

## Chapter 2

# Immunoregulation and *Lycium Barbarum*

Xiaorui Zhang, Wenxia Zhou and Yongxiang Zhang

**Abstract** Widespread pharmacology research has revealed that immune regulation is the main biological effect of *Lycium barbarum*, and the material bases to elicit the effect of *L. barbarum* on the immune system are polysaccharide and glycoprotein complexes. Studies have found that the effect of *L. barbarum* polysaccharide on the activation of macrophages and dendritic cells are important in participating in the immune response. In addition, it was found that TLR4/2 may be closely related to the immunoregulatory activity of *L. barbarum* polysaccharide. Further, the TLR4/2-activated signaling pathways lead to activation of phosphoinositide-3-kinase (PI3K) and LKB1, leading to activation of the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and nuclear factor- $\kappa$ B (NF- $\kappa$ B), p53, C-Jun and AP-1. These pathways lead to induction of gene transcription. *L. barbarum* can induce production of a variety of cytokines, such as the anti-inflammatory factor IL-10, proinflammatory cytokines IL-1 $\beta$  and IL-6, chemokines IL-8, antitumor factors cytokine TNF- $\alpha$ , antiviral factor IFN- $\gamma$ , TGF- $\beta$ 1, and lymphocyte activators IL-2 and IL-4. *L. barbarum* polysaccharide could also upregulate CD40, CD80, CD86, and major histocompatibility complex (MHC) class II molecules to various extents, and enhance antibody titers. Ultimately, activation of these transcription pathways induces expression of pro-inflammatory cytokines and immune regulation, survival, and proliferation.

**Keywords** *Lycium barbarum* polysaccharide · Polysaccharide-protein complex · Lymphocytes · Macrophage · TLR4 · NF- $\kappa$ B · AP-1

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## 2.1 Introduction

Widespread pharmacology research has revealed that immune regulation is the main biological effect of *Lycium barbarum*. Research indicates that the main material bases to elicit the effect of *L. barbarum* on the immune system are polysaccharide and glycoprotein complexes, in addition to other substances such as volatile oils, vitamins, and so on, which also exert certain immune effects (Huang et al. 2001; Tian et al. 1995; Zhao et al. 1996). At present, the immune regulation mechanism of *L. barbarum* is known to be mainly related to the following aspects. First, *L. barbarum* polysaccharides or glycoprotein compounds can activate macrophages, dendritic cells, and T cells to achieve cellular and humoral immune responses (Chen et al. 2008b; Nan et al. 2012). Second, a study found that the activity of *L. barbarum* polysaccharide (LBP) is related to TLR4/2 and that LBP can influence the PI3K/Akt/FoxO1, LKB1/AMPK, JNK/c-Jun, MEK/ERK, and nuclear factor kappa B pathways (Xiao et al. 2013). However, thus far, the exact molecular mechanisms underlying the effects of LBP are still unclear, especially the interaction of the polysaccharide with its receptors and its binding molecules. *L. barbarum* has various biological activities, such as modulation of blood vessels and blood flow, antitumor activity, prevention of neurodegeneration from Alzheimer's disease, stimulation of neurogenesis to improve sexual function, neuroprotective effects in ischemic stroke, skin-related effects from oral and topical preparations and improvement of vision and glaucoma (Chan et al. 2007; Ho et al. 2010). Indeed, wide-spectrum for the effects of *L. barbarum* is not surprising. Under physiological conditions, the immune system itself is involved in every system of the body via cytokines and the antibody network, including systems such as the nervous system. Under pathophysiological conditions, all diseases are directly or indirectly associated with the immune responses. To better outline the effects of *L. barbarum* on the immune system and the underlying mechanisms, this section will focus on our current understanding of the target cells of *L. barbarum*, activity-related receptors of LBP, *L. barbarum*-associated intracellular signal transduction, and effects of *L. barbarum* on the production of cytokines, antibodies, and some other functional molecules in the immune system.

## 2.2 Material Basis of Immunomodulation

The *L. barbarum* phytochemical diversity includes polysaccharides, carotenoids, flavonoids, alkaloids (Wang et al. 2011), peptides (Yuan et al. 2008), sterols (Park et al. 2012), organic acids (Inbaraj et al. 2010; Dong et al. 2013), essential oils (Altintas et al. 2006), glycolipids (Gao et al. 2008), polyphenols (Dong et al. 2011) and so on (Wu et al. 2012; Wang et al. 2010a). Presently, only polysaccharides and polysaccharide-protein complex have been reported to possess immune regulation activity.

