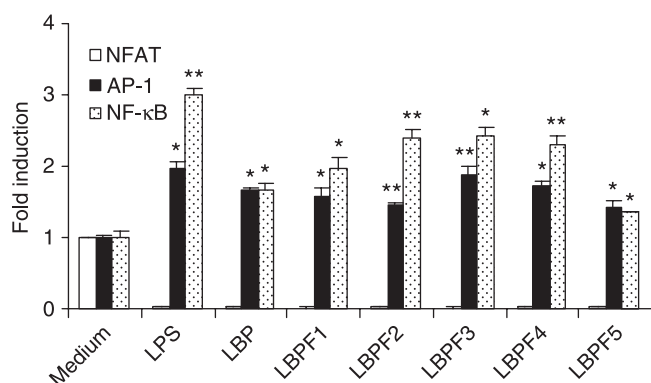


Figure 3. Effects of LBP and LBPF1-5 on the activation of transcription factors. RAW264.7 cells were transiently transfected with luciferase reporter plasmid NFAT-luc, AP-1-luc or NF-κB-luc for 48 h. Transfected cells were stimulated with 100 μg/mL of LBP or LBPF1-5 for 6 h. Luciferase activity was measured by luciferase assay. Results are expressed as fold induction of relative light units (RLU) of treated cells over that of untreated cells. Values are mean ± SD of triplicate. * $p < 0.05$, ** $p < 0.01$, compared with untreated cells.

for 48 h, TNF- α , IL-1 β and IL-12p40 mRNA expression were increased strikingly, of which TNF- α mRNA was increased 1.5- to 2.7-fold (Fig. 4A), IL-1 β mRNA was increased 1600- to 4700-fold (Fig. 4B), and IL-12p40 was increased 1 360 000- to 6 810 000-fold (Fig. 4C). The extremely high levels of relative expression of IL-12p40 and IL-1 β mRNA after LBP stimulation were because

they were expressed at a very low level in the untreated cells (C_T values were 50 and 35, respectively, data not shown).

LBP and LBPF1-5 enhance TNF- α production

As LBP induces TNF- α , IL-1 β and IL-12p40 mRNA expression, it may induce their protein production. To answer this question, RAW264.7 cells were stimulated with LBP or LBPF1-5 at various concentrations, including 1, 10, 100 and 500 μg/mL for 48 h. As shown in Fig. 5, LBP and LBPF1-5 induced TNF- α production in a dose-dependent manner. LBPF3 appeared to be the most potent. The effects were visible at 1 μg/mL and 100 μg/mL of LBP and LBPF1-5 induced 7.6–19.3 ng/mL of TNF- α . In contrast, RAW264.7 cells did not produce any IL-1 β and IL-12p40 and p70 detectable by ELISA after LBP and LBPF1-5 stimulation.

LBP enhances endocytosis and phagocytosis *in vivo*

Endocytosis and phagocytosis are the main functions of macrophages. Macrophages engulf large molecules by endocytosis while destroying microbes by phagocytosis. Animal models were set up to investigate whether LBP enhances macrophage functions. Mice were injected i.p. with LBP daily for 7 days and 30 min prior to killing were injected i.p. with FITC-dextran (endocytosis

