IMMUNOMODULATORY PROPERTIES OF POLYSACCHARIDE-PROTEIN COMPLEX FROM *LYCIUM BARBARUM* L.

CHEN ZHISONG

NATIONAL UNIVERSITY OF SINGAPORE

2008

IMMUNOMODULATORY PROPERTIES OF POLYSACCHARIDE-PROTEIN COMPLEX FROM *LYCIUM BARBARUM* L.

CHEN ZHISONG

B.Med.; M.Med.

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

YONG LOO LIN SCHOOL OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

2008

I would like to dedicate this thesis to

my dear wife, Ma Jin

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my supervisors, Emeritus Professor Chan Soh Ha and Associate Professor Benny Tan Kwong Huat, whose kind support, trenchant critiques, and remarkable patience have made this possible. I cannot thank them enough.

I am also grateful to all the staff and students at the WHO Collaborating Centre for Research and Training in Immunology, including Wee Guan Bock, Meera Chatterji, Nalini Srinivasan, Soo Mei Yun, Loh Mei Fong, Zulaimi Bin Md Nor, Wong Yoke Yon, Chia Jer-Ming, Pang Shyue Wei, and Shen Meixin, with whom I have shared four cherished years in such a cozy environment. This wonderful experience will always be embedded in my mind.

Special thanks are also addressed to Annie Hsu, A/Prof Lu Jinhua, A/Prof Ren Ee Chee, Dr Paul A MacAry, Prof Mary Ng Mah Lee, Prof David Michael Kemeny, Lew Fei Chuin, Chan Yue Ng, Phoon Meng Chee, Ho Lip Chuen, and Lim Ek Wang for their kind help.

I also wish to thank the National University of Singapore and the WHO Collaborating Centre for Research and Training in Immunology for their generous support in making this project possible.

I remain indebted to my family members for their constant understanding and endless love.

TABLE OF CONTENTS

SUMMARY

Lycium barbarum L. (*L. barbarum*), commonly known as wolfberry, is a well-known Chinese herbal medicine with various biological activities, such as hematopoiesis promotion, liver protection, and immunity improvement. The latter has been attributed to the polysaccharides that form the major component of *Lycium* fruit. However, the mechanisms are not fully elucidated yet. In this present study, we isolated and purified polysaccharide-protein complex from *Lycium* fruit (LBP) and investigated its immunomodulatory effects on T cells, macrophages, and dendritic cells (DCs).

L. barbarum fruit was extracted with cold water and precipitated with ethanol, followed by removal of protein by Sevag method. The crude LBP obtained was separated by DEAE-cellulose chromatography and purified by size exclusion chromatography. Five homogeneous fractions, designated as LBPF1, LBPF2, LBPF3, LBPF4, and LBPF5 were obtained. The carbohydrate contents of LBPF1-5 were 48.2%, 30.5%, 34.5%, 20.3%, and 23.5%, respectively. Their protein contents were 1.2%, 4.8%, 4.1%, 13.7%, and 17.3%, respectively. Their molecular weights were 151 kDa, 147 kDa, 146 kDa, 150 kDa, and 290 kDa, respectively. LBP and LBPF1-5 were not contaminated by LPS. LBP was non-toxic or mildly toxic to mice.

T lymphocytes play central roles in adaptive immunity. The results showed that crude LBP, LBPF4, and LBPF5 could significantly stimulate mouse splenocyte proliferation. The proliferation proved to be that of T cells, but not B cells. Cell cycle profile analysis indicated that crude LBP, LBPF4, and LBPF5 could markedly reduce sub-G1 cells.

Crude LBP, LBPF4, and LBPF5 could prompt CD25 expression, inducing IL-2 and IFN-γ gene transcription and protein secretion. Moreover, crude LBP, LBPF4, and LBPF5 could activate transcription factors, NFAT and AP-1, but not NF-κB. Administration of LBP either by i.p. injection or by oral gavage for 7 days induced mouse T lymphocyte proliferation significantly.

Macrophages play crucial roles in innate immunity. The results showed that LBP upregulated the expression of CD40, CD80, CD86, and MHC class II molecules on peritoneal macrophages. LBP and LBPF1-5 activated transcription factors NF-κB and AP-1 by RAW264.7 macrophage cells, induced TNF-α, IL-1-β, and IL-12p40 mRNA expressions, and enhanced TNF-α production in a dose-dependent manner. LBP improved macrophage capacities in endocytosis and phagocytosis.

DC immunogenicity correlates with its maturation. The results showed that LBP induced phenotypic and functional maturation of DCs with strong immunogenicity. LBP upregulated the expressions of CD40, CD80, CD86, and MHC class II molecules by mouse bone marrow-derived DCs (BMDCs) and splenic DCs, downregulated DC uptake of antigen, enhanced DC allostimulatory activity and the production of IL-12p40 and p70 at gene and protein levels. All its five fractions were active. LBP primed Th1 response *in vivo*. LBP-treated BMDCs enhanced Th1 and Th2 response *in vitro* and *in vivo*.

In conclusion, the results showed that LBP is capable of activating macrophages, DCs, and T cells, indicating it can enhance both innate and adaptive immunity. The present

data provdie scientific evidence on potential use of LBP as supplemental treatment for people under poor immune conditions such as cancer, hepatitis, tuberculosis, and aging.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AD	Alzheimer's disease
AGE	advanced glycated end products
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AP-1	activation protein-1
APC	antigen presenting cell
APC dye	allophycocyanin dye
APS	ammonium persulfate
AST	aspartate aminotransferase
BFU-E	burst forming unit-erythroid
BMDC	bone marrow derived dendritic cell
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CAT	catalase
cDC	conventional dendritic cell
cDNA	complementary DNA
CDR	complementarity-determining region
CFU-E	colony forming unit-erythroid
CFU-GM	granulocyte-monocyte colony forming unit
CFU-S	spleen colony forming unit
cGMP	cyclic guanosine monophosphate
CI	confidence level
Con A	concanavalin A
cpm	counts per minute
CRBC	cock red blood cell
C_T	threshold cycle
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T-lymphocyte antigen 4
d	day

DAG	diacylglycerol
DC	dendritic cell
ddH_2O	double distilled water
DEAE-cellulose	diethylaminoethyl-cellulose
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithioithreitol
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
G-CSF	granulocyte-colony stimulating factor
GGT	gamma-glutamyl transferase
GLUT4	glucose transporter 4
GM-CSF	granulocyte monocyte colony stimulating factor
GSH-Px	glutathione peroxidase
h	hour
HDL-c	high-density lipoprotein cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
IFN-γ	interferon gamma
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G

IgM	immunoglobulin M
IKK	IκB kinase
IL-10	interleukin 10
IL-12	interleukin 12
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-2R	interleukin 2 receptor
IL-3	interleukin 3
IL-4	interleukin 4
IL-6	interleukin 6
IP_3	inositol-1,4,5-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
IκB	inhibitor of kappa B
JNK	c-Jun NH_2-terminal kinase
kDa	kilo Dalton
L	liter
L. barbarum	*Lycium barbarum* L.
LAK cell	lymphokine-activated killer cell
LAL	*Limulus* amebocytes lysate
LbGp	*L. barbarum* glycoconjugates
LBP	*Lycium barbarum* polysaccharide-protein complex
LBPF	*Lycium barbarum* polysaccharide-protein complex fraction
LC	Langerhans cell
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LPO	lipid peroxidation
LPS	lipopolysaccharide
LSZ	lysozyme
m	month
MAO	monoamine oxidase
MDA	malondialdehyde

mg	milligram
MHC	major histocompatibility complex
min	minute
ml	milliliter
MLR	mixed leukocytes reaction
mM	millimolar
mRNA	messenger RNA
NFAT	nuclear factor of activated T-cell
NF-κB	nuclear factor kappa B
NIDDM	Non-insulin dependent diabetus mellitus
NK cell	natural killer cell
NO	nitric oxide
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PHA	phytohemagglutinin
PI	propidium iodide
PIP_2	phosphatidylinositol (PI)-4,5-bisphosphate
PKC	protein kinase C
PLCγ	phospholipase C gamma
PMA	phorbol myristate acetate
PRR	pattern recognition receptor
RLU	relative light units
RNA	ribonucleic acid
RNase	ribonuclease
ROI	reactive oxygen intermediate

RPMI	Roswell Park Memorial Institute
RQ	relative quantification
RT	room temperature
RT-PCR	reverse-transcription PCR
s	second
S.I.	stimulation index
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SOD	superoxide dismutase
TCM	traditional Chinese medicine
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TG	triglyceride
TGF-β	transforming growth factor beta
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
v/v	volume per unit volume
VEGF	vascular endothelial growth factor
w	week
WBC	white blood cell
Δ	delta value
μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar

CHAPTER 1

INTRODUCTION

1.1 Advances in *Lycium barbarum* Polysaccharide Research

Lycium barbarum L., commonly known as wolfberry, is a well-known Chinese herbal medicine as well as tonic. *Lycium* is the genus name derived from the ancient southern Anatolian region of Lycia. *Barbarum* is the species name indicating that the wolfberry was of foreign origin outside Anatolia or China where it was first discovered. The end abbreviation, L., refers to Linnaeus who described the species in 1753 in *Species Plantarum*. *L. barbarum* grows mainly in northwestern China, especially in Zhongning county, Ningxia province. In traditional Chinese medicine (TCM), *L. barbarum* fruit possesses the functions of nourishing the kidney and replenishing essence, nourishing the liver and improving eyesight. It has been used in China for thousands of years to treat diseases such as insomnia, liver dysfunction, diabetes, visual degeneration, tuberculosis, hypertension, and cancer. Ancient Chinese believed wolfberry fruits had multiple health benefits and used them to make tea, soup, stew and wine or chewed them like raisins. *L. barbarum* fruit is also a medicinal nutrient which contains many micronutrients and phytochemicals, including 11 essential and 22 trace dietary minerals, 6 essential vitamins, 18 amino acids, 5 unsaturated fatty acids, beta-carotene, zeaxanthin, and polysaccharides (Young et al, 2005; Gross et al, 2006). Polysaccharides are a major constituent of *L. barbarum* fruit, representing up to 31% of pulp weight. Since 1980s, numerous researches have been conducted with modern technology to unveil its bioactive components, of which polysaccharides have been extensively addressed. This review summarizes the isolation and pharmacological properties of *L. barbarum* polysaccharides (LBP).

1.1.1 Isolation, Purification and Characterization

The plant polysaccharides are usually localized in cytoplasmic organelles, plasma membranes, and cell walls (Herman and Lamb, 1992). To effectively isolate LBP from the *Lycium* fruit, it is necessary to first disrupt the cells by grinding or homogenization. Based on the characteristics that LBP is water-soluble but ethanol-insoluble, it can be extracted with water and precipitated with 5 volumes of ethanol. The co-precipitated protein can be removed by repeatedly adding the Sevag reagent (chloroform:n-butanol = 4:1, v:v). Oligosaccharides and other substances with low molecular weight can be removed by dialysis against water. The remaining solution is then lyophilized to obtain crude LBP. To obtain LBP, crude LBP can be fractionated by Diethylaminoethyl (DEAE)-cellulose ion exchange chromatography and purified by size exclusion chromatography (SEC). The carbohydrate content of the purified LBP can be determined by phenol-sulfuric acid assay and the protein content can be measured by the Bradford method. The molecular weight can be determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), SEC, or high performance liquid chromatography (HPLC). The amino acids can be analyzed by β-elimination method. The sugar constituents can be analyzed by gas chromatography and mass spectrometry. Some researchers prefer to remove the lipid and pigments of the *Lycium* fruit by reflux with organic chemicals such as acetone and petroleum with 80% ethanol before extraction with water (Wang and Chen, 1991; Tian et al, 1995; Luo et al, 1999; Gan et al, 2001; Wang et al, 2002). This process can reduce the non-specific precipitates in the subsequent ethanol precipitation, but reflux with hot organic chemicals and high concentration of ethanol may cause irreversible alteration of the polysaccharide structure. Another

approach is to directly extract LBP with water without the preliminary step of removing lipid and pigments (Huang et al, 1998; Peng and Tian, 2001). The disadvantage of this approach is that it increases the non-specific water-soluble substances. This will make the subsequent precipitation and concentration steps more difficult. LBP is commonly extracted with hot water (80ºC) (Luo et al, 1999; Gan et al, 2001; Wang et al, 2002). As LBP is a polysaccharide-protein complex, hot water may denature the protein and affect the bioactivity. Therefore, some researchers prefer to use cold water for extraction (Tian et al, 1995; Huang et al, 1998; Peng and Tian, 2001). The disadvantage is that LBP may not be completely dissolved in cold water.

