

Lycium barbarum polysaccharides induce Toll-like receptor 2- and 4-mediated phenotypic and functional maturation of murine dendritic cells via activation of NF- κ B

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Abstract. Dendritic cells (DCs) are potent antigen-presenting cells that play pivotal roles in the initiation of primary immune responses. *Lycium barbarum* polysaccharides (LBPs) are known to have a variety of immunomodulatory functions. However, the cellular and molecular mechanisms underlying their therapeutic effects are poorly understood. In this study, we report that LBPs induce phenotypic and functional maturation of DCs. LBPs upregulated DC expression of I-A/I-E and CD11c, enhanced DC allostimulatory activity and induced IL-12p40 production. Furthermore, the activity of LBPs on DCs was significantly reduced by treating the cells with anti-TLR2 or anti-TLR4 antibody prior to LBPs, indicating that both are possible receptors of LBPs. Maturation of DCs by LBPs was able to directly activate the nuclear transcription factor NF- κ B p65. The results revealed that LBP stimulation induces the phenotypic and functional maturation of DCs via TLR2- and/or TLR4-mediated NF- κ B signaling pathways.

Introduction

Dendritic cells (DCs), one of the most potent antigen-presenting cells, are important for the initiation of the primary immune response of both helper and cytotoxic T lymphocytes (1-4). After antigen capture, the DC precursors migrate to T cell regions of draining lymph nodes where they mature into functional DCs. The functional DCs further stimulate naive T cells by triggering the signaling pathway involving both major histocompatibility complex molecules presenting

antigen-peptides and costimulatory molecules (5-6). DCs are also highly responsive to inflammatory cytokines, such as tumor necrosis factor (TNF)- α and lipopolysaccharide (LPS), which induce a series of phenotypic and functional changes in DCs (7-8). Similar changes indicative of maturation have also been reported following infection with mycoplasma, viruses, intracellular bacteria and parasites (9-11). In addition, certain plant polysaccharides, including *Astragalus mongholicus* polysaccharides, induce regulatory effects on the phenotypic and functional maturation of DCs (12).

Toll-like receptors (TLRs), which are a family of pattern recognition receptors (PRRs), play an essential role in the recognition of microbial components in mammals. Of the 10 TLRs discovered to date, TLR2 is activated primarily by lipoproteins and glycolipids (13-16), and TLR4 is predominantly activated by LPS and lipoteichoic acid (17-18). Recent studies showed that TLR2 and TLR4, which are the signaling components of LPS and consequently trigger its cellular transduction, led to NF- κ B activation and DC maturation (19-21). The adjuvant activity of bacterial products is important not only for antibacterial responses induced by peripheral DCs but also for vaccine development. LPS is the main cause of septic shock in humans due to its high toxicity; therefore, it is important to find an alternative substance to LPS.

Lycium barbarum, a well-known Chinese traditional medicine and also an edible food, plays multiple roles in pharmacological and biological processes. One of its bioactive components is *Lycium barbarum* polysaccharides (LBPs). LBPs are effective in regulating phenotypic and functional maturation of murine DCs (22). However, there are no studies on the molecular mechanism by which LBPs induce maturation of murine DCs.

Materials and methods

Source of mice. Male or female C57BL/6j (H-2^b) and BALB/c (H-2^d) mice were purchased from the Department of Experimental Animals, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, China. Mice were used at 4-6 weeks of age. The study was approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine, Hangzhou, China.

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Source of drugs. LBPs for clinical application with an endotoxin content <0.1 Eu/mg were purchased from Pharmagenesis Inc. (Newtown, PA, USA). The percentage of LBPs was 84.32% according to the phenol-sulfuric acid colorimetric method. The molecular weights of LBPs were estimated to be 31,000 Da according to high performance gel filtration chromatography. The LBPs mainly consist of mannose, glucose, galactose, arabinose, rhamnose and xylose.