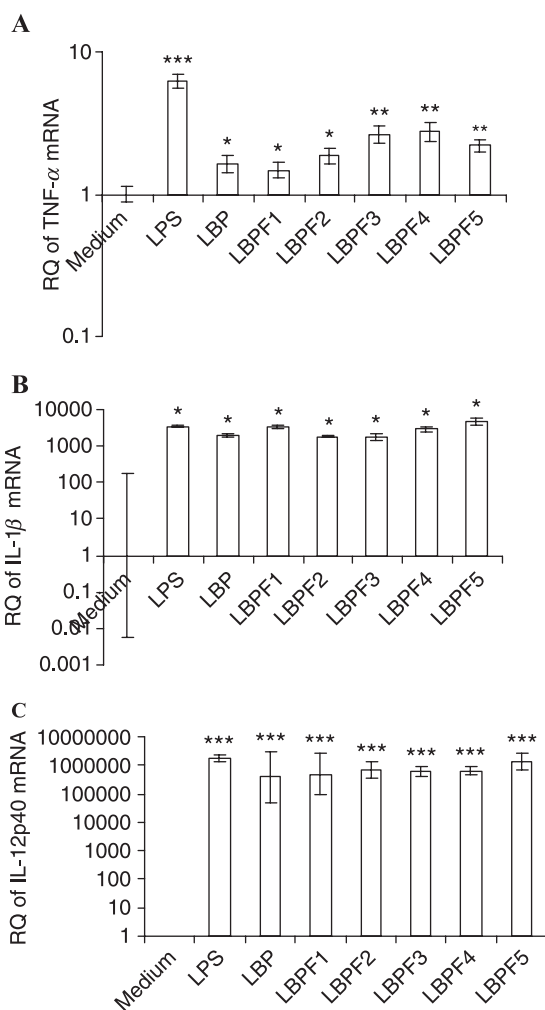


Figure 4. LBP and LBPF1-5 enhance TNF- α , IL-1 β , IL-12p40 mRNA expression. 5×10^5 Raw264.7 cells were stimulated with 100 μ g/mL of LBP, LBPF1-5, or 1 μ g/mL of LPS for 48 h. Cytokine mRNA expression was quantified by RT-PCR relative to that of untreated cells using β -actin gene as endogenous control. Results are represented as 95% CI of triplicate, compared with medium. * 95% CI; ** 99% CI; *** 99.9% CI. (A) TNF- α . (B) IL-1 β . (C) IL-12p40.

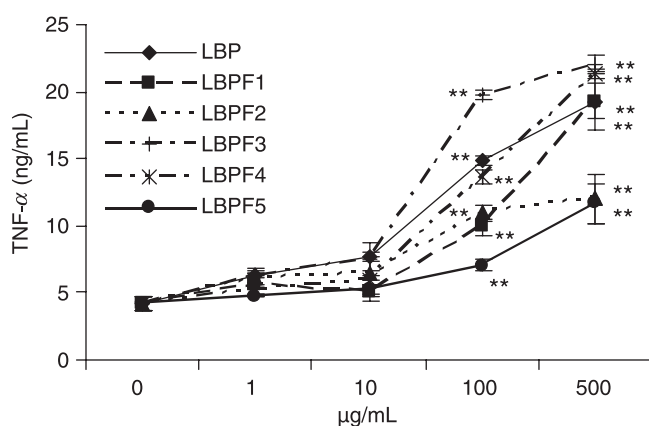


Figure 5. LBP and LBPF1-5 enhance TNF- α production. 5×10^5 Raw264.7 cells were stimulated with 100 μ g/mL of LBP, LBPF1-5 or 1 μ g/mL of LPS for 48 h. TNF- α concentration in the supernatant was measured by ELISA. Values are mean \pm SD of triplicate. ** $p < 0.01$, compared with medium control (untreated cells).

assay) or FITC-*Staphylococcus aureus* (phagocytosis assay). The result showed that FITC-dextran endocytosed by peritoneal macrophages from the LBP-treated mice (Fig. 6B) was much more intensive than that

endocytosed by peritoneal macrophages from the saline control mice (Fig. 6A). Peritoneal macrophages of LBP-treated mice became bigger, irregular, spreading and adherent, whereas peritoneal macrophages of the saline control mice round small, round, loosely adherent. Similarly, the phagocytosis assay showed that fluorescence in peritoneal macrophages from LBP-treated mice (Fig. 6D) was much brighter and more intensive than that in those from the saline control mice (Fig. 6C), indicating many more bacteria were phagocytosed by macrophages after LBP administration. After phagocytosing bacteria, the macrophages subsequently underwent apoptosis characterized as cell shrinking, blebbing and fragmentation (Fig. 6, C and D).

LBP and LBPF1-5 are free of LPS contamination

The LAL results showed that the quantity of endotoxin in crude LBP and LBPF1-5 was less than 0.015 EU/mg (negative). Our previous work has demonstrated that LBP does not stimulate B cell proliferation. Therefore, the study also tested whether LBP was contaminated by LPS during preparation by B cell proliferation assay. Unlike LPS, LBP and LBPF1-5 did not stimulate B cell proliferation (Fig. 7).

DISCUSSION

L. barbarum is believed to possess multiple health benefits. This study demonstrated that one of the pharmacological mechanisms of LBP is through enhancement of innate immunity by activating macrophages. In detail, LBP induced the expression of CD40, CD80, CD86 and MHC class II molecules on mouse peritoneal macrophages. LBP activated transcription factors NF- κ B and AP-1, induced TNF- α , IL-1 β and IL-12p40 mRNA expression, and enhanced TNF- α production by RAW264.7 macrophage cells. All its five fractions were active. LBPF3 appeared to be the most potent in activation of transcription factors and induction of cytokine production. Peritoneal macrophages from LBP-treated mice were very active in endocytosis and phagocytosis. The data indicate that the mechanism of macrophage activation by LBP may be through activation of transcription factors NF- κ B and AP-1 to induce TNF- α production and upregulation of MHC class II costimulatory molecules.