While most researchers use crude LBP for studies, a few laboratories have obtained the purified fractions. Tian et al (1995) isolated crude LBP and separated it by DEAE-cellulose chromatography (eluents: 0.05, 0.1, and 0.5 M $NaHCO_3$) into three fractions, which were designated as LBP1, LBP2, and LBP3, respectively. LBP1 was further purified on Sephadex G-100 column to obtain the homogenous *L. barbarum* glycoconjugates (LbGp) with molecular weight of 88 kDa. It was composed of arabinose, galactose, and glucose in a molar ratio of 2.5:1.0:1.0 and 18 amino acids. Structure analysis indicated that the linkage between the glycan and the core protein backbone may be O-linkage. Five fractions were obtained by changing the elution buffers to H_2O, 0.05, 0.1, 0.25, 0.5 M of $NaHCO_3$ in the step of DEAE-cellulose chromatography, which were designated as LBP1, LBP2, LBP3, LBP4, and LBP5, respectively (Huang et al, 1998). The latter 3 fractions were further purified by SEC to obtain LbGp3, LbGp4, and LbGp5, respectively. It was found that the molecular weights of LbGp3, LbGp4 and LbGp5 were

92.5 kDa, 214.8 kDa and 23.7 kDa, respectively. Carbohydrate contents of LbGp3, LbGp4 and LbGp5 were 93.6%, 85.6%, 8.6%, respectively. LbGp3 was composed of arabinose and galactose in a molar ratio of 1.0:1.0. LbGp4 was composed of arabinose, galactose, rhamnose and glucose in molar ratio of 1.5:2.5:0.43:0.23. LbGp5 was composed of rhamnose, arabinose, xylose, galactose, mannose and glucose in molar ratio of 0.33:0.52:0.42:0.94:0.85:1. The nitrogen contents were 0.83% in LbGp3, 1.72% in LbGp4, and 9.58% in LbGp5. The linkage between the glycan and protein may be of O-linkage in LbGp4 (Huang et al, 1998). LbGp2 was studied by this group later (Peng and Tian, 2001), its carbohydrate content was 90.71%. The molecular weight was 68.2 kDa as determined by SEC. The glycan possessed a backbone consisting of (1→6)-beta-galactosyl residues, about fifty percent of which are substituted at C-3 by galactosyl or arabinosyl groups and the major non-reducing end being made of arabinose (Peng and Tian, 2001). The number of LBP fractions obtained was determined by the concentrations of eluent. Four fractions of LBP (LBP-I, -II, -III, -IV) were obtained by successive elutions with H_2O, 0.05, 0.1, 0.5 M NaCl in the DEAE-cellulose chromatography step and further purified on Sephadex G-25 (LBP-I) or Sephacryl S-100 column (LBP-II, -III, -IV) (Tian and Wang, 2006). They contained 6 kinds of monosaccharides (rhamnose, galactose, glucose, arabinose, mannose, and xylose), galacturonic acid and 18 kinds of amino acids with molecular weight of 152.4 kDa (Tian and Wang, 2006). LBP was complex polysaccharide consisting of acidic heteropolysaccharides and polypeptide or protein with Glycan-O-Ser glycopeptide structures (Tian and Wang, 2006).

Another group at Huazhong Agricultural University, China, obtained five fractions of LBP by successive elution with H_2O, 0.05, 0.1, 0.25, and 0.5 M of NaCl in the DEAE-cellulose chromatography step. The first fraction was further purified on Sephacryl S-300 column and designated as LBP-I (He et al, 1996). It was a glycoprotein composed of protein and acidic heteropolysaccharide consisting of galacturonic acid and neutral saccharides. The neutral saccharides contained galactose, glucose, rhamnose, arabinose, mannose, and xylose in molar ratio of 5.17:4.13:3.15:1.00:0.84:0.48 (He et al, 1996). The contents of neutral saccharides, galacturonic acid and proteins were 81.37%, 3.69%, 9.24%, respectively. Its molecular weight was greater than 20 kDa. Infrared spectrum analysis showed that the main chain was an α-pyranglycoside linkage (He et al, 1996). The second fraction (eluted with 0.05 M NaCl) was purified to LBP2a on a Sephadex-G200 column (Wang et al, 2002b). It contained neutral sugar (69.3%), galacturonic acid (23.8%), and protein (5.3%) with molecular weight of 77.5 kDa. Monosaccharide residues included rhamnose, xylose, arabinose, mannose, glucose, and galactose in molar ratio of 2.62:42.85:2.13:1.00:4.36:22.80. Linkages between sugars and amino acids were glycan-O-Ser (Wang et al, 2002b). The third fraction (eluted with 0.1 M NaCl) was further purified to LBP3p on a Sephadex G-200 column (Wang et al, 2002b; Gan et al, 2003; Gan et al, 2004). It was composed of 63.56% neutral sugars, 24.8% acidic sugars, and 7.63% proteins with molecular weight of 157 kDa. The monosaccharides were galactose, glucose, rhamnose, arabinose, mannose, xylose in molar ratio of 1.00:2.12:1.25:1.10:1.95:1.76. The linkage between glycan and protein was through glycan-O-Ser as shown by β-elimination method (Wang et al, 2002b; Gan et al, 2003; Gan at al 2004). The fourth fraction (eluted with 0.25 M NaCl) was further purified to

LBP4 on a Sephadex G-200 column (Zhang et al, 2003a; Zhang et al, 2003b). It was composed of 28.72% of neutral sugars, 60.45% of galacturonic acid and 6.01% of protein with molecular weight of 156.9 kDa. The monosaccharides were xylose, galactose, mannose, glucose, arabinose, and rhamnose in molar ratio of 2.05:1.20:1.00:0.90:0.85:0.38 (Zhang et al, 2003a; Zhang et al, 2003b). This group also studied another fraction of LBP designated as LBP-X, which contained 33.33% of galacturonic acid and 8.46% of protein (Luo et al, 1999; Gan et al, 2001; Gan and Zhang, 2002; Gan and Zhang, 2003). The monosaccharides were rhamnose, galactose, glucose, arabinose, mannose, and xylose in molar ratio of 4.22:2.43:1.38:1.00:0.95:0.38.

Unlike the above methods which precipitate LBP with 4-5 volumes of absolute ethanol and fractionate with gradient salts ($NaHCO_3$ or NaCl), Zhao et al (1996; 1997) extracted LBP with water and successively precipitated with 1, 4, and 7 volumes of 95% ethanol. By this method, they obtained three crude fractions designated as LBPA, LBPB, and LBPC. The proteins were removed by the Sevag method, dialyzed against water, and further purified on DEAE-cellulose column (successively eluted with H_2O, $Na_2B_4O_7$, and NaOH) and Sephadex G-50 column to four homogenous fractions, designated as $LBPA_3$ (from LBPA, eluted with NaOH), $LBPB_1$ (from LBPB, eluted with H_2O), $LBPC_2$ (from LBPC, eluted with H_2O), and $LBPC_4$ (from LBPC, eluted with NaOH). $LBPC_4$ was peptidoglycan composed of glycan with molecular weight of 10 kDa. $LBPA_3$, $LBPB_1$, and $LBPC_2$ were peptidoglycans composed of heteroglycan with molecular weight of 66, 18, and 12 kDa, respectively. Qin et al (2001) extracted polysaccharides from the fruit of *Lycium chinense* Mill. with cold and hot water. After separation by DEAE-cellulose

chromatography, three main fractions (Cp-1, -2, and -3) were obtained from cold water extraction. Another three main fractions (Hp-2, -3, and -4) were also obtained by hot water extraction from the residue after cold water extraction. Cp-1 proved to be a mixture of 4 kinds of polysaccharides (designated as Cp-1-A, -B, -C, -D), of which Cp-1-A was arabinoxylan (Ara:Xyl = 1:1), Cp-1-B was arabinan, and Cp-1-C and Cp-1-D were arabinogalactan-protein (AGP) (Qin et al, 2001). Cp-2 and Hp-2 were further purified to Cp-2-B and Hp-2-C, respectively. Both of them were AGPs. The average molecular weight was 71 kDa for Cp-2-B and 120 kDa for Hp-2-C. The ratio of arabinose to galactose was approximately 1:1 in both samples, and the carbohydrate was linked O-glycosidically to serine in Cp-2-B, and to both serine and threonine residues of the protein in Hp-2-C (Qin et al, 2001). Both samples also contained non-reducing terminal 3-O- and 4-O-substituted galacturonic acids. The ratio of 6-O-substituted galactose (linear part) and 3,6-di-O-substituted galactose (branching point) was almost unity in both samples (Qin et al, 2001). Different from the common methods in LBP isolation, a new approach has been developed (Pan et al, 2002). The procedure included: supercritical CO_2 extraction, water extraction, electrodialysis, ultra-filtration, reverse osmosis, and lyophilization. It was claimed that LBP isolated by this method was more water-soluble and bioactive (Qin et al, 2001).

The nomenclature for LBP fractions has not been standardized. They were named LbGp (Tian et al, 1995), LbGp2, LbGp3, LbGp4, and LbGp5 (Huang et al, 1998; Peng and Tian, 2001) by Tian's group. By contrast, Zhang's group named them LBP-I (He et al, 1996), LBP2a (Wang et al, 2002b), LBP3p (Wang et al, 2002a; Gan et al, 2003; Gan et al, 2004),

LBP4 (Zhang et al, 2003), and LBP-X (Luo et al, 1999; Gan et al, 2001; Gan and Zhang, 2002; Gan and Zhang, 2003). Zhao's group named them $LBPA_3$, $LBPB_1$, $LBPC_2$ and $LBPC_4$ (Zhao et al, 1997). Qin's group named them Cp-1-A, -B, -C, -D, Cp-2-B, and Hp-2-C (Qin et al, 2001). While the common conclusions from these groups are that all LBP fractions are peptidoglycan and the glycan and protein are linked O-glycosidically, the oligosaccharide constituents and their molar ratios and the molecular weights deduced are varied among these groups. For example, LbGp4 was 214.8 kDa and composed of arabinose, galactose, rhamnose and glucose in a molar ratio of 1.5:2.5:0.43:0.23 by Tian's group (Huang et al, 1998). In contrast, LBP4 was 156.9 kDa composed of xylose, galactose, mannose, glucose, arabinose, and rhamnose in molar ratio of 2.05:1.20:1.00:0.90:0.85:0.38 by Zhang's group (Zhang et al, 2003a; Zhang et al, 2003b). These variances could be due to the difference in extraction conditions used by the two groups. Tian's group used $NaHCO_3$ as eluent while Zhang's group used NaCl. The ionic strength and pH value of the eluents may affect the separation as well. In addition, the species and source of *L. barbarum* and the fruit maturity status may also influence the experimental results.

The composition, structure and molecular weight of LBP fractions from different labs are summarized in Table 1.

Table 1. LBP composition, structure and molecular weight

Fraction	Eleunt	Sugar compostion and molar ratio	Sugar Content (%)	Protein content (%)	MW (kDa)	Structure	Ref.
LbGp	0.05M $NaHCO_3$	Ara:Gal:Glu =2.5:1.0:1.0		18 amino acids		O-linkage	Tian et al, 1995
LbGp2	0.05M $NaHCO_3$	Ara:Gal = 4:5	90.7		68.2	Beta-glycan, O-linkage	Peng and Tian, 2001
LbGp3	0.1M $NaHCO_3$	Ara:Gal = 1:1	93.6	0.83	92.5		Huang et al, 1998
LbGp4	0.25M $NaHCO_3$	Ara:Gal:Rha:Gal = 1.5:2.5:0.43:0.23	85.6	1.72	214.8	O-linkage	Huang et al, 1998
LbGp5	0.5M $NaHCO_3$	Rha:Ara:Xyl:Gal:Man:Glu =0.33:0.52:0.42:0.94:0.85:1	8.6	9.58	23.7		Huang et al, 1998
LBP-I	H_2O	Gal:Glu:Rha:Ara:Man:Xyl =5.17:4.13:3.15:1:0.84:0.48	81.37	9.24	> 20	α-pyranglycoside linkage	He et al, 1996
LBP2a	0.05M NaCl	Rha:Xyl:Ara:Man:Glu:Gal =2.62:42.85:2.13:1:4.36:22.8	69.3	5.3	77.5	Glycan-O-Ser	Wang et al, 2002b
LBP3b	0.1M NaCl	Gal:Glu:Rha:Ara:Man:Xyl =1:2.12:1.25:1.1:1.95:1.76	63.56	7.63	157	Glycan-O-Ser	Gan et al, 2003
LBP4	0.25 NaCl	Xyl:Gal:Man:Glu:Ara:Rha = 2.05:1.2:1:0.9:0.85:0.38	28.72	6.01	156.9		Zhang et al, 2003a,b
LBP-X		Rha:Gal:Glu:Ara:Man:Xyl = 4.22:2.43:1.38:1:0.95:0.38	33.33	8.46			Luo et al, 1999
$LBPC_2$	H_2O				12		Zhao et al, 1996
$LBPC_4$	NaOH				10		Zhao et al, 1996
$LBPA_3$	NaOH				66		Zhao et al, 1996
$LBPB_1$	H_2O				18		Zhao et al, 1996