### 2.2.1 Polysaccharides

Polysaccharides are the main active components of *L. barbarum* and also form the basis of its immunoregulatory activity. In previous studies, the calculated extraction yield of polysaccharides from the fruit of *L. barbarum* was approximately 9.43~13% (Archer and Mench 2014; Li et al. 2007), and the yield of polysaccharides from the leaves of *L. barbarum* was 16.2% (Liu et al. 2012). *L. barbarum* polysaccharides (LBPs) are known to have a variety of immunomodulatory functions (Zhu et al. 2007). LBPs are capable of promoting both the phenotypic and functional maturation of murine bone marrow-derived dendritic cells (BMDC) in vitro. LBPs can also increase peripheral WBC counts of chemotherapy-induced myelosuppressive mice to some extent, but there are no significant differences when compared with controls, and LBPs can obviously stimulate human PBMCs to produce G-CSF (Amagase et al. 2008). Moreover, it has been reported that LBPs can be used in compensating for the decline in total antioxidant capacity (TAOC), immune functions, and the activities of antioxidant enzymes and thereby reducing the risks of lipid peroxidation accelerated by age-induced free radicals (Li et al. 2007). These studies indicate that polysaccharides are one of the main immunoregulatory components of *L. barbarum*.

### 2.2.2 Polysaccharide-Protein Complex

Five homogenous polysaccharide-protein complexes have been obtained from *L. barbarum* fractions, designated LBPF1, LBPF2, LBPF3, LBPF4, and LBPF5. The carbohydrate contents of LBPF1–5 were 48.2, 30.5, 34.5, 20.3, and 23.5%, respectively, as measured by phenol-sulfuric acid assays using glucose as the standard. The protein contents were 1.2, 4.8, 4.1, 13.7, and 17.3%, respectively, as measured by the Bradford method (Chen et al. 2008a). Research has found that LBPF1–5 can activate T lymphocytes and enhance the Th1 response, and polysaccharide–protein complex-treated DCs displayed enhanced Th1 and Th2 responses in vitro and in vivo (Chen et al. 2008b, 2009a). Another fraction, LBP3p, at 10 mg/kg upregulate phagocytic activities of macrophages, altered the antibody type secreted by spleen cells, increased proliferation of spleen lymphocytes, increased CTL activity, increased the mRNA expression level for IL-2 and reduced the lipid peroxidation in S180-bearing mice (Gan et al. 2004). A polysaccharide–protein complex isolated from *L. barbarum* can also enhance innate immunity by activating macrophages (Chen et al. 2009b). These reports indicate that polysaccharide-protein complex is another important immunoregulation component of *L. barbarum*.



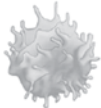

### 2.2.3 Others

Polyphenols and essential oil have immunoregulatory functions. Several biological activities have been described for polyphenolic compounds, including a modulator effect on the immune system. The effects of these biologically active compounds on the immune system are associated with processes such as differentiation and activation of immune cells (Cuevas et al. 2013). The essential oil constituents from aromatic herbs and dietary plants include monoterpenes, sesquiterpenes, oxygenated monoterpenes, oxygenated sesquiterpenes and phenolics, among others. Various mechanisms, such as enhancement of immune function and surveillance, are responsible for their chemopreventive properties (Bhalla et al. 2013). Polyphenols and essential oil from *L. barbarum* have been reported. However, it is unclear of whether the polyphenols and the essential oil are the immune regulation active component of *Lycium barbarum*.

## 2.3 Immunomodulatory Mechanism

The immunostimulatory activity of *L. barbarum* was studied and described almost 13 years ago (Huang et al. 2001; Peng et al. 2001). Shortly after those reports, the effects of *L. barbarum* polysaccharide-protein complex (LBP3p) on the expression of interleukin-2 and TNF- $\alpha$  in human peripheral blood mononuclear cells were investigated by reverse transcription polymerase chain reaction (RT-PCR) and bioassay (Gan et al. 2003). Then, modulation of a polysaccharide-protein complex from *L. barbarum* (LBP3p) on the immune system in S180-bearing mice was investigated (Gan et al. 2004). The neuroprotective effects of *L. barbarum* in a rat chronic ocular hypertension model via immunomodulation of macrophages/microglia was observed (Ip et al. 2006). LBPs were then reported to regulate phenotypic and functional maturation of murine dendritic cells (Zhu et al. 2007). In 2008, the first report of immune modulation by a standardized *L. barbarum* fruit (Goji) juice in randomized, double-blind, placebo-controlled clinical studies was published (Amagase et al. 2008). After that, many studies observed the target cells of *L. barbarum*, including T lymphocytes, macrophages, and dendritic cells (Chen et al. 2008b, 2009a, b; Zhang et al. 2011). More recently, the molecular mechanism and activity-related receptors were further reported, including I kappa B phosphorylation, as well as NF- $\kappa$ B, p65, p50, TLR4, and TLR2 (Chen et al. 2012; Wu et al. 2012; Zhu et al. 2013; Zhang et al. 2014b). A summary of the cellular targets and signal transduction pathways of *L. barbarum* is provided in Table 2.1 and Fig. 2.1. As many studies have reported the action of *L. barbarum* in activating innate immunity, affecting adaptive immunity, and inducing humoral and cell-mediated immune responses to better understand the immunoregulatory mechanisms of *L. barbarum*, we will discuss the issue of the target cells of *L. barbarum*, the active related receptors for LBP, the *L. barbarum*-associated signal transduction molecules, and also the changes of cytokines, antibodies, and leukocyte differentiation antigens affected by *L. barbarum* or LBP.