Generation of bone marrow-derived murine myeloid DCs. DCs were prepared as described previously in detail, with minor modifications (23). Briefly, bone marrow cells were flushed from the femur and tibiae of C57BL/6j mice and depleted of red blood cells by hypotonic lysis using Tris-NH₄Cl (pH 7.2). Cells with a starting counting number of 2x10⁶ cells/ml were cultured in RPMI-1640 medium in six-well flat bottom plates (Orange Scientific, Braine-l'Alleud, Belgium) at 37°C, in 5% CO₂, supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, 30 ng/ml rmGM-CSF (Peprotech, Inc., Rocky Hill, NJ, USA) and 20 ng/ml rmIL-4 (Peprotech, Inc.). On day 3, the old medium was replaced with fresh medium. On day 5, cells were purified by MACS columns (Miltenyi Biotec, Auburn, CA, USA). CD11c⁺ DCs were acquired and divided into 5 groups. In parallel, 5 groups of DCs were incubated at a concentration of 5x10⁵/ml with 100 µg/ml LBPs, serum-free RPMI-1640 media, 100 ng/ml LPS (Sigma, St. Louis, MO, USA) and 100 µg/ml LBPs preincubated with antibody (anti-TLR2 or anti-TLR4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h, respectively. The serum-free RPMI-1640 media group and 100 ng/ml LPS group were used as controls. On day 7, cells and culture supernatants were collected for further experiments and analysis.

Flow cytometric analysis. Cell surface expression of I-A/I-E or CD11c was determined by immunofluorescence staining. On day 7, cells were harvested, washed twice with PBS, and resuspended in washing buffer (PBS containing 2% FCS and 0.1% sodium azide). Cells were first blocked with 20% mixed serum of mice and rat (Bohua Company, Shanghai, China) for 15 min at 4°C to reduce the background and then stained with R-PE conjugated anti-mouse I-A/I-E antibody (BD Pharmingen, San Diego, CA, USA) and FITC-conjugated anti-mouse CD11c antibody (BD Pharmingen) at 4°C in the dark. Thirty minutes later, the antibody-treated cells were washed twice with washing buffer. Cell surface co-expression of I-A/I-E and CD11c was detected using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Histogram and density plots were produced by the CellQuest software package (Becton Dickinson).

Cytokine assay. On day 7, DC culture supernatants were collected and then the concentration of mouse IL-12 p40 was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Bethesda, MD, USA) according to the manufacturer's instructions. Cytokine concentrations were determined according to absorbance readings at 450 nm on a universal microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Mixed lymphocyte reaction (MLR) induced by DCs. Responder mononuclear lymphocytes from H-2^d BALB/c splenocytes were isolated by Ficoll-Urografin density gradient. On day 7, mature DCs were harvested as inducers, treated with 25 µg/ml mitomycin C (AppliChem, Darmstadt, Germany) for 45 min, then 5x10³ cells were added to allogeneic lymphocytes (1x10⁵ cells per well) in flat-bottom 96-well tissue culture plates for 120 h. Cell proliferation was estimated according to the cellular reduction of tetrazolium salt MTT (Sangon, Shanghai, China) by the mitochondrial dehydrogenase of viable cells into a blue formazan product that can be measured spectrophotometrically.

DC ultrastructure. On day 7, DCs were harvested and fixed in methanol and air-dried, and then processed with scanning electron microscopy (Cambridge Scientific Instruments Ltd., Cambridge, UK) according to the manufacturer's instructions.