1.1.2 Pharmacological Functions

1.1.2.1 Immunomodulation

1.1.2.1.1 T Lymphocytes

A pilot study has shown that LBP could stimulate thymocyte proliferation in a dose-dependent manner (Geng et al, 1987). In the ^{3}H-thymidine uptake assay, it was found that the number of ^{3}H-thymidine-incorporated thymocytes harvested from the mice injected with LBP (50-200 mg/kg, i.p., × 7 d) were significantly increased (Geng et al, 1987). The dosage of 100 mg/kg showed the best effect. At low dosages (5-10 mg/kg), LBP did not have such an effect, but it could increase Con A-induced thymocyte proliferation (Geng et al, 1987). *In vitro* experiments also demonstrated that LBP markedly promoted concanavalin A (Con A)-induced thymocyte proliferation (Ma et al, 1996). The amount of ^{3}H-thymidine incorporated into the mouse thymocytes stimulated with Con A (3 and 5 μg/ml) and LBP (156 μg/ml) were significantly more than that with Con A alone (Ma et al, 1996). Furthermore, it was found that LBP could promote T lymphocytes release from the thymus to the peripheral blood (Geng et al, 1987). T lymphocytes in peripheral blood were increased from 65% to 81% in the mice injected with LBP (5-50 mg/kg, i.p., × 7 d) (Geng et al, 1987). It was found that LBP has bidirectional regulatory effects on mouse splenic T lymphocytes (Qian et al, 1988; Wang et al, 1990). At high concentration (1 mg/ml), LBP inhibited mouse splenic T lymphocyte proliferation, whereas it promoted mouse splenic T lymphocyte proliferation at low concentrations (Qian et al, 1988). These results were reproducible *in vivo*. Wang et al (1990) reported that LBP (5 and 10 mg/kg, i.p., × 7 d) significantly improved Con A-induced mouse splenocyte proliferation. The cpm values were increased from 28410 ± 3110 to 64870 ± 2571 when mice were injected

with 10 mg/kg of LBP, showing a 3-fold increase compared to the control, whereas LBP seriously inhibited Con A-induced mouse splenocyte proliferation at 25 and 50 mg/kg. Similar results were reported by Gan et al (2004). Mice were treated with LBP3p (5, 10, 20 mg/kg, p.o., × 10 d). A total of 10 mg/kg dose was more effective than 5 and 20 mg/kg doses in induction of mouse splenic lymphocyte proliferation (Gan et al, 2004). In addition, LBP also has regulatory functions on T lymphocyte subsets. Hu et al (1995) reported that while *L. barbarum* water extract significantly improved phytohemagglutinin (PHA) (50 μg/ml)- and phorbol myristate acetate (PMA) (25 ng/ml)-induced human tonsil lymphocyte proliferation, it markedly decreased the percentage of the $CD_4^-CD_8^+$ and $CD_4^+CD_8^+$ T cells from 2.51 ± 1.81% and 6.33 ± 2.85% to 0.63 ± 0.62% and 1.57 ± 1.13%, respectively, and upregulated the $CD_4^+CD_8^-$ T cells from 39.32 ± 4.10% to 46.55 ± 3.65%. LBP also has effect on cytotoxic T lymphocytes (CTLs) (Wang et al, 1990). LBP (5 mg/kg, i.p., × 7 d) improved the CTLs of P815-bearing mice in specific killing of P815 target cells from 33% to 67% (Wang et al, 1990). Furthermore, LBP (5 and 10 mg/kg, i.p.) could antagonize the inhibition of CTLs by cyclophosphamide. CTL inhibition was reduced from 51% to 19% (10 mg/kg) and 36% (5 mg/kg) (Wang et al, 1990).

1.1.2.1.2 Natural Killer Cells

Wang et al (1990) found that LBP could improve the natural killer (NK) cell function in killing target cells. LBP (5 mg/kg, i.p., × 3 d) improved mouse splenic NK cells in killing target cells from 12.4% to 17.7%). LBP (5 and 10 mg/kg, i.p., × 3 d) could antagonize the inhibition of NK cells by cyclophosphamide. NK cell killing of target cells was increased

from 9.5% to 15% (5 mg/kg) and 16% (10 mg/kg). LBP could improve the NK cell activity in tumor-bearing mice. LBP (10, 20, 50 mg/kg, i.p., × 3 d) increased the NK cell killing of target cells from 32.6 ± 5.9% to 48.4 ± 11.6%, 46.7 ± 11.4%, and 54.2 ± 20.2%, respectively.

1.1.2.1.3 Macrophages

Macrophages are key participants in innate immunity to kill pathogenic organisms. They perform a variety of complex microbicidal functions, including surveillance, chemotaxis, phagocytosis and destruction of targeted organisms (Beutler, 2004). In addition, macrophages can function as antigen-presenting cells and interact with T lymphocytes to modulate the adaptive immune response (Bryant and Ploegh, 2004). Furthermore, macrophages are involved in tissue remodeling during embryogenesis, injury, clearance of apoptotic cells and hematopoiesis (Diegelmann and Evans, 2004). Previous studies have shown that LBP could activate macrophages. Zhang et al (1994) injected LBP (10, 100 mg/kg) i.p. to mice daily for 4 days. It was found that in the LBP-injected mice the number of peritoneal macrophages and their pseudopods were significantly increased, the cellular volume was enlarged and the activity of phagocytosis was enhanced (Zhang et al, 1994). The contents of intracellular DNA, RNA and glycogen in the peritoneal macrophages harvested from the LBP-treated mice were increased as well (Zhang et al, 1994). The activities of intracellular acid phosphatase (AcPase), triphosphatase (ATPase), acid α-naphthyl acetic esterase (ANAE) and succinate dehydrogenase (SDH) were also significantly enhanced after LBP treatment (Zhang et al, 1994). These enymes play important roles in the process of killing microbes (Zhang et al, 1994). Zhang et al (1989)

studied the effects of LBP on mouse peritoneal macrophage inhibition of tumor cell growth. The results showed that LBP (40 mg/kg, i.p., × 7 d) could improve the inhibition of tumor cell growth by Con A-activated macrophages (Zhang et al, 1989). LBP (100 mg/kg, i.p.) significantly increased the phagocytic index of mouse peritoneal macrophages, indicating that their phagocytic ability were improved (Ma and Zhao, 2003). Gan and Zhang (2003) gave LBP3p (5, 10, 20 mg/kg) p.o. to S180-bearing mice daily for 10 days. The capacity of macrophages to phagocytoze cock red blood cells (CRBCs) was markedly improved in the LBP3p-treated mice (Gan and Zhang, 2003). LBP-X was reported having similar effects (Gan et al, 2004). A clinical trial was carried out to investigate the effects of LBP on 60 cancer patients on radiotherapy. It was shown that the number of white blood cells (WBCs) and the rate of macrophage phagocytosis were significantly increased after LBP treatment (Liu et al, 1996). LBP can significantly enhance the expression of C3b and Fc receptors on peritoneal macrophages and antagonize the inhibition of the expression by hydrocortisone acetate (immunosuppressive agent) (Li et al, 1990). Macrophages produce cytotoxic factors after activation. Wang et al (1997) reported that LBP could stimulate macrophages to produce cytotoxic factors. They found the supernatant harvested from LBP-stimulated macrophages could noticeably lyse target cells. Wang et al (1998) stimulated rat peritoneal macrophages *in vitro* with LBP alone or combined with LPS. The result showed LBP (0.32-20 μg/ml) enhanced the capacity of macrophages to phagocytoze neutral red dye in a dose-dependent manner. LBP (2.5 μg/ml) increased the phagocytic rate 2.6-fold. In addition, LBP (5-100 μg/ml) promoted LPS-activated macrophages to produce IL-1 and TNF-α in a dose-dependent manner (Wang et al, 1998). LBP (p.o. or

i.p.) significantly increased the amount of nitric oxide (NO) and enhanced the activities of intracellular lysozyme (LSZ) and superoxide dismutase (SOD) produced by mouse peritoneal resting macrophages (Zhou et al, 2000). Moreover, LBP could stimulate thioglycerol (TG)-activated macrophages to produce these parameters to a higher level, indicating that LBP has effects on both resting and activated macrophages (Zhou et al, 2000). Activation of macrophages by LBP may be related to the calcium signaling pathway. Qi et al (1999), who treated macrophages with LBP *in vitro*, found that the concentration of free calcium in the cytoplasm of macrophages was rapidly increased after LBP stimulation. It was reported that LbGp4 and LbGp4-OL (LbGp4-O-Linkage) (10-100 μg/ml) markedly increased the contents of neutral red dye phagocytozed by resting macrophages and the CRBC phagocytic index of starch-activated macrophages was also elevated (Qi et al, 2005). The levels of NO, IL-1 β and TNF-α produced by resting macrophages were also promoted after incubation of resting macrophages with LbGp4 or LbGp4-OL (Qi et al, 2005). The biological activities of IL-1β and TNF-α were augmented toward L929 cells and mouse thymocyte target cells, respectively (Qi et al, 2005). These results indicated that LbGp4 and LbGp4-OL could enhance macrophage phagocytic functions, suggesting that macrophages are the main immune effective target cells of LbGp4 and LbGp4-OL (Qi et al, 2005).

1.1.2.1.4 Lymphokine Activated Killer Cells

Lymphokine activated killer (LAK) cells are WBCs that help to identify and destroy cancer cells in the body, which can be produced by cultivation of peripheral lymphocytes with interleukin-2 (IL-2) and used experimentally to shrink malignant tumors (Winter

and Fox, 1999). It was shown that LBP could improve the activity of LAK cells (Cao and Du, 1993). A single injection (i.p.) of LBP (5, 10 mg/kg) caused splenocytes of adult C57BL/6 mice to proliferate significantly more in number than saline control. Splenocytes of LBP-treated aged mice were 4 times more than those of the saline control. LAK cells were induced by incubation of mouse splenocytes with 125-1000 U/ml of recombinant mouse IL-2 (rIL-2) for 4 days *in vitro*. The LAK cell cytotoxicity was tested by the 18 h-[^{125}I]-UdR-release assay. It was found that the cytotoxicity caused by LAK cells from the splenocytes of LBP-treated adult mice were 26% and 80% higher respectively than that of the saline control. The dose of rIL-2 used to induce LAK cells was reduced 50%. The cytolytic activities of LAK cells from the splenocytes of LBP-treated aged mice were 120% and 200% higher than those of the saline control, and the dosage of rIL-2 was reduced more than 75% *in vitro* (Cao and Du, 1993). This approach was applied to a clinical trial later, in which seventy-nine patients with advanced cancers were treated with LAK/IL-2 in combination with LBP (1.7 mg/kg, p.o., × 3 m) (Cao et al, 1995). Initial results of the treatment from seventy-five patients indicated that objective regression of cancer was achieved in patients with malignant melanoma, renal cell carcinoma, colorectal carcinoma, lung cancer, nasopharyngeal carcinoma, and malignant hydrothorax (Cao et al, 1995). The response rate of patients treated with LAK/IL-2 plus LBP was 40.9% while that of patients treated with LAK/IL-2 was 16.1% ($P < 0.05$). The mean remission duration in patients treated with LAK/IL-2 plus LBP was also significantly longer. This treatment led to a marked increase in NK and LAK cell activities than LAK/IL-2 alone. The results indicated that LBP can be used as an adjuvant in the biotherapy of cancer (Cao et al, 1995).

1.1.2.1.5 Humoral Immunity

Humoral immunity is mediated by secreted antibodies, which are produced by cells of the B lymphocyte lineage. Secreted antibodies bind to antigens on the surfaces of invading microbes, involving pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination. Previous studies have shown that LBP can enhance humoral immunity. Qi et al (2001) reported that crude LBP significantly promoted LPS-induced splenoctye proliferation. It was shown that LBP (p.o.) not only promoted splenocyte proliferation, but also increased the number of anti-SRBS plaque-forming cells (PFC) of LACA mouse splenocytes. Furthermore, LBP enhanced the level of IgG production by splenocytes of SAM mice, indicating it can improve humoral immunity (Qi et al, 2001). Wang et al (1995) studied the effect of LBP2 (i.p., × 7 d) on the recovery of immune function of the irradiation-damaged mice. The result showed that splenocytes harvested from the mice irradiated with ^{60}Co and treated with LBP2 responded more strongly to LPS stimulation than those harvested from the irradiation control mice. Fu et al (2007) treated 28 sodium fluoride-exposed workers with LBP for 7 days. The result showed that the IgG, IgA and IgM contents in the serum were significantly increased after treatment, indicating that LBP can enhance the humoral immune function. Wolfberry tea has a similar function. It was found to increase immunoglobins (especially IgM) and complement in Wistar rats by p.o. administration (Xing, 1989).

