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Polysaccharide-Protein Complex from *Lyceum barbarum* L. Is a Novel Stimulus of Dendritic Cell Immunogenicity

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Dendritic cell (DC) immunogenicity correlates with its maturation, which can be induced by toxic microbial products such as LPS. In this study, we report that a nontoxic polysaccharide-protein complex isolated from a Chinese medicinal herb, *Lyceum barbarum* (LBP), induces phenotypic and functional maturation of DCs with strong immunogenicity. LBP up-regulated DC expression of CD40, CD80, CD86, and MHC class II molecules; down-regulated DC uptake of Ag; enhanced DC allostimulatory activity; and induced IL-12p40 and p70 production. All of its five fractions were active. LBP developed enhanced Th1 response, and LBP-treated DCs enhanced Th1 and Th2 responses in vitro and in vivo. Our study provides evidence and rationale on using LBP in various clinical conditions to enhance host immunity and suggests LBP as a potent adjuvant for the design of DC-based vaccines. *The Journal of Immunology*, 2009, 182: 3503–3509.

*L. barbarum* (L. barbarum), also known as wolfberry, is a common Chinese herbal medicine as well as tonic. In thousands of years of traditional medicine in East Asia, it has been used in the treatment and prevention of diseases such as insomnia, liver dysfunction, diabetes, and visual degeneration. It is also used as a food supplement. One of its bioactive components is *L. barbarum* polysaccharide-protein complex (LBP),† which consists of six monosaccharides (galactose, glucose, rhamnose, arabinose, mannose, and xylose) and 18 aa (1, 2). It is a β-glycan possessing a backbone of (1→6)-β-galactosyl residues, about one-half of which are substituted at C-3 by galactosyl or arabinosyl groups. The carbohydrate is linked O-glycosidically to serine/threonine residues of the protein part (3, 4). The β-glycan structural aspect may contribute to their biologic function (5). Previous studies have shown that LBP can enhance the immune function (1, 2, 6), antagonize aging and oxidation (7–10), protect liver injury (11), lower blood glucose level (12), reduce the side effects of chemotherapy and radiotherapy (13, 14), and act against cancer (2, 15, 16). We speculate that the immunostimulatory activity of LBP is through activating dendritic cells (DCs).

DC represents a heterogeneous population of APCs that initiate primary immune response (17). These cells take up Ag in peripheral tissues and migrate to secondary lymphoid organs, where they become mature and competent in presenting Ag to T cells, thus initiating Ag-specific immune responses or immunological tolerance (18). DC immunogenicity correlates with its functionally mature state, which is characterized by high levels of expression of MHC and T cell co-stimulatory molecules, acute decrease in Ag uptake, and the ability to present Ag captured in the periphery to T cells (19). DC maturation can be induced by microbial products (such as LPS) or inflammatory cytokines (such as TNF) (20–22). Although these mediators are potent stimuli of DC maturation, they are toxic and have limited applications. In this regard, nontoxic vehicles that are able to induce DC maturation and immunogenicity are useful.

In the present study, we report that LBP induces DC maturation and enhances its immunogenicity. LBP stimulated DC production of IL-12p40 and p70 and promoted DC stimulation of Ag-specific Th1 and Th2 cells in vitro and Th1 immunity in vivo.

Materials and Methods

**Animals**

Female BALB/c and C57BL/6 mice, 6 wk old, were obtained from the Singapore Laboratory Animal Centre. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee, National University of Singapore.

**Preparation of LBP**

LBP was isolated from *L. barbarum*, as described previously (23). Briefly, *L. barbarum* dried fruit was extracted in water. The water extract was precipitated with ethanol, followed by removal of free proteins by Sevag reagent (CHCl3:n-BuOH = 4:1), dialysis against water, and freeze drying. LBP was separated by DEAE-cellulose ion exchange chromatography (successively eluted with water, followed by 0.05, 0.1, 0.2, and 0.5 M NaCl) and further purified by size exclusion chromatography (eluted with water). Five homogenous fractions, designated LBP fraction (LBPF)1, LBPF2, LBPF3, LBPF4, and LBPF5, were obtained (23). The molecular masses of LBPF1–4 were ~150 kDa, determined by SDS-PAGE. The molecular mass of LBPF5 was 293 kDa, determined by gel filtration (3, 23). The carbohydrate contents were 48.2, 30.5, 34.5, 20.3, and 23.5%, respectively, as determined by phenol-sulfuric acid assay (23, 24). The protein contents were 1.2, 4.8, 4.1, 13.7, and 17.3%, respectively, as measured by the Bradford method using protein assay kit (23). 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.