**Table 2.1** Effect of *L. barbarum* on immune target cell

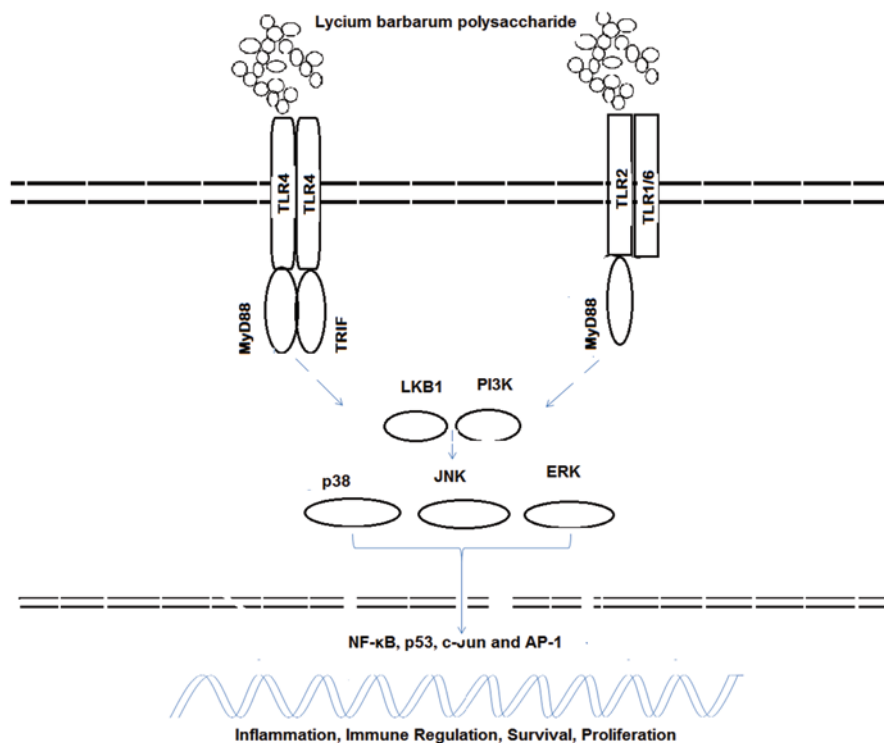
<div><b>Dendritic Cell</b></div> <div></div> <div><div>Promotes: Maturation Antigen presenting</div><div>Increases: IL-12p40 IL-12p70 CD11c I-<math>\beta</math>-E</div><div>CD40 CD80 CD86</div></div>	<div><b>T lymphocyte</b></div> <div></div> <div><div>Promotes: Activation Proliferation</div><div>Increases: IL-2 IFN-<math>\gamma</math> NFAT AP-1 CD25</div></div>
<div><b>Macrophage</b></div> <div></div> <div><div>Promotes: Maturation Antigen presenting</div><div>Increases: IL-12p40 TNF-<math>\alpha</math> IL-1 <math>\beta</math> Nitric oxide NF-<math>\kappa</math>B AP-1</div><div>CD40 CD80 CD86 CD11c I-<math>\beta</math>-E</div></div>	<div><b>NK cell</b></div> <div></div> <div><div>Promotes: Cytotoxicity</div><div>Increases: IFN-<math>\gamma</math> perforin</div><div>Inhibits: Apoptosis Necrosis</div></div>

2.3.1 Target Cells

2.3.1.1 Dendritic Cells

Dendritic cells (DCs) represent a heterogeneous population of antigen-presenting cells that initiate the primary immune response (Banchereau et al. 2000). These cells take up antigens in peripheral tissues and migrate to secondary lymphoid organs where they become mature and competent in presenting antigens to T cells, thus initiating antigen-specific immune responses or immunological tolerance (Guermonprez et al. 2002). DC immunogenicity correlates with the DC functionally mature state, which is characterized by high-level expression of MHC and T cell costimulatory molecules, acute decreases in antigen uptake, and the ability to present antigens captured in the periphery to T cells (Wilson and Villadangos 2005). DC maturation can be induced by microbial products (such as LPS) or inflammatory cytokines (such as TNF) (Winzler et al. 1997). Although these mediators are potent stimuli of DC maturation, they are toxic and have limited applications. In this regard, as biological response modifiers (BRMs), polysaccharides are able to induce DC maturation and immunogenicity.