1.1.2.1.6 Cytokines and Their Receptors

Cytokines are a group of secreted proteins and polypeptides which mediate and regulate immunity, inflammation and hematopoiesis. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins, proliferation and secretion of effector molecules (Khawli et al, 2008). Researchers found that LBP could regulate the production of a number of cytokines such as IL-2, IL-3, IL-6, and TNF-α. Qian et al (1988) reported that LBP had bidirectional regulatory effect on IL-2 production. At 10 μg/ml, it promoted mitogen-induced T and B cell proliferation and IL-2 production, whereas at 1 mg/ml, it inhibited IL-2 production. Clinically, it was found that in aged people (average age: 54 years) who consumed 20 g of *Lycium* fruit daily for 3 weeks, the T cell transformation rate was increased 3.28-fold while the IL-2 activity was increased 2.26-fold in more than one third of the cases (Qian et al, 1989). Hu et al (1995) reported that *L. barbarum* water extract significantly promoted IL-2 secretion and IL-2 receptor (IL-2R) (α, β) expression by PHA-induced human tonsil lymphocytes. Similar results were found when LBP was given to naturally occurring senile mice and D-galactose-induced senile mice (Chen et al, 2001; Qiu et al, 2001). Gan et al (2003) reported that LBP3p (5, 10, 20, 40 μg/ml) significantly upregulated IL-2 and TNF-α mRNA expression and protein secretion by human peripheral blood mononuclear cells in a dose-dependent manner. IL-2 and TNF-α productions peaked at 12 h and 8 h after stimulation, respectively. Another fraction LBP-X prepared by this group also had similar activities (Gan and Zhang, 2002). It was found that LBP had bidirectional regulatory effect on IL-3

production (Qian et al, 1989). At low concentration (10 μg/ml), LBP promoted IL-3 production, whereas at high concentration (1 mg/ml) it inhibited IL-3 production (Qian et al, 1989). Du et al (1994) used LBP (0.5 μg/ml) alone or plus LPS to stimulate human tonsil cells *in vitro*. After 48 hours the supernatant was harvested and the cytokines, IL-6 and TNF-α, were tested. It was shown that LBP induced IL-6 production, whereas it failed to induce TNF-α production (Du et al, 1994). But LBP could significantly promote both IL-6 and TNF-α production by LPS-activated human tonsil cells (Du et al, 1994). He et al (2005) treated H22 tumor-bearing mice with LBP (p.o.). After 2 weeks, tumor was weighed and the cytokines, vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β), in the serum were measured by ELISA. The results showed that tumor growth was inhibited and the VEGF and TGF-β secretions were significantly down-regulated in the mice treated with LBP, indicating that LBP can prevent cancer cells from immune escape and protect the body against cancer (He et al, 2005).

1.1.2.1.7 Signal Transduction

Immune cell activation and proliferation and cytokine secretion are all related to signal transduction, which is carried out largely by membrane receptors such as G-protein couple receptors and receptor tyrosine kinases and second messengers such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), diacylglycerol (DAG), inositol-1,4,5-trisphosphate (IP_3), Ca^{2+} (Zenner et al, 1995; Pawson, 1994; Luttrell et al, 1997). Previous researches have found that LBP can influence immune cell signal transduction pathways. Zhang et al (1997b) reported that

LBP (50, 400 μg/ml) increased the intracellular levels of cAMP and cGMP in mouse lymphocytes. 50 μg/ml of LBP increased the cGMP level of the PMA-activated lymphocytes. LBP-X fraction also has similar effects (Du, 2005). Alternatively, LBP (100 μg/ml) enhanced the membrane protein kinase C (PKC) activity of the lymphocytes activated by Con A. The results suggested that the immunomodulatory mechanism of LBP involves cAMP/cGMP system as well as PKC signaling pathways (Zhang et al, 1997b). This group also found that LBP (100 μg/ml) markedly promoted the membrane mobility of rabbit RBCs and enhanced the membrane mobility induced by Con A (10 μg/ml) (Zhang et al, 1997a). Ca^{2+} functions as an ubiquitous intracellular messenger and plays crucial roles in signal transduction pathways (Feske, 2007). Qi (1999) reported that LBP could upregulate free Ca^{2+} level in mouse lymphocytes rapidly in a dose-dependent manner. LBP-X could increase the free Ca^{2+} concentration in the cytoplasm of mouse splenocytes and macrophages within 2-3 minutes (Du, 2005).

1.1.2.2 Anti-aging, Anti-oxidation, and Anti-peroxidation

Aging is the process of growing older and includes both biological and psychological changes. There are more than 300 theories to explain the aging phenomenon. Among all the theories, the free radical theory of aging, postulated first by Harman, is the most popular and widely tested (Ashok and Ali, 1999). Free radicals are atoms with unpaired electrons. The basic concept of the free radical theory includes that radicals damage cells in an organism and cause aging (Harman, 1956), and mitochondria, regions of the cell that manufacture chemical energy, produce free radicals and are the primary sites for free radical damage (Harman, 1972).

Previous studies have shown that LBP has anti-aging, anti-oxidation and anti-peroxidation effects. A model for studying aging is to investigate the life span of *Drosophila melanogaster* (fruit fly). It has been strikingly demonstrated that *L. barbarum* fruit and LBP can significantly prolong the average lifespan of fruit fly (Dai et al, 1994). Their study in humans showed that the anti-aging effect of LBP may be related to the regulation of some parameters (Dai et al, 1987). They found that *L. barbarum* fruit significantly improved lysozyme activity, IgG and IgA levels, T lymphocyte transformation rate, IFN-γ and IL-2 levels, and SOD contents, while dramatically reducing the level of lipid peroxidation (LPO) and triglycerides (TG) (Dai et al, 1987). It was observed that endogenous lipid peroxidation was increased, while the antioxidant activities, as assessed by SOD, catalase (CAT), glutathione peroxidase (GSH-Px) and total antioxidant capacity (TAOC), were decreased in aged mice (Li et al, 2007). LBP (200, 350 and 500 mg/kg, p.o., × 30 d) could restore these parameters to normal (Li et al, 2007). One of the experimental models for aging is an injection of D-galactose into rats for six to eight weeks (Ho et al, 2003). The metabolism of D-galactose as well as non-enzymatic glycation on D-galactose will gradually exert oxidative stress to the whole body including the brain, bone, liver and the immune system (Ho et al, 2003). LBP could inhibit non-enzymatic glycation in D-galactose-induced mouse aging model *in vivo* (Deng et al, 2003). Decreased levels of serum advanced glycated end products (AGE), hydroxyproline concentration in mouse skin and spontaneous motor activity in D-galactose-induced aging mouse were detected after treatment with LBP, while lymphocyte proliferation and IL-2 activity, learning and memory abilities, SOD activity of erythrocytes, were enhanced (Deng et al, 2003). An *in vivo* model was to investigate

the effect of *L. barbarum* on hypoxia-induced free radicals in mice. It was found that LBP treatment could not prolong mice survival time ($P > 0.05$), but the activities of SOD, CAT and total anti-oxidative capacity were increased as compared with the control group ($P < 0.05$). The study indicated that LBP could not increase hypoxic tolerance, but might have a protective effect on free radical injury caused by hypoxia (Li et al, 2002).

LBP also has effect on skin aging. It (p.o.) significantly increased the water content of skin, epidermis and hypodermis thickness, and fibroblast count in the aged skin induced by D-galactose, suggesting that it can improve the functioning of the internal organs to resist senility (Liang and Zhang, 2007). It was found that LbGp significantly reduced the level of matrix metalloproteinase (MMP)-1, but not that of MMP-3 or -13, in the whole human skin system, without compromising the viability of the skin (Zhao et al, 2005). Consistently, LbGp inhibited skin expansion under mechanical stress. One of *L. barbarum* glycoconjugates, the LbGp5, promoted the survival of human fibroblasts cultured in sub-optimal conditions. Furthermore, in the presence of LbGp5, these cultures also contained higher levels of the MMP-1 substrate-collagen type I. Together these results suggested that *L. barbarum* glycoconjugates in general, and LbGp5 in particular, may have important skin-protective properties (Zhao et al, 2005).

L. barbarum water extract possessed strong inhibition on malondialdehyde (MDA) formation in rat liver homogenate, and superoxide anion scavenging and anti-superoxide formation activities, suggesting that it is a good source of antioxidants as a daily dietary supplement (Wu et al, 2004). An *in vitro* study found that LBP could clear the free radicals and restrain DNA damage caused by the oxidative stress in testicular cells

(Huang et al, 2003). Pretreatment with LBP (50, 100, 200, 400 μg/ml) significantly decreased the frequencies of cells with tail movement and the tail length of testicular cells induced by H_2O_2 (Huang et al, 2003). The result was replicable using a mouse model (Luo et al, 2006). LBP had a dose-dependent protective effect against H_2O_2-induced oxidative damage to the DNA of mouse testicular cells (Luo et al, 2006). *In vivo* studies also showed similar findings (Wu et al, 2006). LBP treatment (10 mg/kg.d) for 4 weeks led to decreased levels of blood glucose, MDA and NO in the serum of fasting rats with type 2 diabetes mellitus; and to increased serum level of SOD. Furthermore, LBP could reduce cellular DNA damage in peripheral lymphocytes of type 2 diabetic rats (Wu et al, 2006). It was recently demostrated that LBP has protective effect on streptozotocin-induced oxidative stress in diabetic rats (Li, 2007). Administration of LBP in drinking water through oral gavage for 30 days could restore the abnormal oxidative indices in the blood, liver and kidney to nearly normal levels (Li, 2007). Aging is the primary risk factor for Alzheimer's disease (AD) and other prevalent neurodegenerative disorders (Yankner et al, 2007).

Recently it has been shown that LBP has neuroprotective effects. LBP (i.p.) could enhance the spontaneous electrical activity of the hippocampus, implying that LBP can improve cognitive functions (Peng et al, 2002). β-amyloid (Abeta) peptide is one of the toxic factors triggering progressive neuronal loss in AD (Sullivan et al, 2005). It was found that extracts from *L. barbarum* have neuroprotective effects against toxicity of fibrillar Abeta (1-42) and Abeta (25-35) fragments (Yu et al, 2005). Primary rat cortical neurons exposed to Abeta peptides resulted in apoptosis and necrosis. Pretreatment with

extract isolated from *L. barbarum* significantly reduced the release of lactate dehydrogenase (LDH) (Yu et al, 2005; Ho et al, 2007). In addition, it attenuated Abeta peptide-activated caspases-3-like activity (Yu et al, 2005; Yu et al, 2007). Pretreatment of LBP effectively protected neurons against Abeta-induced apoptosis by reducing the activity of both caspase-3 and -2, but not caspase-8 and -9 (Yu et al, 2007). The extract elicited a typical dose-dependent neuroprotective effect. Effective dosage of this extract was wider than that of a well-known western neuroprotective medicine, lithium chloride (Yu et al, 2005). Abeta peptides induce a rapid activation of c-Jun N-terminal kinase (JNK) by phosphorylation. Pretreatment of aqueous extract markedly reduced the phosphorylation of JNK-1 (Thr183/Tyr185) and its substrates, c-Jun-I (Ser73) and c-Jun-II (Ser63) (Yu et al, 2005). Glaucoma is one of the major neurological disorders in the eye, leading to irreversible blindness in the elderly (Chan et al, 2007). Oral administration of *L. barbarum* in Sprague-Dawley rats (250-280 g) significantly reduced the loss of retinal ganglion cells (RGCs), suggesting it may be a potential candidate for the development of neuroprotective drug against the loss of RGCs in glaucoma (Chan et al, 2007). Monoamine oxidase (MAO), consisting of MAO-A and MAO-B subtypes, catalyzes the oxidative deamination of biogenic amines accompanied by the release of H_2O_2 (Lin et al, 2003). The regulation of MAO-B activity is important in the treatment of neurodegenerative diseases (Lin et al, 2003). It was shown that the water-methanol extract of *Lycium chinense* (same family as *L. barbarum*) fruit significantly inhibited MAO-B activity in rat brain homogenates, indicating it can delay the neurodegeneration in neurological disease states (Lin et al, 2003).