LBP and LBPF1-5 significantly activated transcription factors AP-1 and NF- κ B using the RAW264.7 macrophage cell line. In contrast, NFAT was completely inhibited. The reason that NFAT induction was suppressed after LBP treatment is not known, probably because it mainly participates in the regulation of T-cell function and development (Macian, 2005). Unlike NFAT, NF- κ B plays crucial roles in macrophage activation. NF- κ B induction is essential for the expression of a wide variety of immune response genes, including proinflammatory cytokines, chemokines and adhesion molecules (Beinke and Ley, 2004). AP-1 proteins have been implicated in invasive cell growth and matrix metalloprotease production and in cell line models, and have been suggested to mediate the induction of inflammatory genes such as TNF (Hu *et al.*, 2007).

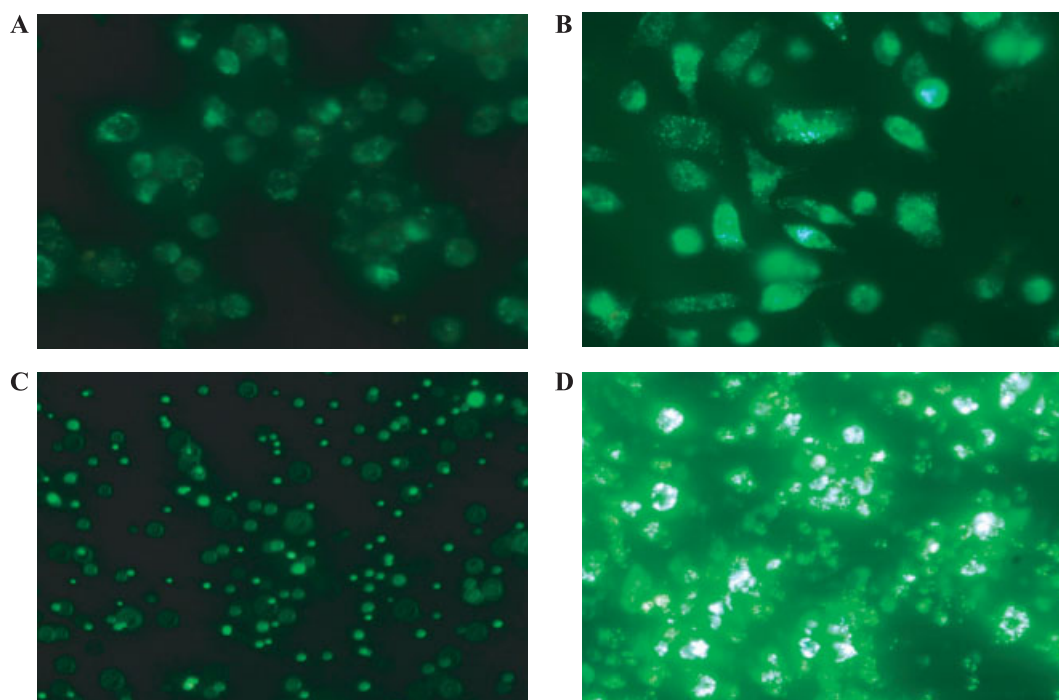


Figure 6. LBP enhances endocytosis and phagocytosis *in vivo*. BALB/c mice were injected with LBP (50 mg/kg) i.p. daily. Saline was control. Mice were killed 7 days later. 30 min prior to killing, mice were injected i.p. with 1 mL of FITC-dextran (1 mg/mL) (A, B) or 1 mL of FITC-*Staphylococcus aureus* (1 mg/mL) (C, D). Peritoneal cells were harvested, washed and incubated in 24-well tissue culture plates with a microscope glass cover slip in the bottom for 6 h. The cover slip with adherent cells were observed and photographed under a fluorescent microscope (Olympus BX-60, magnification, $\times 40$). (A) Saline + dextran. (B) LBP + dextran. (C) Saline + *Staphylococcus aureus*. (D) LBP + *Staphylococcus aureus*.