**Test of LPS contamination**

LPS contamination was tested by Limulus amebocytes lysate (LAL) assay and B-cell proliferation assay. LAL assay was performed using E-TOX-ATE kit (Sigma-Aldrich), according to the manufacturer’s instruction. Briefly, 100 μl of samples (10 mg/ml), LPS standards (0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 EU/ml), or endotoxin-free water (negative control) was added into each well of 96-well microtiter plate containing endotoxin-free water (100 μl). After 30 min at 37°C, 100 μl of LAL reagent (CHCl3:n-BuOH = 4:1) was added into each well. Absorbance was measured at 570 nm. The LPS contamination was determined by the 1.0 EU/ml level of LPS standard.

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was mixed with 100 μL of LAL in a microcentrifuge tube. The mixture was incubated at 37°C for 1 h and observed for gelation. A positive test is the formation of hard gel that permits complete inversion of the tube or vial without disruption of the gel. All other results (soft gels, turbidity, increase in viscosity, or clear liquid) are considered negative. B cell proliferation assay was performed by incubation of isolated BALB/c mouse spleen B cells (2 × 10^5/well) with LPS (0.01–10 μg/ml; Sigma-Aldrich; Escherichia coli serotype 026:B6), LBP (100 μg/ml), or LBPF1–5 (100 μg/ml) for 72 h. Cell proliferation was measured by ^[3]Hthymidine (GE Healthcare) incorporation.

**DC culture and activation**

Mouse bone marrow-derived DCs (BMDCs) were prepared, as previously described, with some modifications (25, 26). Briefly, bone marrow cells were harvested from the femurs and tibias of BALB/c mice and filtered through a 70-μm cell strainer (BD Falcon). RBC was lysed with Tris-NC, lysis buffer. Cells were washed and cultured in six-well plates at 10^6 cells/ml (3 ml/well) in RPMI 1640 (Sigma-Aldrich) supplemented with 5% FBS, 50 μM 2-ME, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin, in the presence of mouse GM-CSF (10 ng/ml; Pierce) and IL-4 (10 ng/ml; Pierce). On day 3, floating and loosely adherent cells were harvested and cultured in fresh medium. On day 6, nonadherent cells were harvested and cultured in 60-mm petri dishes at 10^6 cells/ml (5 ml/dish).

**Day 6 BALB/c BMDCs (10^6/ml) were pulsed with OVA (100 μg/ml) in the ELISPOT assay for IL-4 and IFN-γ measurement.**

**DC stimulation with LBP in vivo**

LBP (20 mg/kg) was administered s.c., i.p., or by mouth (p.o.) to BALB/c mice in groups of four. Naive mice without LBP injection were control. After 24 h, splenic DCs were isolated and examined for CD40, CD80, and CD86, and I-A/I-E expression by flow cytometry.

**Th cell response to OVA plus LBP in vivo**

BALB/c mice in groups of four were injected s.c. with OVA (10 μg) three times at 1-wk intervals, and were also given s.c., i.p., or p.o. LBP (20 mg/kg) daily. Mice given OVA or LBP alone were controls. After 21 days, splenocytes were harvested and restimulated with OVA (10 μg/ml) for 48 h in ELISPOT plates. IL-4- and IFN-γ-producing cells were determined.

**Real-time RT-PCR**

Total RNA was extracted from BMDCs using the Qiagen RNeasy mini kit and reverse transcribed to cDNA using the Invitrogen SuperScript first-strand synthesis system. PCR primers and fluorogenic probes for the target gene (IL-12p40) and endogenous control (β-actin) were purchased as TaqMan Gene Expression Assays (Applied Biosystems). PCR was performed in optical 96-well reaction plate on the ABI 7900 Fast Real-Time PCR System. Experiments were performed in triplicate. The thermal cycle conditions were 20-s hold at 95°C, followed by 50 cycles of 1 s at 95°C (denaturation) and 20 s at 60°C (annealing/extension). The relative expression of the target genes was calculated by comparative cycle threshold (C_T) method using the SDS 1.3.1 software. Values reported have a 95% confidence interval as determined by the software.