Nuclear protein extraction and western blot analysis. On day 7, DCs were harvested and the medium was removed, 5 groups of DCs (5x10⁶) were suspended in 1 ml of ice-cold PBS (pH 7.2), centrifuged at 1,000 x g for 5 min, resuspended in 400 µl of ice-cold hypotonic buffer (10 mM HEPES-KOH pH 7.9, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 1 µg/ml leupeptin and 1 mM PMSF), left on ice for 10 min, vortexed and centrifuged at 15,000 x g (at 4°C for 30 sec). Pelleted nuclear protein was resuspended in 50 µl of ice-cold saline buffer (50 mM HEPES-KOH pH 7.9, 10% glycerol, 300 mM NaCl, 1.5 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 µl/ml leupeptin and 1 mM PMSF), left on ice for 20 min, vortexed and centrifuged at 15,000 x g (at 4°C for 10 min). The protein concentration was determined and aliquots were stored at -70°C. Thirty micrograms of nuclear protein was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then was transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in TBST for 1 h at room temperature and then incubated with rabbit anti-murine NF-κB p65 antibody (Rockland Immunochemicals, Gilbertsville, PA, USA) for 1 h. After washing three times in TBST, the membrane was incubated with HRP-conjugated goat anti-rabbit-IgG (Rockland Immunochemicals) for 1 h and the antibody-specific protein was visualized by an enhanced chemiluminescence detection system.

Statistical analysis. The results were expressed as the means ± standard deviation (SD) of the indicated number of experiments. The statistical significance was estimated using a t-test for unpaired observations. P<0.05 was considered to indicate a statistically significant difference.

Results

DCs treated by LBPs show a more mature morphology in a TLR2- and 4-mediated manner. On day 7, DCs were harvested and processed for scanning electron microscopy, as shown in Fig. 1. LBPs (100 µg/ml)- or LPS (100 ng/ml)-treated DCs showed a more mature morphology with long protrusions. Untreated DCs, LBPs plus anti-TLR2 and LBPs plus anti-TLR4-treated DCs had shorter protrusions than the LBPs- or LPS-treated DCs.

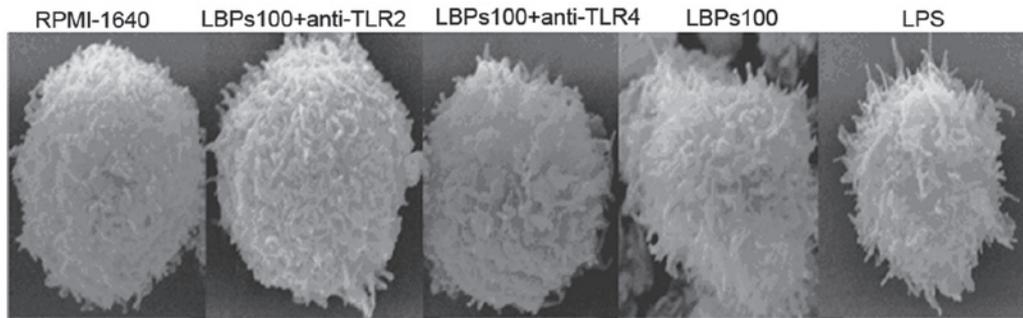


Figure 1. Compared with the RPMI-1640, LBP- or LPS-treated DCs showed the characteristic morphology of mature DCs. On day 7 when analyzed by a scanning electron microscope (Cambridge Scientific Instruments Ltd.), LBP- or LPS-treated DCs exhibited evident needle-like protrusions. LBP, *Lycium barbarum* polysaccharides; LPS, lipopolysaccharide; DC, dendritic cells.