1.1.2.3 Anticancer

It has been shown that LBP can inhibit the growth of a number of cancer cells *in vitro*, including leukemia, liver cancer, lung cancer, prostate cancer, and cervical cancer. One of the LBP fractions, LBP-X was proved to remarkably inhibit the growth of K562 cells after stimulation for 48 h (Cui et al, 2006). K562 cell apoptosis was dose- and time-dependent. Apoptotic characteristics, such as anomalistic, condensed and fragmented nuclei, DNA ladder, apoptotic peaks were observed on K562 cells after LBP-X stimulation (Cui et al, 2006). Similarly, LBP-X (20, 100, 500, 1000 μg/ml) inhibited the growth of human leukemia HL-60 cells in a dose-dependent manner (Gan et al, 2001). HL-60 cells treated with LBP-X underwent apoptosis (Gan et al, 2001). In addition, LBP-X could decrease the membrane fluidity of HL-60 cells (Gan et al, 2001). Crude LBP also possesses such effects. LBP could inhibit the growth of human hepatoma QGY7703 cells with cycle arrest in S phase. The cells underwent apoptosis after LBP treatment (Zhang et al, 2005). It was shown that after LBP treatment, the amount of RNA in cells and the concentration of intracellular Ca^{2+} were increased. Moreover, the distribution of calcium in cells was changed, suggesting that the induction of cell cycle arrest and the increase of intracellular calcium in apoptotic system may participate in the anti-proliferative activity of LBP in QGY7703 cells (Zhang et al, 2005). These results were reproducible using a lung cancer cell line. LBP could inhibit the growth of human lung cancer A549 cells in a dose-dependent manner and induced apoptosis (Xiao et al, 2006). The 1evel of intracellular free calcium concentration in LBP-treated A549 cells was significantly higher than that of the PBS control, indicating that one of its molecular

mechanisms may be associated with the increase in the level of intracellular free calcium concentration (Xiao et al, 2006).

LBP has bidirectional effects on the growth of human pulmonary giant cell carcinoma PG cells (Lu et al, 2002). At low dose, LBP promoted PG cell growth, whereas at high dose it inhibited the growth. Furthermore, LBP induction of PG cell apoptosis was time- and dose-dependent (Lu et al, 2002). LBP could significantly inhibit the growth of human prostate cancer PC-3 cells. The inhibition rate was up to 87% with about 40% of the cells becoming apoptotic (Li et al, 2005; Li et al, 2006; Cui et al, 2006). The ratio of Bcl-2/Bax protein was decreased significantly after LBP treatment (Li et al, 2005). Bcl-2 can inhibit apoptosis while Bax promotes apoptosis. Hot water-extracted crude LBP (2-5 mg/ml) could inhibit proliferation of hepatocellular carcinoma cells and induce p53-mediated apoptosis (Chao et al, 2006). LBP could inhibit the growth of cervical carcinoma Hela cells in a dose-dependent manner. The inhibition rate was up to 97% and the apoptosis rate was 36.8% (Cui, 2006).

LBP also has anticancer effect *in vivo*, which may be through the activation of immune cells such as T cells, macrophages, NK cells, and CTLs to eradicate cancer cells. LBP (10, 20, 50 mg/kg, i.p., × 7 d) could inhibit the growth of S180 tumor cells in a dose-dependent manner. T cell proliferation, NK cell activity, and TNF-α secretion were recovered in the tumor-bearing mice after LBP treatment, indicating that enhancement of the immunity of the tumor-bearing mice is one of its anticancer mechanisms (Liu et al, 1996). Oral administration of LBP also showed similar effects (Gan et al, 2004). LBP3p

(5, 10, 20 mg/kg, p.o., × 10 d) significantly inhibited the growth of transplantable sarcoma S180 and improved macrophage's capacity in phagocytosis, the form of antibody secreted by spleen cells, spleen lymphocyte proliferation, CTL activity, IL-2 mRNA expression level and reduced the lipid peroxidation in S180-bearing mice (Gan et al, 2004). LBP (5, 10, 20 mg/kg, p.o., × 14 d) could inhibit the growth of hepatoma H-22 up to 62.7%. NK cell activity was enhanced up to 35% (Zhu and Zhang, 2006b). SOD activity was increased 11 units (Zhu and Zhang, 2006a). LBP (p.o., × 14 d) remarkably down-regulated the secretions of immunosuppressive factors VEGF and TGF-β1 in hepatoma H-22 bearing mice, indicating that its anticancer effect is related to the regulation of VEGF and TGF-β1 production (He et al, 2005; He et al, 2006). LBP has synergic effect with cyclophosphamide on tumor inhibition. Cyclophosphamide (25 mg/kg, s.c., × 2 d) combined with LBP (10 mg/kg, i.p., × 7 d) increased the S180 tumor inhibition rate to 47%, whereas the inhibition rate was 31% when cyclophosphamide was used alone. Furthermore, the synergic effect was more significant when cyclophosphamide was at a low dosage of 12.5 mg/kg. At this dose, the tumor inhibition rate was 14%. When combined with LBP, it was increased to 54% (Cao and Du, 1992). In an *in vivo* model of Lewis lung cancer transplanted on C57BL/6 mice to investigate the radiosensitizing effects of LBP, it was found that when LBP alone was administered, it was not obvious that LBP inhibited the growth of Lewis lung cancer. The significant radiosensitizing effects were obtained by combination of LBP and radiation. The mean numerical value of the dose modifying factors (DMF) was 2.05 (Lu and Cheng, 1991). In a clinical trial, LBP (1.7 mg/kg, p.o.) could improve the effect of LAK/IL-2 therapy for advanced-stage cancer patients. The response rate of patients treated with LAK/IL-2 plus

LBP was 40.9% while that of patients treated with LAK/IL-2 was 16.1% ($P < 0.05$) (Cao et al, 1995). LBP in combination with allicin (isolated from garlic) could markedly improve the general status of tumor-bearing mice, increase the life span of fluid type mouse cervical carcinoma U14- and fibrosarcoma S180-bearing mice to 41% and 85%, respectively, and inhibit the growth of solid type cervical carcinoma U14 and fibrosarcoma S180 up to 81.5% and 73%, respectively (Dong et al, 1997). This treatment also significantly inhibited the growth and proliferation of human cervical carcinoma Hela cells and human gastric adenocarcinoma MGC-803 cells *in vitro* (Dong et al, 1997).

1.1.2.4 Reduction of Side-Effects of Chemotherapy and Radiotherapy

While chemotherapy and radiotherapy are effective in cancer treatment, they often cause side effects, such as nausea, diarrhea, fatigue, dry skin, hair loss, and suppression of the hematopoietic system and immune system. It has been shown that LBP can reduce the side effects of chemotherapy and radiotherapy. A clinical study was done to investigate the effect of LBP on 171 radiotherapeutic cancer patients (Liu et al, 1996). The results showed that the ratio of $CD4^+$ T cells to $CD8^+$ T cells, lymphocyte transformation rate, and macrophage phagocytic activity in the patients treated with LBP were markedly improved after radiotherapy. After radiotherapy, the total counts of WBCs and lymphocytes, the ratio of $CD4^+$ T cells to $CD8^+$ T cells, the rate of lymphocyte transformation, and the macrophage phagocytic activity in the LBP-treated group were significantly higher than those in the radiotherapy control group ($P < 0.01$), indicating that LBP can enhance the immunity of radiotherapeutic cancer patients (Liu et al, 1996). Another clinical trial was to observe the effect of LBP combined with chemotherapy on

treatment of 20 primary liver cancer patients (Gu, 1990). This treatment could reduce the side effects, such as suppression of hematopoietic system, nausea, diarrhea, and fatigue caused by chemotherapy (Gu, 1990). LBP2 could remarkably restore the immune function of ^{60}Co-irradiated mice (Wang et al, 1995). 30 days after irradiation, some parameters, including thymus index, splenocyte proliferation, mix lymphocyte reaction, and delayed type hypersensitivity (DTH) reaction, were better restored in the LBP-treated mice than in the irradiation control mice (Wang et al, 1995). In an *in vivo* experiment, LBP (50, 100, and 200 mg/kg, s.c., × 6 d) significantly ameliorated the decrease of peripheral WBCs, RBCs, and platelets in irradiation- or chemotherapy-induced myelosuppressed mice (Gong et al, 2005). In an *in vitro* experiment, LBP markedly stimulated human PBMCs to produce granulocyte-colony stimulating factor (G-CSF), suggesting that the improvement in peripheral blood parameters may be related to the stimulation of G-CSF production (Gong et al, 2005). Similarly, LBP was effective on peripheral RBCs and platelet recovery in mitomycin C-induced myelosuppressed mice (Hai et al, 2004).

1.1.2.5 Anti-diabetes

It was found that crude LBP and the purified fraction, LBP-X, could significantly reduce blood glucose levels and the concentrations of serum total cholesterol (TC) and TG in alloxan-induced diabetic or hyperlipidemic rabbits, and at the same time markedly increase high density lipoprotein cholesterol (HDL-c) levels after 10 days treatment in tested rabbits, indicating that LBP has substantial hypoglycemic and hypolipidemic effects (Luo et al, 2004). The hypoglycemic effect of LBP may be related to improved

insulin sensitivity. In a diabetes model induced by high-fat diet and streptozotocin (50 mg/kg, i.p.), LBP (p.o., × 21 d) significantly reduced the concentration of plasma triglyceride and weight in type 2 diabetic rats. Furthermore, LBP markedly decreased the plasma cholesterol and fasting plasma insulin levels, as well as the postprandial glucose level at 30 min during the oral glucose tolerance test. LBP also significantly increased the insulin sensitive index in the type 2 diabetic rats. Under insulin stimulus, cell-surface level of glucose transporter 4 (GLUT4) content in plasma membrane in type 2 diabetic control rats was significantly lower than that of control ($p < 0.01$), and GLUT4 content in the plasma membrane in LBP-treated diabetic rats was higher than that of diabetic control ($p < 0.01$). These results indicated that LBP can ameliorate insulin resistance, and the mechanism may be via increased cell-surface level of GLUT4, improving GLUT4 trafficking and intracellular insulin signaling (Zhao et al, 2005). The hypoglycemic effect of LBP may also be related to its inhibition of α-glucosidase. LBP is a potent α-glucosidase inhibitor (Tian et al, 2005; Tian et al, 2006). LBP (0.4 mg and 4 mg) could inhibit α-glucosidase up to 52% and 88%, respectively (Tian et al, 2006). The mechanism is via non-competitive inhibition (Tian et al, 2005; Tian et al, 2006). LBP (20, 40 mg/kg, p.o., × 4 w) could reduce fasting blood glucose (FBG) level, increase insulin level, and increase the density, nuclear area, and the ratio of nucleus to cytoplasm of islet β cells in high fat diet- and streptozotocin-induced type 2 diabetic mice, while the islet α cells were reduced (Zhao et al, 2007). These results suggested that LBP possesses the action of lowering blood glucose, improvement of morphogeny and function of pancreatic islet β cells and increase of insulin secretion in type 2 diabetic mice (Zhao et al, 2007). In an *in vitro* experiment, it was shown that LBP could protect the alloxan-induced rat pancreatic

islet damage (Zhang and Wang, 2005). LBP could restore glucokinase and SOD activity, insulin synthesis and secretion inhibited by alloxan, and reduce NO and MDA production in alloxan-induced islets, suggesting that LBP could protect glucose-induced insulin synthesis and insulin secretion through reducing NO production and maintaining glucokinase and SOD activities in pancreatic β cells (Zhang and Wang, 2005). LBP could alleviate the oxidative stress of diabetic nephropathy and has protective effect on the kidney of diabetic rats (He and Liu, 2006). LBP (250 mg/kg, p.o.) could lower the levels of blood glucose, TG, cholesterol, low-density lipoprotein (LDL), blood urea nitrogen, serum creatinine, and urine microalbumin of type 2 diabetic rats (He and Liu, 2006).

1.1.2.6 Cytoprotection

A study was done to investigate the protective effect of *Lycium chinense* Miller (Solanaceae) fruit (LFE) against CCl_4-induced hepatotoxicity in rats and the mechanism underlying this protective effect (Ha et al, 2005). Pretreatment of LFE was shown to cause a significant protection by lowering the serum aspartate and alanine aminotransferase (AST and ALT) and alkaline phosphatase (ALP). This hepatoprotective action was confirmed by histological observation (Ha et al, 2005). In addition, pre-treatment of LFE prevented the elevation of hepatic MDA formation and the depletion of reduced glutathione (GSH) content and catalase activity in the liver of CCl_4-injected rats. LFE also displayed hydroxyl radical scavenging activity in a dose-dependent manner. The expression levels of cytochrome P450 2E1 (CYP2E1) mRNA and protein were significantly decreased in the liver of LFE-pretreated rats, suggesting that the hepatoprotective effects of the LFE might be related to anti-oxidative activity and

regulation of CYP2E1 expression (Ha et al, 2005). In an independent experiment to study the preventive effects of LBP on the development of alcoholic fatty liver (AFL) in rats and its possible mechanisms, LBP was also found to reduce ALT, AST and gamma-glutamyl transferase (GGT) content in the serum, lower the MDA, H_2O_2 and CYP2E1 levels in the liver, while enhancing the activity of SOD and GSH-PX in the liver (Gu et al, 2007). The prevention of AFL by LBP may be due to its effects in inhibiting hepatocyte CYP2E1 expression as well as prevention of lipid peroxidation (Gu et al, 2007). LBP was also shown to have a protective effect on doxorubicin-induced cardiotoxicity (Xin et al, 2007). Pretreatment with LBP significantly prevented the loss of myofibrils and improved the heart function of the doxorubicin-treated rats as evidenced from lower mortality (13%), normalization of anti-oxidative activity and serum AST and creatine kinase, as well as improving arrhythmias and conduction abnormalities (Xin et al, 2007). LBP exhibits cytoprotective effects against reducing stress by lowering the dithiothreitol (DTT)-induced LDH release and caspase-3 activity (Yu et al, 2006). DTT can trigger endoplasmic reticulum (ER) stress leading to PKR-like ER kinase (PERK) activation. It was also shown that *L. barbarum* glycans (LBG) attenuated DTT-induced PERK phosphorylation (Yu et al, 2006). LBP provided a protective effect against testicular tissue damage induced by heat exposure (Luo et al, 2006). When compared with negative control, LBP significantly increased testis and epididymis weight, improved SOD activity, and raised sexual hormone levels in the damaged rat testes. LBP had a dose-dependent protective effect against DNA oxidative damage of mouse testicular cells induced by H_2O_2 (Luo et al, 2006).