LBP are known to exhibit immunomodulatory functions, including activation of B cells and natural killer (NK) cells. However, little is known about the immunomodulatory effects of LBP on DC. The effects of LBP on the phenotypic and



**Fig. 2.1** Schematic model illustrating the signaling pathways involved in macrophage activation by *Lycium barbarum* polysaccharides. *L. barbarum* polysaccharides can activate macrophages via Toll-like receptor 4 (TLR4) and TLR2. TLR4/2-activated signaling pathways lead to activation of phosphoinositide-3-kinase (PI3K) and LKB1, leading to activation of the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and nuclear factor-κB (NF-κB), p53, C-Jun, and AP-1. Ultimately, these pathways lead to induction of gene transcription. TLR4 ligation leads to the activation of IL-1R-associated kinase (IRAK) via adaptor myeloid differentiation protein 88 (MyD88), with subsequent activation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF-6), MAP kinases (e.g., p38 and JNK) and NF-κB. Activation of these transcription pathways induces expression of pro-inflammatory cytokines and immune regulation, survival and proliferation

functional maturation of murine BMDC were investigated *in vitro*. Compared with BMDC in the control group that was exposed to RPMI 1640 only, the co-expression of I-A/I-E, CD11c, and secretion of IL-12 p40 from BMDC were increased by LBPs (100 µg/ml). In addition, the endocytosis of FITC-dextran by LBPs-treated BMDC (100 µg/ml) was impaired, whereas the activation of proliferation of allogenic lymphocytes by BMDC was enhanced. The results strongly suggest that LBPs are capable of promoting both the phenotypic and functional maturation of murine BMDC (Zhu et al. 2007). Both LBPs and polysaccharide-protein complex isolated from *L. barbarum* can induce phenotypic and functional maturation of DCs with strong immunogenicity. Research has demonstrated that LBPs upregulate DC expression of CD40, CD80, CD86, and MHC class II molecules; down-regulate

DC uptake of antigens; enhance DC co-stimulatory activity; and induce IL-12p40 and p70 production. Of all five fractions, LBPF1–5 has been demonstrated to be active. *L. barbarum* polysaccharide-protein complex enhances Th1 responses, and polysaccharide-protein complex-treated DCs enhance Th1 and Th2 responses in vitro and in vivo. The research provides evidence and a rationale for using *L. barbarum* in the treatment of various clinical conditions to enhance host immunity and suggests *L. barbarum* is a potent adjuvant in the design of DC-based vaccines (Chen et al. 2009a).

### 2.3.1.2 Macrophages

Macrophages play a major role in the host defense against infection. Macrophages express a broad range of pattern recognition receptors (PRRs) to bind the conserved structures of pathogens, ingest and microbes into vesicles, and produce reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (mainly nitric oxide) to destroy microbes (Aderem and Underhill 1999). Activated macrophages also secrete the cytokines TNF- $\alpha$  and IL-1 and chemokines to induce inflammatory reactions to microbes (Pylkkanen 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 (Krysko et al. 2006).

LBPF4-OL is the glycan part of *L. barbarum* polysaccharide-protein complex fraction 4 (LBPF4). A study demonstrated that the LBPF4-OL markedly induced proliferation of spleen cell but could not induce proliferation of purified T and B lymphocytes. Further study revealed that the proliferation of B cell took place in the presence of activated macrophages or LPS. Multiplex bead analysis indicated that LBPF4-OL can obviously induce production of IL-6, IL-8, IL-10 and IFN- $\alpha$  by spleen cells in a concentration-dependent manner. Flow cytometric analysis indicated that LBPF4-OL (i.p.) triggers expression of CD86 and MHC-II on macrophages. An enzyme-linked immunosorbent assay (ELISA) assay demonstrated that LBPF4-OL could greatly stimulate macrophages to secrete TNF- $\alpha$  and IL-1 $\beta$ . These results suggest that the glycan LBPF4-OL plays an important role in the immunopharmacological activity of *L. barbarum* polysaccharide-protein complex; and macrophages, rather than T and B cells, are the principal target cells of LBPF4-OL (Zhang et al. 2011).

It has been found that polysaccharide-protein complex from *L. barbarum* fruit (50 mg/kg, i.p.) markedly upregulated the expressions of CD40, CD80, CD86, and MHC class II molecules on peritoneal macrophages. In vitro studies demonstrated that *L. barbarum* polysaccharide-protein complex activated transcription factors NF- $\kappa$ B and AP-1 in RAW264.7 macrophages; induced mRNA expression for TNF- $\alpha$ , IL-1 $\beta$ , IL-12p40; and enhanced production of TNF- $\alpha$  in a dose-dependent manner. Furthermore, *L. barbarum* polysaccharide-protein complex (50 mg/kg, i.p.) significantly enhances endocytic and phagocytic capacities of macrophages



in an in vivo study. These results indicate that *L. barbarum* polysaccharide-protein complex enhances innate immunity by activating macrophages. The mechanism may be mediated via activation of transcription factors NF- $\kappa$ B and AP-1 to induce production of TNF- $\alpha$  and upregulation of MHC class II (Chen et al. 2009b). The comparisons of immune activities of polysaccharides and polysaccharide-protein complex from *L. barbarum* on macrophages have also been reported. Experiments using in vitro assays found that LBPF4-induced proliferation of splenocytes was dependent on both B and T cells. However, LBPF4-OL-induced splenocyte proliferation was mainly dependent on B cells. The ELISA results indicated that both LBPF4 and LBPF4-OL significantly induced production of TNF- $\alpha$ , IL-1 $\beta$ , and NO from macrophages. Furthermore, electrophoretic mobility shift assay (EMSA) studies suggest that LBPF4 100  $\mu$ g/ml can be more effectively to increase NF-kappa B activity than that of LBPF4-OL. The results demonstrate that LBPF4 can enhance T, B cells, and macrophage functions, but LBPF4-OL can only enhance B cells, and macrophage functions. This is partly due to LBPF4 being able to more significantly enhance lymphocytes NF- $\kappa$ B activity (Zhang et al. 2014a).