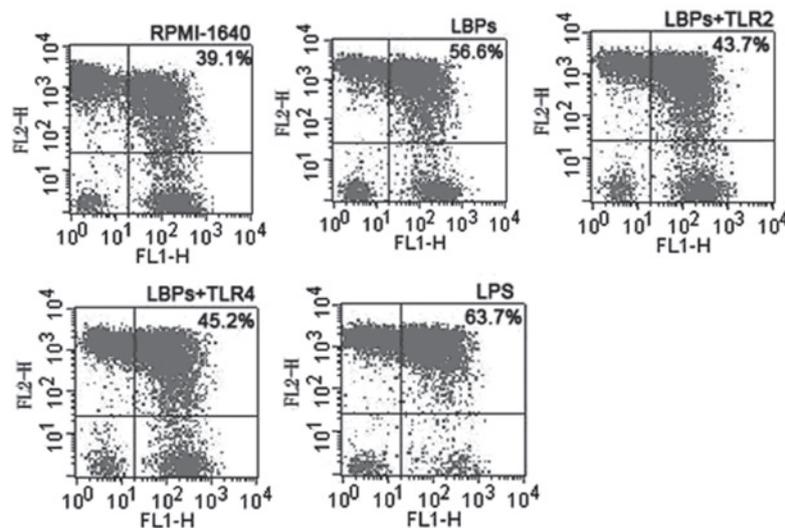


Figure 2. Representative flow cytometric analysis of DCs treated with RPMI-1640, LBPs, LBPs plus anti-TLR2 or anti-TLR4 Ab, and LPS. On day 5 of DC culture, cells were incubated at a concentration of 1×10^5 cells per well with 100 ng/ml LPS, 100 $\mu\text{g/ml}$ LBPs, LBPs plus anti-TLR2 and anti-TLR4 Ab and RPMI-1640. On day 7, cells were stained with R-PE conjugated anti-mouse antibody. DCs, dendritic cells; LBP, *Lycium barbarum* polysaccharides; LPS, lipopolysaccharide.

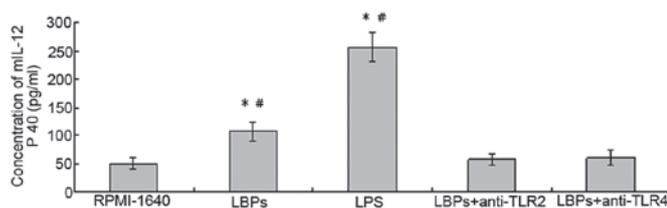


Figure 3. Production of IL-12 p40 in culture supernatants of DCs treated with RPMI-1640, LBPs, LBPs plus anti-TLR2, LBPs plus anti-TLR4 and LPS. On day 7, culture supernatants were collected for detecting the level of mouse IL-12 p40 (by ELISA). Cytokine concentration was determined with a standard curve derived from known amounts of the IL-12 p40, $n=5$. * $P < 0.01$ vs. RPMI-1640, # $P < 0.01$ vs. LBPs plus anti-TLR2 or anti-TLR4. DCs, dendritic cells; LBPs, *Lycium barbarum* polysaccharides; LPS, lipopolysaccharide.

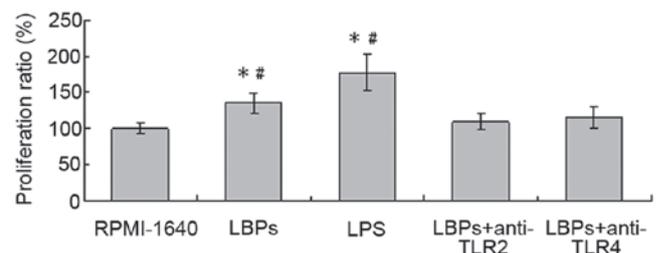


Figure 4. Lymphocyte proliferation of MLR induced by DCs. On day 5 of DC culture, cells were incubated with 100 ng/ml LPS and LBPs (100 $\mu\text{g/ml}$). RPMI-1640 was used as control. On day 7, mature DCs were treated with 25 $\mu\text{g/ml}$ mitomycin C and mononuclear lymphocytes from splenocytes were isolated by Ficoll-Urografin density gradient. H-2^d BALB/c responder spleen lymphocytes (1×10^5 cells per well) were cultured with H-2^b C57BL/6j inducer mature LBPs-treated DCs, RPMI-1640-treated DCs, LPS-treated DCs and LBPs plus anti-TLR2 or LBPs plus anti-TLR4 treated DCs (all 5×10^3 cells per well). Cell proliferation was estimated by the MTT method. $n=5$. * $P < 0.05$ vs. RPMI-1640. MLR, mixed lymphocyte reaction; DCs, dendritic cells; LBPs, *Lycium barbarum* polysaccharides; LPS, lipopolysaccharide.