1.1.2.7 Promotion of Hematopoiesis

LBP (10 mg/kg, i.p., × 3 d) could increase the burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) in mouse bone marrow by 342% and 192%, respectively (Zhou et al, 1991). The number of peripheral blood reticulocytes was increased 218% on day 6 after LBP injection. In addition, LBP could promote mouse splenoctyes to secrete colony stimulating factors and improve their activity (Zhou et al, 1991). LBP could stimulate the spleen colony forming unit (CFU-S) proliferation, markedly increase the number of granulocyte-monocyte colony forming unit (CFU-GM), and promote them to differentiate toward granulocytes (Zhou, 1991). A model was to investigate the therapeutic effects of LBP on irradiation- and chemotherapy-induced myelosuppressed mice (Gong et al, 2005). Mice were irradiated with sublethal dose of 550 cGy X-ray or single injection of carboplatin (125 mg/kg, i.p.) to induce severe myelosuppression. It was found that LBP (50, 100, 200 mg/kg, i.p., × 6 d) could significantly promote the recovery of peripheral blood, such as WBC, RBC, and platelet counts in the myelosuppressed mice (Gong et al, 2005).

1.1.2.8 Hypertension Prevention

A study showed that LBP could prevent hypertension (Jia et al, 1998). In the study, the effects of LBP on endothelial function in the two-kidney, one clip model of hypertension were observed. The results showed that the increase of blood pressure in hypertensive rats (HR) could be prevented significantly by treatment with 10% LBP. In isolated aortic rings of LBP-treated rats, the contraction of phenylephrine (PE) was reduced as compared with HR rats. Removal of the endothelium abolished the difference of PE-

induced vasoconstriction among groups. *In vitro* incubation of aortic rings from LBP-treated rats with methyl blue (MB) or N-nitro-L-arginine methyl ester (L-NAME) increased the magnitude of PE-induced contraction. Meanwhile the response to acetylcholine (ACh) was significantly increased in LBP-treated rats, but the response to nitroprusside was not significantly different among the different groups. Pretreatment with L-arginine partially restored ACh-induced relaxation in HR rats, but had no effect in LBP-treated rats. These results suggested that the role of LBP in decreasing vasoconstriction to PE may be mediated by increase of endothelium-derived relaxation factor (EDRF) production. Increased formation of EDRF by LBP may be related to increase in the substrate of EDRF (Jia et al, 1998).

1.2 T Cell Activation

T lymphocytes are a group of white blood cells that play a central role in cell-mediated adaptive immunity. They originate from hematopoietic stem cells in the bone marrow and migrate to the thymus, where they undergo positive selection, lineage commitment, and negative selection, and become mature $CD4^+$ or $CD8^+$ cells (Li, 2006; Ye and Graf, 2007; Miosge and Zamoyska, 2007; Chen, 2004). The functionally competent T lymphocytes then emigrate to the periphery. T cells consist of two subsets, helper T lymphocytes ($CD4^+$) and CTLs ($CD8^+$). When T cells recognize antigens in peripheral lymphoid organs or tissues, they are activated and then become effector cells to perform functional responses such as cytokine secretion, proliferation, and differentiation. Effector $CD4^+$ T cells differentiate into Th1 subset, which produces IFN-γ, Th2 subset, which produces IL-4, (Farrar et al, 2002), and Th17 subset, which produces IL-17 (Dong, 2008). $CD8^+$ T cells differentiate into functional CTLs. Some of the antigen-stimulated T cells develop into memory cells (Khanolkar et al, 2007; Lefrançois, 2006; Foulds et al, 2006). T cell activation is composed of a cascade of events, including TCR/CD3 recognition of peptide-MHC (I or II) complex, formation of immunological synapse (IS), and triggering of multiple signaling pathways.

1.2.1 TCR/CD3 Recognition of Peptide-MHC Complex

1.2.1.1 T Cell Receptor Complex

The T cell receptor (TCR) is a clonally distributed receptor which recognizes the peptide-major histocompatibility complex (MHC) displayed on antigen presenting cells (APCs). There are two types of TCRs, αβTCR and γδTCR. Most T cells express αβTCR, which is

composed of two covalently linked α and β chains with complementarity-determining regions (CDRs) to bind peptide-MHC complexes (Davis et al, 1998). The α and β heterodimer is non-covalently associated with the CD3 and ζ chains to form the TCR complex. The CD3 chains consist of two heterodimers designated as γε and δε. The ζ chain consists of one homodimer ζζ. Both CD3 and ζ chains contain the immunoreceptor tyrosine-based activation motif (ITAM), where communication of αβTCR engagement by peptide-MHC to the intracellular signaling machinery takes place (Kane et al, 2000). The characteristic of the ITAM is a pair of tyrosine residues separated by 9-11 amino acids. These tyrosines become rapidly phosphorylated by the Src-family kinase Lck following TCR stimulation, a required event for initiating TCR signaling (Huang and Wange, 2004).

1.2.1.2 Role of Costimulators in T Cell Activation

T cell activation requires engagement of the TCR with the peptide-MHC complex presented on the cell surface of APCs (Greenwald et al, 2005). In addition to this antigen-specific interaction, a second interaction involving costimulatory receptors on T cells and their respective ligands on APCs is required for optimal T cell activation (Bhatia et al, 2006). This pathway consists of two B7 family members, B7-1 (CD80) and B7-2 (CD86), both of which are expressed on activated APCs and bind to the same receptor CD28, which is constitutively expressed on the surface of T cells (Wang and Chen, 2004). CD28 is the best understood of the costimulatory molecules, and delineating the pathways by which it enhances T-cell activation will be central for the design of T-cell costimulatory therapeutics. The most discernible effects of CD28 ligation are observed when this signal is given in concert with TCR stimulation. Ligation of CD3 and CD28 (CD3/28

costimulation) promotes increases in glucose metabolism, high levels of cytokine/chemokine expression including a unique ability to produce very high levels of IL-2, resistance to apoptosis, and long-term expansion of T cells (Riley and June, 2005). Engagement of CD28 on naïve T cells by either B7-1 or B7-2 ligands on APCs also confers critical survival signals to activated T cells through the Bcl-xL pathway (Wang and Chen, 2004).

1.2.1.3 TCR Binding of Peptide-MHC Complex

Clonotypic αβTCRs recognize peptides presented by either class I or class II MHCs. Class II MHCs present peptides that originate from proteolysis of extracellular antigens in endosomal-type compartments, whereas class I MHCs present peptides primarily derived from intracellular degradation of proteins in the cytosol. TCRs that recognize these MHCs are found on two distinct cytotoxic and T-helper cell lineages, depending on the class of the MHC to which they are restricted (Rudolph et al, 2006). The αβTCRs bind peptide-MHC through CDRs present in their variable domains (Davis et al, 1998). Like many cell surface receptors that interact with ligands on other cell surfaces, αβTCRs bind with generally very low peptide-MHC complexes (about 1-50 μM). This is 99.9-99.99% weaker than that of most affinity-matured antibodies and reflects the fact that the confined space between two cell membranes and polyvalency mandate much lower affinities with no apparent loss of specificity (Krogsgaard and Davis, 2005). In addition to their cognate TCRs, class I and class II MHCs are recognized by their respective co-receptors, CD8 and CD4. The CD8αα homodimer binds primarily to the α3 domain of the MHC molecule in an antibody-like fashion, with the MHC α3 CD loop wedged between

two corresponding CDR-like loops from the CD8αα dimmers (Rudolph et al, 2006). While both domains of CD8 cooperate to bind class I MHCs, only one domain (the N-terminal variable-like region) of CD4 makes contact with the MHC with the second tandem CD4 domain being distal to the interface (Rudolph et al, 2006).

1.2.2 Formation of the Immunological Synapse

Following the initial engagement of TCRs with peptide-MHC complex, several T cell surface proteins and intracellular signaling components rapidly cluster at T cell-APC contacts. This accumulation of receptors is referred to as an immunological synapse (IS) (Irvine and Doh, 2007). In the mature synapse, a central structure called the central supramolecular activation cluster (cSMAC) is notable for being enriched with the TCR complex (TCR, CD3, and ζ chains) and other signaling molecules (CD4 or CD8 co-receptors, CD28 costimulatory molecules, CD2, PKCθ, etc) (Bromley et al, 2001; Cemerski and Shaw, 2006). Surrounding the cSMAC is the peripheral SMAC (pSMAC), which is enriched mainly with leukocyte function-associated antigen 1 (LFA-1), talin, very late antigen 4 (VLA-4), adhesion- and degranulation-promoting adaptor protein (ADAP) and transferring receptor (Bromley et al, 2001; Cemerski and Shaw, 2006). The formation of the IS brings signaling molecules into proximity to one another and to the receptors that activate these molecules to initiate and amplify TCR-induced signals. In addition, the formation of IS ensures that the molecules that T cells use to communicate with APCs are brought close to the target molecules on the APCs. Accumulation of fusion proteins at this site is easily and reliably scored and has been productively used as an indicator of T cell reactivity (Richie et al, 2002). The IS, although not required for

initiating TCR signaling, is required for sustained signaling, IL-2 production, and proliferation (Huppa and Davis, 2003). It was proposed that the IS functions as an adaptive controller, dampening strong signals and enhancing weak signals (Lee et al, 2003).

1.2.3 Activation and Recruitment of Kinases and Adaptor Proteins

Antigen engagement by the TCR results in the recruitment of Src family kinases, Lck and Fyn, in the proximity of the IS. Lck and Fyn may be activated by autophosphorylation, and in turn phosphorylate the tyrosine residues in the ITAMs of the CD3 and ζ chains (Palacios and Weiss, 2004). The phosphorylated tyrosine residues then serve as docking sites for the Syk-family kinase (SFR) ZAP-70. SFKs then phosphorylate and activate the recruited ZAP-70 (Palacios and Weiss, 2004). Activated ZAP-70 autophosphorylates at tyrosines 292, 315, and 319, which serve as docking sites to recruit various positive and negative signaling effectors to the TCR complex (Huang and Wange, 2004). In addition to serving as a scaffold via self-phosphorylation, ZAP-70 also phosphorylates a restricted set of substrates following TCR stimulation, including α-tubulin, Sam-68, Vav-1, VHR, Shc, Gab2, LAT, and SLP-76 (Huang and Wange, 2004). The latter two substrates in particular have been recognized to play a pivotal role in TCR signaling. The adapter protein, LAT, was identified as an integral transmembrane protein of 36-38 kDa (Zhang et al, 1998). SLP-76 is a cytosolic protein and refers to SH-2 binding leukocyte phosphoprotein of 76 kDa (Clements, 2003). When phosphorylated, both LAT and SLP-76 act as linker/adapter proteins, leading to the binding with Grb2, Grap, Gads-SLP-76, phospholipase Cγ-1 (PLC-γ1), Vav, Cbl and the regulatory subunit of PI-3K.

Consequently, upon their binding to LAT, these proteins can themselves be activated by tyrosine phosphorylation and find higher concentrations of their substrates in the plasma membrane (Aguado et al, 2006). Acting in concert, LAT and SLP-76 regulate the activation of PLCγ-1 and the subsequent hydrolysis of PIP_2 to generate DAG and IP_3, second messengers in PKC and Ras activation (via DAG) and calcium mobilization (via IP_3) (Huang and Wange, 2004).