**ELISA**

IL-12p40 and p70 in the cell culture supernatant were quantified by sandwich ELISA using BD Pharmingen OptiEIA sets, according to the manufacturer’s instruction. The absorbance was measured at 450 nm with a reference of 570 nm using a spectrometer (Tecan Sunrise).

**Results**

LPS contamination was excluded by LAL assay and B cell proliferation assay. LAL assay is the most common method to test LPS contamination. The principle is that LAL will form hard gel when exposed to minute quantities of LPS. As shown in Table I, the lowest concentration of LPS capable of inducing gel formation was 0.03 EU/ml (i.e., 20 pg/ml), and at 0.015 EU/ml (i.e., 10
pg/ml) it did not induce hard gel formation. Gel formation was not induced by LBP or its fractions (LPBF1–5) at 10 mg/ml. In most experiments, LBP and its fractions were used at 100-fold lower concentration (0.1 mg/ml), which means less than 0.1 pg/ml LPS (if any). LPS is a strong B cell stimulus. We previously found that LBP do not activate B cells (23). Therefore, we used B cell proliferation assay to further exclude LPS contamination. Effective B cell proliferation was induced with LPS at 10 ng/ml (Fig. 1). However, at 100 μg/ml, neither LBP nor its fractions caused significant B cell proliferation (Fig. 1). These results indicate that LBP and LPBF1–5 are free of LPS contamination.

LBP induces DC maturation in vitro and in vivo
To determine whether LBP induces DC maturation, we generated DCs from BALB/c mouse bone marrow and stimulated them with LBP or its individual fractions. The expression of CD40, CD80, CD86, and MHC class II molecules was examined. Twenty-four-hour treatment with LBP or LPBF1–5 up-regulated all of these molecules to various extent, compared with medium control (Fig. 2A). The increase in mean fluorescence intensity was most

**FIGURE 1.** Test of LPS contamination by B cell proliferation assay. B cells were negatively selected from BALB/c mouse spleen and stimulated with LBP (100 μg/ml), LPBF1–5 (100 μg/ml), or LPS (0.01–10 μg/ml) for 72 h. Cell proliferation was measured by [3H]thymidine incorporation. Values are represented as mean ± SD of four replicates.

**FIGURE 2.** LBP induces DC maturation in vitro and in vivo. A, LBP induces DC maturation in vitro. Day 6 BMDCs were stimulated with LBP (100 μg/ml), LPBF1–5 (100 μg/ml), or LPS (1 μg/ml) for 24 h. B, LBP induces DC maturation in vivo. BALB/c mice were administered s.c., i.p., or p.o. with LBP (20 mg/kg) or LPS (2.5 mg/kg). Naive mice were negative control. Splenic DCs were isolated 24 h later. BMDCs and splenic DCs were stained with anti-CD11c (allophycocyanin) and anti-CD40, CD80, CD86, or I-A/I-E (FITC) mAbs, and analyzed by flow cytometry, with CD11c+ cells being gated. Results are representative of three independent experiments.
prominent for MHCII and CD86 by LBP, BLPF4, or LBPF5 stimulation. CD40 expression was weak, but there was still \( \frac{1}{2} \)-fold increase after treatment. Increase of CD80 was \( \frac{1}{2} \)-fold in all fractions except LBPF1. Overall, LBPF4 and LBPF5 appeared to be more potent than the other three fractions. These results indicate that DCs are phenotypically mature after LBP and LBPF1–5 stimulation in vitro.

Due to the fact that LBP is effective in inducing DC maturation in vitro and the difficulty in getting enough LBPF1–5 for in vivo study, we tested whether LBP (s.c., i.p., or p.o.) causes DC maturation in vivo. LPS (s.c., i.p., or p.o.) was positive control. Twenty-four hours after administration, mouse splenic DCs were isolated and analyzed. LBP administration via s.c. route was the most effective, inducing \( \frac{1}{2} \)-fold increase of all molecules (Fig. 2B). As a positive control, LPS (s.c) induced \( \frac{1}{2} \)-fold increase in all molecules. When LBP or LPS was applied through i.p. or p.o. route, the effects were weaker (Fig. 2B). These results indicate that LBP is able to induce DC maturation in vivo.

LBP strengthens DC allostimulatory activity
DC is a potent stimulator of allogeneic T cell proliferation in MLR (27). We next performed MLR using LBP-activated DCs (from BALB/c mice) and CD4\(^{+}\) T cells (from C57BL/6 mice). As a control, fresh bone marrow cells were cocultured with allogeneic CD4\(^{+}\) T cells, which caused 10-fold increase in proliferation. Immature DCs induced 26-fold increase (Fig. 3), but DCs, activated by LBPF fractions, especially LBPF2 and LBPF3, were more potent in allogeneic T cell stimulation (32- to 45-fold).