LBPs upregulate the co-expression of I-A/I-E and CD11c on the DC surface in a TLR2- and 4-mediated manner. LBPs (100 $\mu\text{g/ml}$) or LPS (100 ng/ml)-treated DCs showed an increased co-expression of I-A/I-E and CD11c on the DC surface, and the double positive cell ratios were 57.4 ± 7.2 or $65.2 \pm 9.0\%$, respectively, compared with $38.7 \pm 4.8\%$ in the RPMI-1640 DCs. Additionally, LBPs (100 $\mu\text{g/ml}$) plus

anti-TLR2 or LBPs (100 $\mu\text{g/ml}$) plus anti-TLR4 showed a decreased co-expression of I-A/I-E and CD11c on the DC surface, and the double positive cell ratios were 45.4 ± 6.3 or

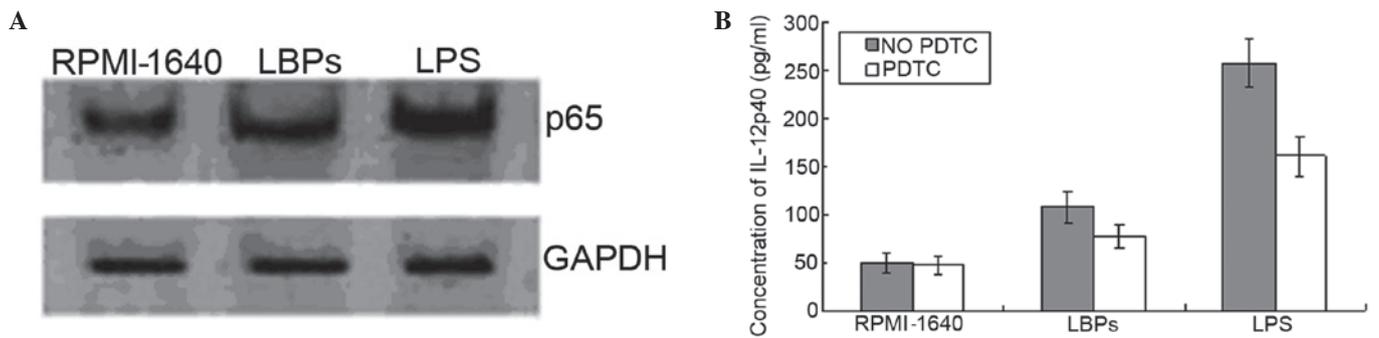


Figure 5. (A) Western blot analysis of NF- κ B in DCs treated with LBPs (100 μ g/ml) or LPS (100 ng/ml). (B) Effects of the NF- κ B-specific inhibitor, PDTC, on LBP-induced IL-12p40 production. NF- κ B, nuclear factor- κ B; DCs, dendritic cells; LBPs, *Lycium barbarum* polysaccharides; PDTC, pyrrolidinedithiocarbamate.

42.3 \pm 5.0%, respectively, compared with cells treated with LBPs only. The difference was significant according to the paired t-test analysis ($n=5$, $P<0.01$). One group of flow cytometric analysis is shown (Fig. 2).

LBPs increase IL-12 p40 production of DCs in a TLR2- and 4-mediated manner. LBPs (100 μ g/ml) or LPS (100 ng/ml)-treated DCs showed an increased production of IL-12 p40 in culture supernatants, and the levels were 107.2 \pm 16.9 and 257.3 \pm 24.9 pg/ml, respectively, compared with 49.6 \pm 10.0 pg/ml for RPMI-1640 control DCs. Additionally, LBPs plus anti-TLR2- or LBPs plus anti-TLR4-treated DCs did not show a marked difference in production of IL-12 p40 in culture supernatants, and the levels were 57.4 \pm 11.4 or 60.2 \pm 14.6 pg/ml, compared with the LBPs only group (107.2 \pm 16.9 pg/ml). The difference was significant according to the paired t-test analysis ($n=5$, $P<0.05$; Fig. 3).