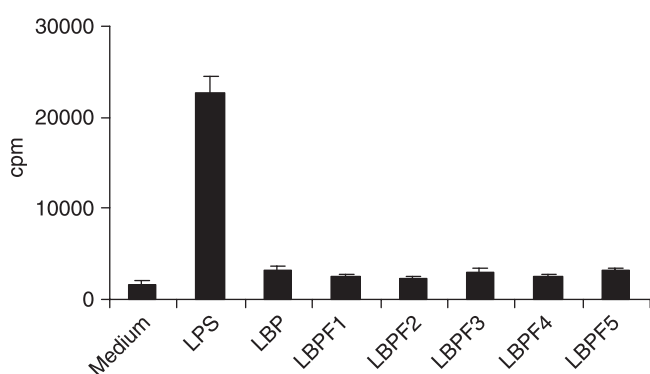


Figure 7. Test of LPS contamination by B cell proliferation assay. B cells were negatively selected from BALB/c mouse spleen and stimulated with LBP (100 $\mu\text{g}/\text{mL}$), LBPF1-5 (100 $\mu\text{g}/\text{mL}$) or LPS (1 $\mu\text{g}/\text{mL}$) for 72 h. B cell proliferation was measured by ^3H -thymidine incorporation assay. Values are mean \pm SD of four replicates.

As expected, the induction of NF- κB and AP-1 triggered TNF- α , IL-1 β and IL-12p40 mRNA expression. However, only TNF- α production was detectable by ELISA, and RWA264.7 cells constitutively secreted a substantial level of TNF- α . The lack of IL-1 β and IL-12p40 and p70 production could be because their absolute mRNA expression was low. IL-12, secreted from dendritic cells (DCs) and macrophages, is a crucial cytokine that can direct Th1 cell differentiation. Nevertheless, it was found that LBP induced IL-12p40 and p70 production from DCs, and T cells stimulated with LBP-treated DCs produced a higher level of IFN- γ , indicating that LBP can induce Th1 response (manu-

script in preparation). Furthermore, LBP also stimulates T cells to produce IFN- γ (Chen *et al.*, 2008). As the principal physiological function of TNF- α is to stimulate the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes, LBP enhancement of TNF- α production may be beneficial to host defense.

One of the main functions of macrophages is to endocytose antigens and phagocytose invading microbes (Watford *et al.*, 2003; Taylor *et al.*, 2005). The results clearly demonstrated that LBP (i.p.) extraordinarily enhanced the peritoneal macrophage's capacity in endocytosis and phagocytosis (Fig. 6, B and D), indicating that LBP-treated macrophages are activated and functional. Macrophages recognize pathogens through PRRs, which include scavenger receptors (SRs), mannose receptor, Toll-like receptors (TLRs), Dectin-1 and complement receptor type 3 (CR3) (Underhill and Ozinsky, 2002). Macrophage activation by plant polysaccharides is thought to be mediated primarily through the recognition of polysaccharide polymers (Schepetkin and Quinn, 2006). PRRs. It will be interesting to identify the LBP corresponding receptors on macrophages in the future.

Endotoxin (LPS) is a known immunomodulator and is often a contaminant in biological preparations. Thus, one of the principal concerns in the field is that the immune stimulating properties of botanical polysaccharides might be due to contamination from bacterial endotoxin (Schepetkin and Quinn, 2006). Probably, the most common approach to detecting LPS contamination involves the use of LAL assay (Hase *et al.*, 1997; Sanzen *et al.*, 2001). Therefore, the study tested whether LBP and its five fractions were contaminated with LPS

by the LAL assay. The result showed that the samples were not contaminated with LPS, as was further supported by the fact that LBP and its five fractions did not stimulate B cell proliferation, whereas LPS did. This result demonstrated that macrophage activation is by LBP, but not by LPS.