LBP down-regulates DC endocytosis
Efficient Ag uptake is a specific attribute of immature DCs, which are down-regulated upon maturation (20). In this experiment, DCs with or without LBP and LBPF1–5 activation were incubated with soluble FITC-dextran. Result shown in Fig. 4 is representative of three independent experiments. LBP- and LBPF1–5-treated DCs showed \( \frac{1}{2} \)-fold decrease in soluble dextran uptake (Fig. 4). When data from all three experiments were analyzed by Student’s t-test, \( p \leq 0.05; **, p \leq 0.01; ###, p \leq 0.001; \), compared with iDCs.

LBP induces IL-12 production at gene and protein level
Day 6 BMDCs were stimulated with LBP (1 \( \mu \)g-1 mg/ml), LBPF1–5 (100 \( \mu \)g/ml), or LPS (1 \( \mu \)g/ml) for 48 h. A, Dose response of LBP on IL-12p40 mRNA expression. B, Effects of LBP and LBPF1–5 on IL-12p40 mRNA expression. IL-12p40 mRNA expression was quantified by real-time RT-PCR relative to untreated DCs using \( \beta \)-actin as endogenous control. Values are represented as 95% confidence level of triplicate. C and E, Dose response of LBP on IL-12p40 and p70 production, respectively. D and F, Effects of LBP and LBPF1–5 on IL-12p40 and p70 production, respectively. IL-12p40 and p70 productions were measured by ELISA. Values are mean \( \pm \) SD of triplicate. *p \leq 0.05; **, p \leq 0.01; ###, p \leq 0.001, compared with medium control.

FIGURE 3. LBP strengthens DC allostimulatory activity. Day 6 BMDCs from BALB/c mice were stimulated with LBP, LBPF1–5 (100 \( \mu \)g/ml), or LPS (1 \( \mu \)g/ml) for 24 h, and then treated with mitomycin C (50 \( \mu \)g/ml) for 1 h. After wash, DCs (5 \( \times \) \( 10^6 \)) were cocultured with the allogeneic T cells (2 \( \times \) \( 10^6 \)) isolated from C57BL/6 mice for 72 h. T cell proliferation was measured by \( [\text{H}] \text{thymidine incorporation assay. Values are mean \( \pm \) SD of four replicates. FBMC, Fresh bone marrow cells from BALB/c mice; iDC, immature DC. *p \leq 0.05; **, p \leq 0.01, compared with iDCs.}

FIGURE 4. LBP down-regulates DC endocytotic capacity. Day 6 BMDCs were stimulated with LBP, LBPF1–5 (100 \( \mu \)g/ml), or LPS (1 \( \mu \)g/ml) for 24 h, followed by incubation with FITC-dextran (1 mg/ml) for 1 h at 37°C. As a control, cells were also incubated at 4°C. Cells were stained anti-CD11c (allophycocyanin) mAb and analyzed for FITC-dextran uptake by flow cytometry. Results are representative of three independent experiments.
LPS, mice injected with OVA-pulsed and LBP-stimulated DCs measured IFN-γ and IL-4 secretion of Th1 and Th2 cells in this experiment was determined by quantitative RT-PCR. At 100 µg/ml, LBP-induced IL-12p40 secretion from BMDCs in a dose-dependent manner (Fig. 5A). At 100 µg/ml, LBP induced p40 mRNA expression 2.6-fold. At 1 mg/ml, a 10-fold increase was observed. LBP at 100 µg/ml was as potent as LPS at 1 µg/ml in the induction of IL-12p40 mRNA (Fig. 5B). Among the five fractions, LBPF3 was most potent; it induced more IL-12p40 mRNA than crude LBP and LPS. LBPF2 and LBPF4 showed no difference from crude LBP. However, LBPF1 and LBPF5 showed markedly more IL-12p40 mRNA induction. In line with the RT-PCR results, LBP also induced IL-12p40 secretion from BMDCs in a dose-dependent manner (Fig. 5C). At 100 µg/ml, LBP stimulated BMDCs to produce 8.6 ng/ml IL-12p40, which is highly significant compared with unstimulated BMDCs (p < 0.01). IL-12p40 secretion was markedly increased when LBP was used at 0.5 and 1 mg/ml (Fig. 5C). Among the fractions, LBPF3 was most potent in inducing IL-12p40 secretion (Fig. 5D). It was noted that LBP and its fractions were less potent than LPS in inducing IL-12p40 secretion. This is particularly true when IL-12p70 secretion was examined. At 100 µg/ml, LBP significantly increased IL-12p70 production by DCs, but it requires 0.5–1 mg/ml to induce IL-12p70 to a level that is comparable to that induced by LPS (Fig. 5, E and F). Nonetheless, LBP indeed induced IL-12p70 from DCs.