LBPs facilitate the allostimulatory capacity of DCs in a TLR2- and 4-mediated manner. The effects of LBPs on the MLR induced by DCs were illustrated in Fig. 4. LBPs (100 μ g/ml)-treated DCs or LPS (100 ng/ml)-treated DCs stimulated proliferative responses more effectively than the RPMI-1640-treated DCs, and the proliferation ratio of the lymphocytes was 135.7 \pm 13.8 or 177.3 \pm 24.9%, respectively, compared with 100 \pm 7.0% for RPMI-1640-treated cells only. Additionally, LBPs-treated DCs stimulated proliferative responses more effectively than LBPs plus anti-TLR2- or LBPs plus anti-TLR4-treated DCs, and the proliferation ratio of the lymphocytes was 109.9 \pm 11.7 or 114.9 \pm 15.1% (Fig. 4). The difference was significant according to the paired Student's t-test analysis ($n=5$, $P<0.05$). RPMI-1640 control served as 100%.

Activation of NF- κ B in DCs treated with LBPs. To elucidate whether the NF- κ B pathway was activated by LBPs in DCs, DCs were treated with LBPs (100 μ g/ml) or LPS (100 ng/ml), and activation of the NF- κ B pathway was measured as nuclear translocation of the NF- κ B p65 subunit by western blot analysis. The content of p65 in the nuclear extract increased significantly in response to LBPs (100 μ g/ml) treatment as compared with that in the nuclear extract of the untreated DCs (Fig. 5).

Discussion

It has been known for decades that LBPs are biologically active components of *Lycium barbarum* that have potential pharmacological and biological functions. DCs are potent antigen-presenting cells that initiate primary immune responses and antigen-specific adaptive immunity (1). DC immunogenicity correlates to its functionally mature state. DCs can differentiate from immature to mature stages by stimulation with microbial products (such as LPS) or inflammatory cytokines (such as TNF) (24–26). However, these stimulators are toxic and have limited applications. It has been reported that a number of polysaccharides in plants, including *Ganoderma lucidum* polysaccharides, *Phellinus linteus* polysaccharides and *Coriolus versicolor* polysaccharides, may stimulate the induction of DC maturation (27–29).

In the present study, we have showed that LBPs are capable of inducing the maturation of BM-derived DCs. LBPs significantly increased membrane molecules, including CD11c and I-A/I-E, and IL-12 in DCs. LBPs could also help DC to strengthen the activation of the proliferation of allogenic lymphocytes and make DC demonstrate a characteristic morphology. However, the molecular basis of the signal transduction pathway activated by the polysaccharides is not clearly understood. In particular, it is unknown which cell surface receptors played a role in signal transduction. TLRs are a class of proteins that play a key role in the recognition of invading pathogens and activation of cytokine production by DCs and macrophages. The members of the TLR family play different roles in PRR signaling. Furthermore, it has been suggested that different mechanisms may control IL-12 and TNF- α production (30–32). The stimulation of TLRs leads to the activation of several transcription factors, including NF- κ B. In this study, it was shown that the stimulation of murine DCs by LBPs via TLR2 and/or TLR4 resulted in the activation of NF- κ B. NF- κ B activation is necessary for the expression of a variety of cytokines in the proinflammatory cytokine and LPS responses. At the same time, we used PDTC, a potent NF- κ B inhibitor, to inhibit LBPs-induced NF- κ B p65 subunit nuclear translocation, as well as to cause the downregulation of IL-12 secretion. This suggested that the NF- κ B pathway is important in the LBPs-mediated TLR signaling in DCs.

LBP have been widely used as an injection in clinical patients in China to improve immune functions. Our study makes the mechanism involved in this process clearer than has previously been known. According to the results of this study, LBPs are effective in the induction of the phenotypic and functional maturation of DCs, and are effective for anti-tumor DC-based therapy.

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