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REFERENCES

- Aderem A, Underhill DM. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* **17**: 593–623.
- Beinke S, Ley SC. 2004. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J* **382**: 393–409.
- Chao JC, Chiang SW, Wang CC *et al.* 2006. Hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa* inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells. *World J Gastroenterol* **12**: 4478–4484.
- Chen Z, Tan BK, Chan SH. 2008. Activation of T lymphocytes by polysaccharide–protein complex from *Lycium barbarum* L. *Int Immunopharmacol* **8**: 1663–1671.
- Gan L, Zhang SH, Liu Q *et al.* 2003. A polysaccharide–protein complex from *Lycium barbarum* upregulates cytokine expression in human peripheral blood mononuclear cells. *Eur J Pharmacol* **471**: 217–222.
- Gan L, Zhang SH, Yang XL *et al.* 2004. Immunomodulation and antitumor activity by a polysaccharide–protein complex from *Lycium barbarum*. *Int Immunopharmacol* **4**: 563–569.
- Gong H, Shen P, Jin L *et al.* 2004. Therapeutic effects of *Lycium barbarum* polysaccharide (LBP) on mitomycin C (MMC)-induced myelosuppressive mice. *J Exp Ther Oncol* **4**: 181–187.
- Gong H, Shen P, Jin L *et al.* 2005. Therapeutic effects of *Lycium barbarum* polysaccharide (LBP) on irradiation or chemotherapy-induced myelosuppressive mice. *Cancer Biother Radiopharm* **20**: 155–162.
- Guha M, Mackman N. 2001. LPS induction of gene expression in human monocytes. *Cell Signal* **13**: 85–94.
- Ha KT, Yoon SJ, Choi DY *et al.* 2005. Protective effect of *Lycium chinense* fruit on carbon tetrachloride-induced hepatotoxicity. *J Ethnopharmacol* **96**: 529–535.
- Hancock WW, Sayegh MH, Zheng XG *et al.* 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc Natl Acad Sci USA* **93**: 13967–13972.
- Hase K, Basnet P, Kadota S *et al.* 1997. Immunostimulating activity of Celosian, an antihepatotoxic polysaccharide isolated from *Celosia argentea*. *Planta Med* **63**: 216–219.
- Hu X, Chen J, Wang L *et al.* 2007. Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. *J Leukoc Biol* **82**: 237–243.
- Huang LJ, Lin Y, Tian GY *et al.* 1998. Isolation, purification and physico-chemical properties of immunoactive constituents from the fruit of *Lycium barbarum* L. *Acta Pharm Sin* **33**: 512–516.
- Krysko DV, D'Herde K, Vandenabeele P. 2006. Clearance of apoptotic and necrotic cells and its immunological consequences. *Apoptosis* **11**: 1709–1726.
- Leung MY, Liu C, Koon JC *et al.* 2006. Polysaccharide biological response modifiers. *Immunol Lett* **105**: 101–114.
- Luo Q, Cai Y, Yan J *et al.* 2004. Hypoglycemic and hypolipidemic effects and antioxidant activity of fruit extracts from *Lycium barbarum*. *Life Sci* **76**: 137–149.
- Macian F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* **5**: 472–484.
- Omarsdottir S, Freysdottir J, Barsett H *et al.* 2005. Effects of lichen heteroglycans on proliferation and IL-10 secretion by rat spleen cells and IL-10 and TNF-alpha secretion by rat peritoneal macrophages *in vitro*. *Phytomedicine* **12**: 461–467.
- Peng XM, Tian GY. 2001. Structural characterization of the glycan part of glycoconjugate LbGp2 from *Lycium barbarum* L. *Carbohydr Res* **331**: 95–99.
- Pylkkänen L, Gullstén H, Majuri ML *et al.* 2004. Exposure to *Aspergillus fumigatus* spores induces chemokine expression in mouse macrophages. *Toxicology* **200**: 255–263.
- Qin X, Yamauchi R, Aizawa K *et al.* 2001. Structural features of arabinogalactan-proteins from the fruit of *Lycium chinense* Mill. *Carbohydr Res* **333**: 79–85.
- Sanzen I, Imanishi N, Takamatsu N *et al.* 2001. Nitric oxide-mediated antitumor activity induced by the extract from *Grifola frondosa* (Maitake mushroom) in a macrophage cell line, RAW 264.7. *J Exp Clin Cancer Res* **20**: 591–597.
- Schepetkin IA, Faulkner CL, Nelson-Overton LK *et al.* 2005. Macrophage immunomodulatory activity of polysaccharides isolated from *Juniperus scopolorum*. *Int Immunopharmacol* **5**: 1783–1799.
- Schepetkin IA, Quinn MT. 2006. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *Int Immunopharmacol* **6**: 317–333.
- Taylor PR, Martinez-Pomares L, Stacey M *et al.* 2005. Macrophage receptors and immune recognition. *Annu Rev Immunol* **23**: 901–944.
- Tsirogianni AK, Moutsopoulos NM, Moutsopoulos HM. 2006. Wound healing: immunological aspects. *Injury* **37**: S5–S12.
- Tzianabos AO. 2000. Polysaccharide immunomodulators as therapeutic agents: structural aspects and biologic function. *Clin Microbiol Rev* **13**: 523–533.
- Underhill DM, Ozinsky A. 2002. Phagocytosis of microbes: complexity in action. *Annu Rev Immunol* **20**: 825–852.
- Wasser SP. 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl Microbiol Biotechnol* **60**: 258–274.
- Watford WT, Moriguchi M, Morinobu A. 2003. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev* **14**: 361–368.
- Zhang M, Chen H, Huang J *et al.* 2005. Effect of *Lycium barbarum* polysaccharide on human hepatoma QGY7703 cells: inhibition of proliferation and induction of apoptosis. *Life Sci* **76**: 2115–2124.