LBP induces IL-12 production from DCs

Bioactive IL-12 is a disulfide-linked p35/p40 heterodimer, known as IL-12p70 (28). mRNA for p35 is constitutively expressed in many cell types, whereas the expression of the p40 mRNA is highly restricted and appears to be expressed only by cells that produce IL-12 (29, 30). To examine whether LBP induces IL-12 from DCs, BMDCs were stimulated with LBP or LBPF1–5 for 48 h. IL-12p40 mRNA was determined by quantitative RT-PCR. LBP induced IL-12p40 mRNA expression in BMDCs in a dose-dependent manner (Fig. 5A). Among the five fractions, LBPF3 was most potent; it induced more IL-12p40 mRNA than crude LBP and LPS. LBPF2 and LBPF4 showed no difference from crude LBP. However, LBPF1 and LBPF5 showed markedly reduced IL-12p40 mRNA induction. In line with the RT-PCR results, LBP also induced IL-12p40 secretion from BMDCs in a dose-dependent manner (Fig. 5C). At 100 µg/ml, LBP stimulated BMDCs to produce 8.6 ng/ml IL-12p40, which is highly significant compared with unstimulated BMDCs (p < 0.01). IL-12p40 secretion was markedly increased when LBP was used at 0.5 and 1 mg/ml (Fig. 5C). Among the fractions, LBPF3 was most potent in inducing IL-12p40 secretion (Fig. 5D). It was noted that LBP and its fractions were less potent than LPS in inducing IL-12p40 secretion. This is particularly true when IL-12p70 secretion was examined. At 100 µg/ml, LBP significantly increased IL-12p70 production by DCs, but it requires 0.5–1 mg/ml to induce IL-12p70 to a level that is comparable to that induced by LPS (Fig. 5, E and F). Nonetheless, LBP indeed induced IL-12p70 from DCs.

LBP promotes Th1 and Th2 responses in vitro

To investigate whether LBP-activated DCs exhibit augmented capacity in Ag presentation, BALB/c BMDCs were pulsed with OVA Ag and then activated with LBP and LBPF1–5. These cells were then cocultured with BALB/c CD4+ splenic T cells. Stimulation of Th1 and Th2 cells in this experiment was determined by measuring IFN-γ and IL-4-producing cells using ELISPOT assay. More IFN-γ and IL-4-producing cells were detected in wells in which T cells were cocultured with LBP- or LBPF1–5-stimulated DCs compared with wells in which T cells were cocultured with unstimulated DCs (Fig. 6) (p < 0.01–0.05). There was no difference in IL-2 induction among LBP, LBPF1–5, and medium control, but LPS reduced its production (p < 0.05). IFN-γ and IL-4 are characteristic of Th1 and Th2 cells, respectively. Therefore, DCs activated by LBP in vitro augment T cell differentiation to both Th1 and Th2 cells.

DCs activated by LBP in vitro enhance Th1 and Th2 responses in vivo

To investigate the immunogenicity of LBP-activated DCs in vivo, BMDCs were pulsed with OVA, activated with LBP in vitro, and then injected s.c. into mice. After 7 days, spleen cells were isolated and examined for IFN-γ and IL-4 production. Splenocytes from mice injected with OVA-pulsed and LBP-stimulated DCs produced more IFN-γ and IL-4 spots than those from mice injected with saline, OVA, untreated DCs, LBP-stimulated DCs, or OVA-pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5 pulsed DCs (Fig. 7) (p < 0.01–0.05, compared with untreated DCs). Splenocytes from mice injected with saline or single dose of OVA produced a few IFN-γ and IL-4 spots. A total of 2 × 10^5
LBP- and OVA-treated mice produced more IFN-γ and examined by ELISPOT assay. We found that splenocytes from LBP was given s.c., i.p., or p.o. daily. Splenic cells were isolated and immunogenicity both in vitro and vivo. Because LBP is a strong stimulus of DC activation, we first of all excluded its contamination in the sample by LAL assay and B cell proliferation assay; LAL assay showed that the amount of LPS in the sample, if any, was less than 0.015 EU (0.01 ng/mg sample) (negative). LBP or LBPF1–5 (100 μg/ml) failed to cause mouse B cell proliferation. In contrast, LPS, as low as 10 ng/ml, strongly activate mouse B cells. In addition, we previously found that only LBPF4 and LBPF5 are able to induce mouse splenocyte proliferation, not only LBPF1–5 and LBPF5. Overall, these results indicate that LBP and LBPF1–5 are free of LPS contamination. The effect of LBP on DCs is truly on its own.