1.2.4 Activation of Signaling Pathways

1.2.4.1 Ras-MAP Kinase Signaling Pathway

The Ras pathway is activated in T cells on TCR clustering, leading to the activation of MAP kinases and eventually transcription factors. Ras proteins are molecular switches that cycle between inactive, GDP-bound, and active, GTP-bound, forms. Signal-induced conversion of the inactive to active state is mediated by guanine nucleotide-exchange factors (GEFs) that stimulate the exchange of GDP for GTP (Vetter and Wittinghofer, 2001). When LAT is phosphorylated, it recruits another adaptor protein Grb-2 through binding the SH2 domain, which is then activated by ZAP-70 and serves as the docking site for the SH3 domain of the GTP/GDP exchange factor Sos. Sos then converts the inactive Ras-GDP to the active Ras-GTP (Zebisch et al, 2007). Once Ras is activated at the membrane, it recruits and activates the serine/threonine kinase Raf-1, which then phosphorylates and activates MEK (MAPK/Erk kinase), a dual specificity tyrosine/threonine kinase, that in turn phosphorylates and activates Erk1 and Erk2. Phospho-Erk forms dimers that are transported into the nucleus, where they phosphorylate the Ets family of transcription factors, including Elk-1. The

phosphorylated Elk-1 stimulates transcription of Fos, a component of the activation protein-1 (AP-1) transcription factor (Mor and Philips, 2006). In parallel with Ras-MAP pathway is the JNK pathway, which represents one sub-group of MAP kinases that is activated primarily by cytokines and exposure to environmental stress (Davis, 2000; Weston and Davis, 2002). Besides Grb-2 and Sos, the phosphorylated adaptor LAT also recruits and activates a GTP/GDP exchange protein called Vav that acts on another small 21-kDa guanine nucleotide-binding protein called Rac. The Rac-GTP activates MAP3Ks, which then phosphorylate and activate the MAP2K isoforms MKK4 and MKK7, which in turn phosphorylate and activate JNK. Activated JNK then phosphorylates c-Jun, the second component of AP-1 (Davis, 2000; Weston and Davis, 2002; Weston and Davis, 2007).

1.2.4.2 Calcium-Dependent Signaling Pathway

Engagement of TCR leads to the recruitment of adaptor proteins and kinases, resulting in tyrosine phosphorylation of PLCγ, a cytosolic enzyme specific for inositol phospholipids that is recruited to the plasma membrane by tyrosine phosphorylated LAT (Huang and Wange, 2004; Aguado, 2006). Recruited PLCγ is phosphorylated by ZAP-70 and other kinases such as the Tec family kinase Itk, resulting in the hydrolysis of PIP_2 to the second messengers, DAG and IP_3 (Savignac et al, 2007). IP_3 binds to IP_3 receptors in the ER and induces the release of Ca^{2+} into the cytoplasm. Depletion of Ca^{2+} from intracellular stores triggers the entry of Ca^{2+} across channels in the plasma membrane (Lewis, 2001). Ca^{2+} influx through these channels elevates the intracellular (Ca^{2+}) ($[Ca^{2+}]i$) for a period of minutes to hours (Quintana et al, 2005). Cytosolic free calcium acts as a signaling

molecule by binding to calmodulin, an ubiquitous calcium-dependent regulatory protein. Calcium-calmodulin complexes then activate a serine/threonine phosphatase calcineurin, which then dephosphorylates the nuclear factor of activated T cells (NFAT). The dephosphorylated NFAT then translocates to the nucleus and triggers the expression of genes that control activation, proliferation, differentiation, and effector functions of activated T lymphocytes (Savignac et al, 2007).

1.2.4.3 Protein Kinase C-Mediated Signaling Pathway

PKC has several isoforms classified as conventional PKCs (cPKCs; α, β, and γ), which are activated by Ca^{2+} and the second messenger DAG, and the atypical PKCs (aPKCs; ζ and λ), which are not activated by Ca^{2+} or DAG, and novel PKCs (nPKCs; θ, ε, and η), which are Ca^{2+} independent (Hayashi and Altman, 2007). cPKCs, which are activated by the PIP_2 breakdown product DAG, participate in the generation of active transcription factors. Ionomycin (Ca^{2+} mobilizer) in combination with phorbol esters (PKC activators) mimics the signals required for T cell activation, indicating that IP_3-induced Ca^{2+} influx and DAG-mediated PKC activation cooperate with each other to mediate T cell activation (Macian et al, 2003). Among all of the PKCs, PKCθ plays the most important role in T cell activation. This Ca^{2+} independent serine/threonine kinase is selectively expressed in T cells and skeletal muscle and has been revealed in several studies as an essential member of the NF-κB activation cascade in T cells (Schmitz et al, 2003). PKCθ is indispensable for NF-κB activation and its enzymatic activity depends on recruitment to the IS. TCR engagement leads to the activation of PLC-γ1, which hydrolyzes PIP_2 to IP_3 and DAG. DAG activates PKCθ, which is then translocated into the IS through a

mechanism requiring the activation of PI-3K by the engagement of CD28. Several adaptor proteins, including caspase recruitment domain (CARD), membrane-associated guanylate kinase (MAGUK), caspase recruitment domain-containing MAGUK protein-1 (CARMA1, also termed CARD11), B-cell lymphoma-10 (Bcl10) and mucosa-associated lymphoid tissue-1 (MALT1), as well as the IκB kinase (IKK) complex, are also recruited to the IS (Lin and Wang, 2004; Weil et al, 2004; Hayashi and Altman, 2007). Deletion of the *CARMA1* gene in mice or in Jurkat T cells results in impaired receptor- and PKC-mediated T cell proliferation and cytokine production resulting from a selective defect in NF-κB and JNK activation (Hara et al, 2004; Jun et al, 2003; Hayashi and Altman, 2007). Downstream from PKCθ, the CARD proteins CARD11/CARMA1 and Bcl10 relay T cell receptor-derived signals to the IKK complex. A poorly understood mechanism involving phosphorylation of IKKb by TAK1 then leads to the activation of the IKK complex (Siebenlist et al, 2005). Once activated, the IKK kinases phosphorylate the IκB inhibitors of nuclear factor- κB (NF-κB) inducing their ubiquitination and degradation. Following this process, NF-κB translocates to the nucleus and activates its target genes (Siebenlist et al, 2005).

1.2.5 Activation of Transcription Factors

1.2.5.1 NFAT

NFAT protein is a family of transcription factors including NFAT1 (NFATp, NFATc2), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx, NFATc3), and NFAT5. The only NFAT protein not regulated by Ca^{2+}, NFAT5, is a transcription factor crucial for cellular response to hypertonic stress (Savignac et al, 2007; Lopez-Rodriguez

et al, 1999). It was first found as an inducible nuclear factor that could bind the IL-2 promoter in activated T cells (Shaw et al, 1988).

In resting cells, NFAT proteins are phosphorylated and reside in the cytoplasm. They are activated by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin through a rise in $[Ca^{2+}]i$ (Chen et al, 1998), the latter depending strictly on Ca^{2+} influx through calcium-release-activated calcium (CRAC) channels (Feske et al, 2006). Calcium binds receptor calmodulin, which in turn activates calcineurin. Calcineurin-mediated NFAT dephosphorylation induces a conformational change in the NFAT molecule that exposes its nuclear localization signals (NLS), enabling the import of NFAT into the nucleus and the induction of NFAT-mediated gene transcription (Savignac et al, 2007). In addition, calcineurin also enhances the nuclear retention of NFAT by masking the nuclear export signals (NES) and maintaining NFAT in its dephosphorylated state (Savignac et al, 2007).

The importance of NFAT proteins in T-cell activation is underscored by genetic data (Macian, 2005). In two human families, the inability to activate NFAT proteins because of a defect in store operated calcium entry was associated with severe immunodeficiency (Feske et al, 2000). In mice, deficiency in both NFAT1 and NFAT2 in T cells is associated with grossly impaired production of many cytokines, including IL-2, IL-4, IL-10, IFN-γ, GM-CSF, and TNF. IL-5 expression is also notably diminished, as well as the expression of CD40 ligand (CD40L) and CD95 ligand (CD95L) (Peng et al, 2001), which confirms that the activation of NFAT proteins is essential for T cells to carry out many of their effector functions (Macian, 2005).

1.2.5.2 AP-1

The AP-1 transcription factor is composed of proteins Fos, Jun, and activating transcription factor (ATF). While the Fos proteins (Fos, FosB, Fra-1, and Fra-2) can only heterodimerize with members of the Jun family, the Jun proteins (Jun, JunB, and JunD) can both homodimerize and heterodimerize with Fos members to form transcriptionally active complexes (Chinenov and Kerppola, 2001). AP-1 is formed by either Fos-Jun heterodimers or by Jun-Jun homodimers. AP-1 converts extracellular signals of T cells into changes in the expression of specific target genes, which harbor an AP-1 binding site in their promoter or enhancer regions. The activity of AP-1 is modulated by interactions with other transcriptional regulators and is further controlled by upstream kinases that link AP-1 to various signal transduction pathways (Wagner and Eferl, 2005). Activation of AP-1 usually involves synthesis of the Fos protein and phosphorylation of preexisting Jun protein. Fos synthesis is controlled by Ras/ERK pathway, while Jun is phosphorylated by JNK.

AP-1 and NFAT are the main transcriptional partners during T cell activation. Fos and Jun dimers form quaternary complexes with NFAT and DNA on NFAT-AP1 composite sites, which contain two adjacent binding motifs for both transcription factors and are present in many genes that are induced during T-cell activation (Macian et al, 2001; Macian, 2005). These complexes have an extensive network of protein-protein contacts, which explains their stability and cooperative nature (Chen et al, 1998; Macian, 2005). The ternary NFAT/Fos/Jun complex serves as a signal integrator for crosstalk between the Ca^{2+}/calcineurin pathway that activates NFAT and the Ras-MAP kinase pathway that

promotes the expression and activation of the Fos and Jun family (Savignac et al, 2007). NFAT-AP1 cooperation during T-cell activation is responsible for a specific pattern of gene expression, which induces the functional changes that characterize an activated T cell (Macian, 2005). The nature of AP-1 is important for the induction of IFN-γ and IL-4 genes (Savignac et al, 2007). Indeed, JunB is the only Jun family member that is induced in Th2 cells. In transgenic mice, elevated JunB levels cause an increased expression of the Th2 cytokines IL-4, -5, -6, and -10 (Li et al, 1999).

1.2.5.3 NF-κB

The mammalian NF-κB transcription factor family consists of p50 (NF-κB1), p52 (NF-κB2), REL (also known as cREL), REL-A (p65) and REL-B, each encoded by a distinct locus. These proteins dimerize to form functional NF-κB (Siebenlist et al, 2005). In unstimulated T cells, NF-κB is sequestered in the cytoplasm by IκB. T cell activation results in phosphorylation and degradation of IκB, leading to translocation of NF-κB to the nucleus (Lin et al, 2000). Phosphorylation of IκB is mediated by IKK complex, which contains two catalytic subunits, IKKα and IKKβ, and one regulatory subunit, IKK-γ (Manicassamy et al, 2006). Activation of NF-κB by a wide array of stimuli, including cytokines such as IL-1 and TNF-α, byproducts of bacterial and viral infections, radiation, or T cell costimulation, leads to the onset of signaling cascades that ultimately converge at the level of the IKK complex (Schmitz et al, 2003). As discussed above, PKC-θ is crucial for NF-κB activation upon TCR-mediated stimulation. Primary *PKC*-θ -/- T cells displayed defects in NF-κB activation upon TCR stimulation (Pfeifhofer et al, 2003; Sun

et al, 2000). *PKC*-θ -/- T cells failed to activate IKK complex or degrade IκB (Sun et al, 2000).

NF-κB regulates the transcription of genes involved in the inflammatory and immune responses as well as in some aspects of cell growth, survival and differentiation. Aberrant NF-κB activity has been associated with defects in T-cell proliferation, activation and cytokine production (IL-2, IL-6, IL-8, GM-CSF, TNF-α and IFN-γ) (Caamano and Hunter, 2002; Tak and Firestein, 2001). Genetic evidence obtained from mouse models and from NF-κB defects identified in humans demonstrates the importance of NF-κB for an effective mounting of the immune response (Li and Verma, 2002).

The T cell activation pathway is dipicted in Figure 1 (Gaffen and Liu, 2004).

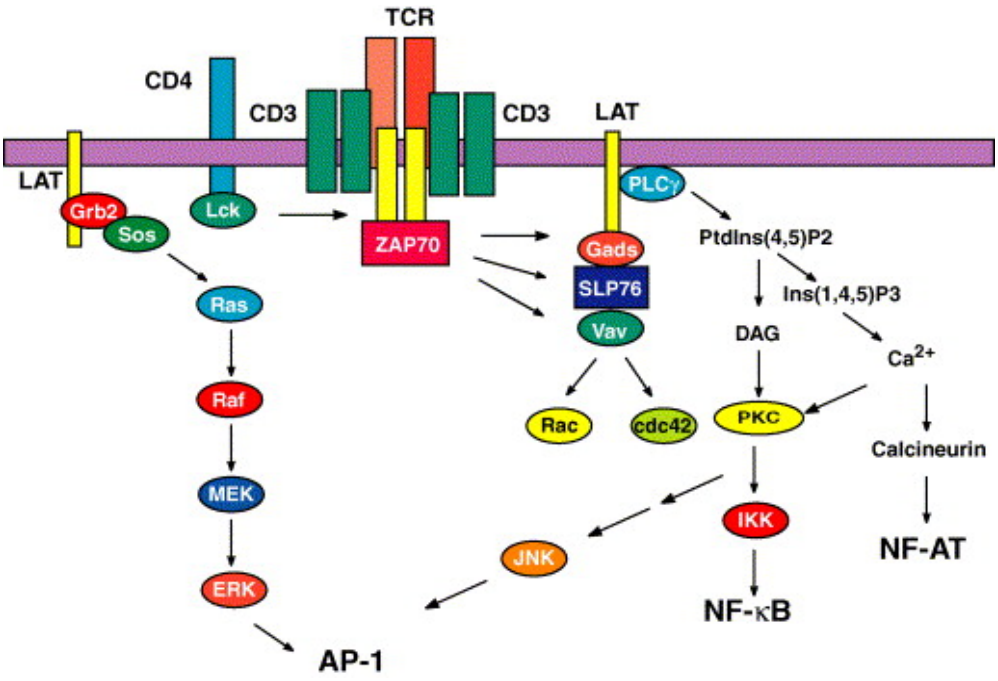

Figure 1. T cell activation pathway (Gaffen and Liu, 2004).