### 2.3.1.3 T Lymphocytes

The present research has revealed the effects of *L. barbarum* in activating T cells. Flow cytometry assays revealed that *L. barbarum* polysaccharide enhanced the proliferation of murine splenic lymphocyte. The combined use of LBP and Con A had synergistic effects. MTT assays demonstrated that LBP significantly promoted proliferation of murine splenic lymphocytes, whereas LBP plus Con A combination also enhanced the lymphocyte proliferation at a high concentration. LBP with Con A had effects on immunocompetence (Amagase and Farnsworth 2011). Another research group found that LBP significantly stimulated proliferation of mouse splenocytes. T cell but not B cell proliferation was observed. Cell cycle profile analysis indicated that LBP5 markedly reduced sub-G1 cell expression. LBP could activate the transcription factors NFAT and AP-1, prompt CD25 expression, and induce IL-2 and IFN- $\gamma$  gene transcription and protein secretion. LBP (i.p. or p.o.) significantly induced proliferation of T cells. The effect of *L. barbarum* glycopeptide 3 (LBGP3) on T cell apoptosis in aged mice has been reported. LBGP3 was purified from *Fructus Lycii* water extracts and identified as a 41 kD glycopeptide. Treatment with 200  $\mu$ g/ml LBGP3 increased the apoptotic rate of T cells from aged mice and produced a similar DNA ladder pattern to that observed in young T cells. The reversal of apoptotic resistance was involved in down-regulating the expression of Bcl-2 and FLIP and upregulating the expression of FasL. *L. barbarum* glycopeptide 3 reverses apoptotic resistance of aged T cells by modulating the expression of apoptosis-related molecules (Yuan et al. 2008). The results suggest that activation of T lymphocytes by *L. barbarum* may contribute to one of its immune enhancement functions (Chen et al. 2008a).



### 2.3.1.4 NK Cells

Polysaccharides are believed to be strong immune stimulants that can promote the proliferation and activity of T cells, B cells, macrophages, and NK cells. A study aimed to investigate the effects of polysaccharides, including *L. barbarum* polysaccharide (LBP), on primary human NK cells under normal or simulated microgravity (SMG) conditions. The results demonstrated that LBP markedly promoted the cytotoxicity of NK cells by enhancing the secretion of IFN- $\gamma$  and perforin and increasing the expression of the activating receptor NKP30 under normal conditions. Moreover, LBP can enhance NK cell function under SMG conditions by restoring the expression of the activating receptor NKG2D and reducing early apoptosis and late apoptosis/necrosis. Additionally, antibody neutralization tests demonstrated that CR3 may be the critical receptor involved in polysaccharide-induced NK cell activation. These findings indicate that polysaccharides may be used as immune regulators to promote the health of the public and even astronauts during space missions (Ting et al. 2014).

### 2.3.1.5 Other Target Cells

Granular leukocytes and mast cells are the main effector cells of food allergies, which cause type I hypersensitivity. *L. barbarum* berries have been introduced into Western diets. Preliminary reports have demonstrated its allergenic capacity (Balarin et al. 2011; Larramendi et al. 2012). A study investigated the frequency of sensitization and the allergens. In this study, 566 individuals with respiratory or cutaneous symptoms were skin prick tested with *L. barbarum* berry extract. Thirty-three individuals were positive (5.8%), and 94% were sensitized to other allergens. The specific IgE to *L. barbarum* berries, peaches, tomatoes, and a nut mix was measured. Thirteen individual serum samples out of 24 available serum samples (54.2%) had positive specific IgE. In addition, 92.3% of *L. barbarum* berry-positive patients were positive to peaches. Seven individuals recognized eight bands, and six recognized a 7-kDa band. This band was identified as a lipid transfer protein by mass spectrometry (MS/MS). Cross-reactivity was demonstrated with tomato, tobacco, nut mix, Artemisia pollen, and purified Lyce3 and Pru p3. These results indicate that *L. barbarum* berries are a new allergenic source with a high prevalence of sensitization (Carnes et al. 2013). Some other reports found that the isolated active component of LBP3a, combined with a DNA vaccine encoding the major outer membrane protein of *Chlamydomophila abortus*, induced protection in mice against challenge. A combination of DNA vaccine and LBP3a induced significantly higher antibody levels in mice. MOMP-specific IgG1, IgG2a, and IgG2b antibodies were found in the pool of sera postvaccination on day 42. IgG2a and IgG2b became the predominant isotypes in 12.5, 25, and 50 mg/kg LBP3a-adjuvanted groups (Ling et al. 2011). It has also been reported that mice fed *L. barbarum* had higher influenza antibody titers (Du et al. 2014). These studies indicate that granular leuko-

cytes, mast cells, and B lymphocytes are also related to the *L. barbarum* activity. However, more experiments are required to clarify whether these cells are the direct target cells of *L. barbarum*.