We demonstrated that LBP and its five homogeneous fractions LBPF1–5 up-regulated the expression of MHC class II molecules, CD40, CD80, and CD86, on BMDCs, suggesting that LBP induces phenotypically mature DCs. Surprisingly, the result was reproducible in vivo. Administration of LBP via s.c., i.p., or p.o. routes induced splenic DC phenotypic maturation. LBP (s.c.) is most effective, perhaps because the in situ activated DCs migrate to the spleen more effectively through the lymphatic system. DC phenotypic maturation is related to its immunogenicity. Priming of T cells needs that both peptide-MHC complex and CD80/CD86 on APCs bind to TCR and CD28 on T cells, respectively (31).

LBP induces not only phenotypic, but also functional maturation of DCs, as characterized by decrease in Ag uptake and increase in the ability in allogeneic T cell stimulation and IL-12 production. IL-12 is a functional DC maturation marker with a molecular mass of 70 kDa composed of two subunits, p35 and p40. Neither p40 nor p35 alone appears to be bioactive; only a combination of soluble p40 with soluble p35 to form the heterodimeric p70 exhibits bioactivity (32). We found that LBP and LBPF-5 significantly enhanced the inducible IL-12p40 mRNA expression and protein production, and the functional p70 production by BMDCs. Both IL-12p40 and p70 protein levels were consistent with the p40 mRNA amount. It is noted that the concentration of IL-12p70 was 20 times lower than that of p40, indicating that most p40 is free and does not combine with the p35 subunit to form the heterodimeric p70. This is consistent with the previous findings (33). Cells that secrete bioactive IL-12p70 usually also secrete free p40 chains in ~10-fold excess, whereas secretion of significant amounts of p35 in the absence of p40 has not been found (34).

Most importantly, LBP-treated DCs are highly immunogenic. Immunogenic DCs can induce Th1 cell differentiation, Th2 cell differentiation, and/or CTL priming, depending on the nature of the maturation signal they received, as well as the constraints imposed by ontogeny and/or environmental modifiers (35). LBP-treated BMDCs induced higher levels of IFN-γ and IL-4 production from T cells in vitro, suggesting they are more potent in priming Th1 and Th2 response. Injection of LBP-treated BMDCs s.c. to mice resulted in the production of IFN-γ and IL-4 and examined by ELISPOT assay. We found that splenocytes from LBP-treated mice produced more IFN-γ and IL-4 spots, which were increased to ~500 in the mice injected with OVA-pulsed and LBP-stimulated DCs.

**LBP primes Th1 response in vivo**

Because LBP was shown to induce DC maturation in vivo, we examined whether it enhances DC immunogenicity in vivo. This was tested using two animal models. First, LBP was given s.c., i.p., or p.o. to mice. After 24 h, splenic DCs were isolated and cocultured with CD4+ T cells from naive mice. T cells cocultured with DCs from LBP-treated mice produced more IFN-γ spots than those cocultured with DCs from naive mice (Fig. 8, A and C) (**p < 0.001**). Few IL-4-producing cells were detected. In another experiment, OVA was injected s.c. into mice weekly for three times, and LBP was given s.c., i.p., or p.o. daily. Splenic cells were isolated and examined by ELISPOT assay. We found that splenocytes from LBP- and OVA-pulsed and LBP-stimulated DCs is truly on its own. Administration of LBP via s.c., i.p., or p.o. routes induced splenic DC phenotypic maturation. LBP (s.c.) is most effective, perhaps because the in situ activated DCs migrate to the spleen more effectively through the lymphatic system. DC phenotypic maturation is related to its immunogenicity. Priming of T cells needs that both peptide-MHC complex and CD80/CD86 on APCs bind to TCR and CD28 on T cells, respectively (31).