### 2.3.2 Receptors

Identifying cellular receptors is important to understand how polysaccharides exert their immunomodulatory effects. Several  $\beta$ -glucan receptors have been identified. The reported  $\beta$ -glucan receptors include lactosylceramide (LacCer), Toll-like receptors (TLRs) 2 and 6, and dectin-1 (Zimmerman et al. 1998; Sletmoen and Stokke 2008). In addition, TLR4 has been identified as a receptor of polysaccharides, and many polysaccharide activities involve TLR4. Other polysaccharide-related receptors have been reported, including complement receptor-3 (CR3), scavenger receptor (SR), MR (CD206), CD44/RHAMM and selectins.

A study reported that the activity of the polysaccharide LBPF4-OL, which was purified from LBP, is closely associated with the TLR4-MAPK signaling pathway. Research found that LBPF4-OL could significantly induce production of TNF- $\alpha$  and IL-1 $\beta$  in peritoneal macrophages isolated from wild-type (C3H/HeN) but not TLR4-deficient mice (C3H/HeJ). The study also found that the proliferation of LBPF4-OL-stimulated lymphocytes from C3H/HeJ mice is significantly weaker than that of lymphocytes from C3H/HeN mice. Furthermore, through a bio-layer interferometry assay, it was 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 examined by 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 (Zhang et al. 2014b). Similar results have been observed for dendritic cells. Zhu et al. reported that LBPs induced phenotypic and functional maturation of DCs. LBPs upregulated DC expression of I-A/I-E and CD11c, enhanced DC allostimulatory activity and induced production of IL-12p40. 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- $\kappa$ B p65. The results revealed that LBP stimulation induces the phenotypic and functional maturation of DCs via TLR2 and/or TLR4-mediated NF- $\kappa$ B signaling pathways (Zhu et al. 2013). The above results indicate the immunoactivity of *L. barbarum* is related to TLR4/2. Whether other receptors are related to *L. barbarum* remains unknown.

### 2.3.3 Signal Transduction

Several signal transduction pathways, including the PI3K/Akt/FoxO1, LKB1/AMPK, JNK/c-Jun, MEK/ERK, and PI3K/HIF-1 $\alpha$  pathways, and transcription factors NF- $\kappa$ B, p53, c-Jun, and AP-1 are reported to be *L. barbarum* activity-related signal transduction molecules.

*L. barbarum* polysaccharides (LBPs) from wolfberries have been reported to have antioxidant and neuroprotective derivatives. A study found that LBPs are also a novel hepatoprotective agent against nonalcoholic steatohepatitis (NASH) caused by a diet-induced NASH rat model. The study examined female rats fed with 1 mg/kg LBP daily for 8 weeks and compared with control rats. NASH+ LBPs-cotreated rats displayed (1) improved histology and free fatty acid levels, (2) re-balancing of lipid metabolism, (3) reducing profibrogenic factors through the TGF- $\beta$ /SMAD pathway, (4) improved oxidative stress through the cytochrome P450 2E1-dependent pathway, (5) reducing production of hepatic pro-inflammatory mediators and chemokines, and (6) ameliorating hepatic apoptosis through the p53-dependent intrinsic and extrinsic pathways. All these effects of LBP were partly modulated through the PI3K/Akt/FoxO1, LKB1/AMPK, JNK/c-Jun, and MEK/ERK pathways and the down-regulation of transcription factors in the liver, such as NF- $\kappa$ B and activator protein-1 (AP-1) (Xiao et al. 2013). Moreover, LBPs have also been found to inhibit tumor cell growth by suppressing IGF-1-induced angiogenesis via PI3K/HIF-1 $\alpha$ /VEGF signaling pathways. Studies have reported that a 90 h treatment with 0.50 mg/ml of LBPs resulted in significant inhibition of MCF-7 cell proliferation. Using this same cell type, studies have also observed that LBPs could also affect insulin-like growth factor (IGF)-1 protein accumulation, suppress PI3K activity and p-PI3K protein levels, inhibit accumulation of hypoxia-inducible factor-1 (HIF-1 $\alpha$ ) protein without altering HIF-1 $\alpha$  mRNA levels, and suppress mRNA expression and protein production of VEGF (Huang et al. 2012).

NF- $\kappa$ B has also been found to be one of the most important transcription factors related to *L. barbarum* activity. A study reported that LBP treatment may protect against intestinal ischemia-reperfusion injury (IRI)-induced intestinal damage by inhibiting PMN accumulation and ICAM-1 expression and ameliorating changes in TNF- $\alpha$  level, NF- $\kappa$ B activation, intestinal permeability, and histology (Yang et al. 2013). Other reports indicate that LBPs do not delay primary degeneration of RGCs after either complete optic nerve transection (CONT) or partial optic nerve transection (PONT), but they delay secondary degeneration of retinal ganglion cells (RGCs) after PONT. The study found that LBPs appeared to exert these protective effects by inhibiting oxidative stress and the JNK/c-Jun pathway and by transiently increasing production of insulin-like growth factor-1 (IGF-1) (Li et al. 2013). After investigating the effect of LBP on the differentiation and maturation of healthy human peripheral blood-derived dendritic cells cultured in different tumor microenvironments in vitro and evaluating the molecular and immunological mechanisms of LBP in the treatment of tumors, a study reported that LBPs could increase the expression of the phenotype of DCs, secretion of IL-12p70 and IFN- $\gamma$  in MLR and

enhance NF- $\kappa$ B expression, especially in virus-related peripheral blood-derived dendritic cell precursor cells, suggesting that LBPs play a stronger antitumor role in virus-related environments, and this phenomenon correlates with the NF- $\kappa$ B signaling pathway (Chen et al. 2012). Maturation of DCs by LBPs is able to directly activate the nuclear transcription factor NF- $\kappa$ B p65 (Zhu et al. 2013).

### 2.3.4 Cytokines

As a type of immune regulator, *L. barbarum* can induce production of a variety of cytokines, such as the anti-inflammatory factor IL-10, proinflammatory cytokines IL-1 $\beta$  and IL-6, chemokines IL-8, antitumor factors cytokine TNF- $\alpha$ , antiviral factor IFN- $\gamma$ , TGF- $\beta$ 1, and lymphocyte activators IL-2 and IL-4. LBPF4-OL is the glycan part of *L. barbarum* L polysaccharide-protein complex fraction 4 (LBPF4). A study found that LBPF4-OL can obviously induce production of IL-6, IL-8, IL-10, and INF- $\alpha$  from mouse spleen cells in a concentration-dependent manner in vitro (Zhang et al. 2011). The effects of *L. barbarum* polysaccharide-protein complex (LBP3p) on the expression of IL-2 and TNF- $\alpha$  in human peripheral blood mononuclear cells have been examined by reverse transcription polymerase chain reaction (RT-PCR) and bioassay. A study found that administration of LBP3p increased the expression of IL-2 and TNF- $\alpha$  at both the mRNA and protein levels in a concentration-dependent manner (Gan et al. 2003). LBPs can also increase the secretion of IL-12 p40 of BMDC in vitro (Zhu et al. 2007). LBP, LBPF4, and LBPF5 have been reported to significantly stimulate proliferation of mouse splenocyte and induce gene expression for IL-2 and IFN- $\gamma$  as well as their protein secretion (Chen et al. 2008a). A study also found adoptive transfer of wolfberry-treated bone marrow DCs (loaded with ovalbumin (323–339)-peptide) promoted proliferation of antigen-specific T cells as well as production of interleukin-4 and interferon-gamma in CD4(+) T cells (Du et al. 2014). The above results indicate LBPs can induce cytokine production in vitro.

It has been reported that polysaccharide-rich *L. barbarum* and *Rehmannia glutinosa* treatment increases hepatic anti-inflammatory cytokine IL-10 levels, suppresses liver fibrosis-biomarkers TGF- $\beta$ 1, and reduces hepatic levels of the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  after exposing the rats to carbon tetrachloride (CCl<sub>4</sub>) (Wu et al. 2011). Another study fed adult mice (4 month-old) a milk-based preparation of wolfberries called Lacto-Wolfberry (LWB) for 4 weeks and then infected the mice with influenza A/Puerto Rico/8/34 (HI NI) while continuing the same experimental diet. The LWB-fed mice displayed, overall, significantly higher concanavalin A-induced IL-2 production. Furthermore, the study found positive correlations between weight loss, lung viral titer, pathology score, TNF- $\alpha$ , and IL-6 production, as well as negative correlations with T cell proliferation and IL-2 production (Ren et al. 2012). The aforementioned results indicate LBPs can induce cytokine production in vivo.