LBP induces not only phenotypic, but also functional maturation of DCs, as characterized by decrease in Ag uptake and increase in the ability in allogeneic T cell stimulation and IL-12 production. IL-12 is a functional DC maturation marker with a molecular mass of 70 kDa composed of two subunits, p35 and p40. Neither p40 nor p35 alone appears to be bioactive; only a combination of soluble p40 with soluble p35 to form the heterodimeric p70 exhibits bioactivity (32). We found that LBP and LBPF-5 significantly enhanced the inducible IL-12p40 mRNA expression and protein production, and the functional p70 production by BMDCs. Both IL-12p40 and p70 protein levels were consistent with the p40 mRNA amount. It is noted that the concentration of IL-12p70 was ~20 times lower than that of p40, indicating that most p40 is free and does not combine with the p35 subunit to form the heterodimeric p70. This is consistent with the previous findings (33). Cells that secrete bioactive IL-12p70 usually also secrete free p40 chains in ~10-fold excess, whereas secretion of significant amounts of p35 in the absence of p40 has not been found (34).

Most importantly, LBP-treated DCs are highly immunogenic. Immunogenic DCs can induce Th1 cell differentiation, Th2 cell differentiation, and/or CTL priming, depending on the nature of the maturation signal they received, as well as the constraints imposed by ontogeny and/or environmental modifiers (35). LBP-treated BMDCs induced higher levels of IFN-γ and IL-4 production from T cells in vitro, suggesting they are more potent in priming Th1 and Th2 response. Injection of LBP-treated BMDCs s.c. to mice resulted in the production of IFN-γ and IL-4 and examined by ELISPOT assay. We found that splenocytes from LBP-treated mice produced more IFN-γ and IL-4 spots, which were increased to ~500 in the mice injected with OVA-pulsed and LBP-stimulated DCs.

**LBP primes Th1 response in vivo**

Because LBP was shown to induce DC maturation in vivo, we examined whether it enhances DC immunogenicity in vivo. This was tested using two animal models. First, LBP was given s.c., i.p., or p.o. to mice. After 24 h, splenic DCs were isolated and cocultured with CD4+ T cells from naive mice. T cells cocultured with DCs from LBP-treated mice produced more IFN-γ spots than those cocultured with DCs from naive mice (Fig. 8, A and C) (**p < 0.001**). Few IL-4-producing cells were detected. In another experiment, OVA was injected s.c. into mice weekly for three times, and LBP was given s.c., i.p., or p.o. daily. Splenic cells were isolated and examined by ELISPOT assay. We found that splenocytes from LBP- and OVA-pulsed and LBP-stimulated DCs are free of LPS contamination. The effect of LBP on DCs is truly on its own.

We demonstrated that LBP and its five homogeneous fractions LBPF1–5 up-regulated the expression of MHC class II molecules, CD40, CD80, and CD86, on BMDCs, suggesting that LBP induces phenotypically mature DCs. Surprisingly, the result was reproducible in vivo. Administration of LBP via s.c., i.p., or p.o. routes induced splenic DC phenotypic maturation. LBP (s.c.) is most effective, perhaps because the in situ activated DCs migrate to the spleen more effectively through the lymphatic system. DC phenotypic maturation is related to its immunogenicity. Priming of T cells needs that both peptide-MHC complex and CD80/CD86 on APCs bind to TCR and CD28 on T cells, respectively (31).

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also showed similar effect. Furthermore, splenic DCs matured by LBP in vivo induced Th1 response. Mice given OVA together with LBP also showed higher level of Ag-specific Th1 response, indicating LBP has adjuvant activity.

Admittedly, LBP is not as strong as LPS in terms of inducing IL-12 production. However, it is comparable to LPS with regard to enhancement of Ag presentation. As we know, LPS is toxic and may cause severe inflammation. In contrast, LBP is prepared from food and is nontoxic. Administration of LBP by the safe p.o. route also showed moderate effect. This advantage makes it a good supplement for people whose immunity may be low or suppressed, such as those with cancer, chronic infection, and aging. Improvement of immunity in these cases can be achieved by simply oral consumption of wolfberry.

Overall, the data presented in this study show that LBP induces phenotypic and functional maturation of DCs with strong immunostimulating effects on human lymphocytes. Our study provides scientific support and rationale on the consumption of wolfberry.

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Disclosures
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References