### 2.3.5 Antibodies

*L. barbarum* polysaccharides (LBPs) can moderate immune responses. They could potentially be used as a substitute for oil adjuvants in veterinary vaccines. It has been demonstrated that the isolated active component of LBP3a, combined with a DNA vaccine encoding the major outer membrane protein of *C. abortus*, exerted protection to mice against the challenge. A combination of DNA vaccine and LBP3a significantly induced higher levels of antibodies in mice. MOMP-specific IgG1, IgG2a, and IgG2b antibodies were found in a sera pool 42 days after vaccination. IgG2a and IgG2b became the predominant isotypes in 12.5, 25, and 50 mg/kg LBP3a-adjuvanted groups (Ling et al. 2011). Sulfated *L. barbarum* polysaccharides (sLB-PSs) with different degrees of sulfation (DS), sLBPS (1.5), and sLBPS (1.9) were added into cultured chicken peripheral lymphocytes, and the change of lymphocyte proliferation was compared by MTT assay, taking the nonmodified LBPS as a control. On days 7, 14, 21, and 28, after the first vaccination, the changes in proliferation of peripheral lymphocyte and serum hemagglutination inhibition (HI) antibody titer were determined. The results indicated that two sLBPSs could significantly promote proliferation of lymphocyte and enhance serum antibody titer (Wang et al. 2010b). A 3-month randomized, double-blinded, placebo-controlled study was conducted on 150 healthy community-dwelling Chinese elderly (65–70 years old) supplemented with Lacto-Wolfberry or placebo (13.7 g/day). The immune response to influenza vaccine was assessed in the study, along with inflammatory and physical status. No serious adverse reaction was reported during the trial nor were symptoms of influenza-like infection. No changes in body weight and blood pressure, blood chemistry or cell composition, as well as in autoantibodies levels were observed. The subjects receiving Lacto-Wolfberry had significantly higher levels of serum influenza-specific immunoglobulin G after vaccination and the rate of seroconversion between days 30 and 90 as compared with the placebo group. The postvaccination positive rate was greater in the Lacto-Wolfberry group than the placebo group but did not reach significance (Vidal et al. 2012). It has also been reported that mice fed with wolfberries had higher influenza antibody titers (Du et al. 2014). The effects of *L. barbarum* polysaccharide (LBP) on immune responses in vaccinated chickens were also reported. A total of 600 Hy-Line Brown chickens aged 15 days old were randomly divided into four groups with three replicates per group and 50 chickens per replicate, and all of the chickens were injected with Newcastle Disease (ND) vaccine. Three experimental groups of chickens were injected with 20, 10, and 5 mg/kg LBP (LBPH, LBPM, and LBPL). The results indicated that LBP (10 and 20 mg/kg) could significantly enhance the ND antibody titers (Qin et al. 2012).

### 2.3.6 Leukocyte Differentiation Antigen

DC phenotypic maturation is related to its immunogenicity. The priming of T cells requires that both peptide-MHC complex and CD80/CD86 on APCs bind to TCR

and CD28 on T cells, respectively. To determine whether LBP induces DC maturation, a study generated DCs from BALB/c mouse bone marrow and stimulated them with LBPs or individual fractions. Twenty-four hour treatment with LBPs or LBP1–5 upregulated CD40, CD80, CD86, and MHC class II molecules to various extents compared with the medium control. The increase in mean fluorescence intensity was most prominent for MHC-II and CD86 by LBP, LBP4, or LBP5 stimulations. CD40 expression was weak, but there was still a ~2-fold increase after treatment. Surprisingly, LBP (s.c.) was most effective, perhaps because the in situ activated DCs migrate to the spleen more effectively through the lymphatic system (Chen et al. 2009a). Compared with the BMDC that were only subjected to treatment with RPM11640, the coexpression of I-A/I-E and CD11c by bone marrow-derived dendritic cells stimulated with LBPs (100 µg/ml) were increased (Zhu et al. 2007).

## 2.4 Future Remarks and Conclusions

Thus far, a profound understanding of the immune regulating function of *L. barbarum* has been achieved, especially the antitumor and immune regulation mechanism of LBPs, includes deep knowledge of the receptors and signal transduction levels. Studies have found that the effect of LBPs on the activation of macrophages and dendritic cells are important in participating in the immune response. In addition, it was found that TLR4/2 may be closely related to the immunoregulatory activity of LBPs. In addition, whether other PRRs, which include SRs, mannose receptors, TLRs, Dectin-1, and complement receptor type 3 (CR3), are associated with the activity of *L. barbarum* is not yet known. It will be interesting to identify the receptor of *L. barbarum* on macrophages or dendritic cells in the future.

Little is known about the LBP protective effects on neurons, the anti-neurogenesis activity, and the effect on the drug-induced learning and memory mechanism. A recent study found that LBPs can prevent scopolamine-induced cognitive and memory deficits and reductions in cell proliferation and neuroblast differentiation (Chen et al. 2014). Other research indicates that LBPs can delay secondary degeneration of RGCs, and this effect may be linked to inhibition of oxidative stress and the JNK/c-Jun pathway in the retina and transient increases in production of insulin-like growth factor-1 (IGF-1) (Li et al. 2013). In addition, there is also a research report indicated that LBP treatment may protect against IRI-induced intestinal damage, possibly by inhibiting IRI-induced oxidative stress and inflammation (Yang et al. 2013). Recently, a new study reported that LBPs partially exerted their beneficial neuroprotective effects on ischemia-reperfusion via the activation of Nrf2 and an increase in HO-1 protein expression (He et al. 2014). These studies suggest that antioxidant effects may play a key role in the LBP protective effect on various diseases. Exploration of the effect of *L. barbarum* on the influence of the balance of the body's REDOX reactions, such as key oxidative stress Nrf-2/ARE



pathways, may further reveal the intrinsic relationship of *L. barbarum*'s antioxidant and immune regulation functions.

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