1.3 Macrophage Activation

Macrophages are widely distributed immune system cells that play indispensable roles in both innate and adaptive immunity. In innate immunity, resident macrophages provide immediate defense against foreign pathogens and coordinate leukocyte infiltration (Martinez et al, 2008). Macrophages contribute to the balance between antigen availability and clearance through phagocytosis and subsequent degradation of apoptotic cells, microbes and possibly neoplastic cells (Gordon, 2003). In adaptive immunity, macrophages collaborate with T and B cells, through both cell-to-cell interactions and fluid phase-mediated mechanisms, based on the release of cytokines, chemokines, enzymes, arachidonic acid metabolites, and reactive radicals (Gordon, 2003; Martinez et al, 2008). Macrophage activation can be either pro-inflammatory or anti-inflammatory, contributing to tissue destruction or regeneration and wound healing. Activated macrophages can be broadly classified into two main groups: classically activated macrophages (or M1), whose prototypical activating stimuli are IFN-γ and LPS, and alternatively activated macrophages (or M2), further subdivided into M2a (after exposure to IL-4 or IL-13), M2b (immune complexes in combination with IL-1β or LPS) and M2c (IL-10, TGF-β or glucocorticoids) (Martinez et al, 2008). M1 exhibits potent microbicidal properties and promotes strong IL-12-mediated Th1 responses, whilst M2 supports Th2-associated effector functions. Beyond infection, M2-polarized macrophages play a role in the resolution of inflammation through high endocytic clearance capacities and trophic factor synthesis, accompanied by reduced pro-inflammatory cytokine secretion (Martinez et al, 2008).

1.3.1 Classical Pathway

It is generally accepted that classically activated macrophages develop in response to two signals. The first signal is IFN-γ and the second signal is provided by what has been termed 'pathogen-associated molecular patterns' (PAMPs) (Schnare et al, 2000). IFN-γ is the sole type II IFN and is recognized by an IFN-γ receptor (IFNGR) consisting of two ligand-binding IFNGRα chains associated with two signal-transducing IFNGRβ chains (Schroder et al, 2004). IFN-γ is mainly secreted by Th1 and CTLs, NK cells, and professional antigen-presenting cells, and to a lesser extent, by B cells and NKT cells (Young, 2006). In most descriptions of macrophage activation, the second signal is provided by a microbe that expresses one or more PAMPs, which are small molecular motifs consistently expressed on pathogens. They are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in plants and animals. PAMPs include bacterial LPS, flagellin, lipoteichoic acid, peptidoglycan, and nucleic acid variants normally associated with viruses, such as double-stranded RNA (dsRNA) or unmethylated CpG motifs. The prototypical PAMPs, which stimulate macrophage activation responses, are LPS or LTA from the surface of Gram-negative or -positive bacteria, respectively.

1.3.1.1 IFN-γ Signaling

The most important priming stimulus for macrophages is low-dose IFN-γ. Homodimeric IFN-γ binds with two IFNGR ligand-binding α chains. Dimerized α chains associate with two signaling IFNGR β chains. IFNGR α and β chains are constitutively associated with Janus kinases JAK1 and JAK2, respectively. Ligand binding induces phosphorylation

events, which first lead to binding of latent, cytosolic signal transducer and activator of transcription-1α (STAT1α) followed by its subsequent activation by phosphorylation. Activated STAT1α is released from the IFN-γ /IFNGR complex and forms a homodimer known as IFN-γ activation factor (GAF), which translocates to the nucleus and binds to gamma activated site (GAS) to initiate transcription (Decker et al, 1997). The primary IFN-γ JAK/STAT response is not dependent on *de novo* synthesis of transcription factors, because activation of already available, preexisting components mediates it. Finally, subthreshold concentrations of IFN-γ upregulate its own expression in activated NK cells and increase the sensitivity of macrophages to a subsequent second stimulus (Ma et al, 2003). In most cases IFN-γ acts synergistically with TNF in macrophage activation. TNF signals mainly through its receptor TNF-R1. Ligand binding causes dimerization of TNF-R1 and release of silencer of death domain (SODD) proteins from the cytoplasmic part of the ligand-receptor complex (Tschopp et al, 1999). This leads to ordered binding of several adaptor proteins, including TNF receptor-associated death domain (TRADD), receptor interacting protein (RIP), TNF-R-associated factor 2 (TRAF2), and Fas-associated death domain (FADD). These adapter proteins bind some key enzymes and initiate three different signaling arms, including FADD-dependent binding and activation of caspase-8 (apoptosis), TRAP2-dependent activation of the JNK pathway, and RIP-dependent NF-κB, which promotes production of pro-inflammatory mediators and protects against apoptosis (Ma et al, 2003). The combination of TNF and IFN-γ results in optimal macrophage activation. These classically activated macrophages become strongly microbicidal and they are important immune effector cells (van Ginderachter et al, 2006).

1.3.1.2 TLR Signaling

TLRs are a type of PRRs and recognize molecules that are broadly shared by pathogens but distinguished from host molecules, collectively referred to as PAMPs. They are type I membrane-associated receptors characterized by an extracellular leucine-rich repeat signature, a transmembrane cysteine-rich flanking region, and a cytoplasmic domain homologous with the IL-1 receptor family referred to as the Toll-IL-1 receptor (TIR) (Uematsu and Akira, 2008). To date, 13 TLRs have been identified in mice and 11 in humans (Roach et al, 2005). TLRs collectively recognize conserved PAMPs in lipids, carbohydrates, peptides, and nucleic acids of microbes. Among them, TLR4 recognizes LPS, TLR2 recognizes microbial lipopeptides and peptidoglycans (PGN), TLR3 recognizes double-stranded RNA, TLR5 recognizes bacterial flagellin, whereas TLR9 recognizes unmethylated bacterial CpG DNA (Yan and Hansson, 2007). Except for TLR3, all TIR domains are conserved and associate with other TIR domain-containing molecules, including the adaptors that mediate TLR signaling. The four best-characterized TIR domains containing activating adaptors include myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal), TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR domain-containing adaptor molecule-1 (TICAM-1) and TRIF-related adaptor molecule (TRAM) (O'Neill and Bowie, 2007; Zhang and Mosser, 2008). All of these proteins are expressed in myeloid cells and all play important roles in activating innate signaling events. MyD88 is the primary adaptor for microbial signaling (Krishnan et al, 2007). The early production of inflammatory cytokines is largely dependent on the presence of MyD88.

To initiate efficient signaling, LPS first binds to soluble LPS-binding protein in the blood or extracellular fluid, and this complex serves to facilitate binding of LPS to CD14. Once LPS binds to CD14, LBP dissociates, and the LPS-CD14 complex physically associates with TLR4. Recognition of minute quantities of LPS by TLR4 requires an additional extracellular accessory protein called MD-2, which is expressed as a dimer with TLR4 on the surface of immune cells (Zhang and Mosser, 2008). Ligand binding to TLRs results in the immediate recruitment of MyD88, which also contains a death domain homologous to TNF receptor family signaling molecules. Subsequently a second protein with similar death domain called IL-1 receptor-associated kinase (IRAK) is recruited to the signaling complex and phosphorylated. Once activated, IRAK dissociates from MyD88 and activates TNF-R-associated factor 6 (TRAF-6), which in turn ubiquitinates TGF-β-activated kinase (TAK)-1. TAK-1 then serves as a branch point, leading to the activation of both NF-κB and MAPK signaling pathways (Krishnan et al, 2007; Zhang and Mosser, 2008).

1.3.2 Alternative Pathway

Macrophages activated via alternative pathways are termed M2 cells, which play a critical role in type II inflammation and in the resolution and tissue repair phase (van Ginderachter, 2006). M2 cells are subdivided to M2a or alternatively activated macrophages, which are elicited by type II cytokines IL-4 or IL-13; M2b, corresponding to type II activated macrophages, obtained by triggering of Fcγ receptors in the presence of a Toll receptor stimulus; and M2c which includes deactivation programs elicited by glucocorticoids, IL-10 or TGF-β (van Ginderachter, 2006; Martinez et al, 2008).

1.3.2.1 M2a Activation

M2a activation is triggered in the presence of IL-4 and IL-13, signaling to macrophages in part through a common receptor chain, IL-4Rα (Ma et al, 2003). A number of molecules are regulated upon IL-4 and IL-13 signaling. Actions of IL-4 and IL-13 on macrophages could down-regulate the production of pro-inflammatory mediators such as IL-1β, TNF-α, IL-6, IL-8, IL-12, GM-CSF, IFN-γ, CCL2/MCP-1, and superoxide anions (Martinez et al, 2008). IL-4/IL-13 also regulate molecules such as MHC-II, β2 integrins, the chemokines CCL22/MDC (Mantovani et al, 2002) and CCL18/AMAC-1, tissue-type plasminogen activator and metalloproteinase 1 (Hart et al, 1989; Chizzolini et al, 2000). IL-4/IL-13 also downregulate caspase 1, which is responsible for the proteolytic cleavage of pro-IL-1β into its active mature form (Cerretti et al, 1992) and affects the IL-1β system by enhancing the production of IL-1R receptor antagonist (IL-1ra) and the decoy IL-1β type II receptor (IL-1RII) (Mosser, 2003). In addition, IL-4 decreases CD14 and CCR5 expression (Wang et al, 1998) and was recently found to upregulate several scavenger receptors and C-type membrane lectins, such as MRC1, SR-A, Dectin-1, DC-SIGN, DCIR (CLECSF6), DCL-1, and CLECSF13 (Martinez et al, 2006). M2a cells do not express iNOS in rodents, but express high levels of arginase 1 (ARG1), which skews the metabolic pathway of NO to the production of proline. Consequently, these cells fail to produce NO and are significantly compromised in their microbicidal ability for intracellular pathogens, but they synthesise polyamine and proline that stimulate cell growth, collagen formation and tissue repair (Hesse et al, 2001).

1.3.2.2 M2b Activation

M2b activation is characterized by LPS or IL-1β stimulation through TLR4 or IL-1R and immune complexes recognized by Fcγ receptor (FcγR) (Bowie and O'Neill, 2000). M2b cells produce low IL-12 and high IL-10, favoring the development of type II adaptive immune responses (Anderson and Mosser, 2002a). M2b are distinct from M2a since they produce much higher levels of IL-10, but also produce significant amounts of TNF-α, IL-1β, and IL-6, indicating that these cells are not anti-inflammatory *per se* (Martinez et al, 2008). In terms of B cell responses, M2b cells efficiently sustain antibody production, the majority of which are of the IgG1 isotype, consistently with a type II IgG class switch (Anderson and Mosser, 2002b).

1.3.2.3 M2c Activation

M2c cells represent deactivated macrophages after stimulation with IL-10, TGF-β, or glucocorticoids. Their recognition occurs in the nucleus by the gluococorticoid receptor, resulting in strong repression of proinflammatory cytokines such as TNF-α, IL-4, IL-5, IL-1, IL-6, IL-8, and IL-12 (Martinez et al, 2008). In contrast, the expression of IL-10 and other molecules with anti-inflammatory functions such as the scavenger receptor CD163 are increased. Gluococorticoid finally downregulate a great variety of genes known to be upregulated by IFN-γ, such as the chemokines CXCL10/Ip-10, CXCL11/I-TAC, CCL5/RANTES and CCL24/eotaxin 2, and the chemokine receptor CX3CR1 (Ehrchen et al, 2007).

1.4 Dendritic Cell Maturation and Immunogenicity

Dendritic cells (DCs) consist of a heterogenous population of professional antigen presenting cells, which are derived from multiple lineages from bone marrow with distinctive stages of cell development, activation and maturation and may be immunogenic and tolerogenic (Banchereau and Steinman, 1998; Steinman et al, 2003; Morelli and Thomson, 2007). DCs can be categorized into conventional DCs (cDCs) and precursor DCs (pre-DCs). cDCs already have DC form and function and can be subdivided into migratory DCs (Langerhans cells and dermal DCs), which act as sentinels in peripheral tissues, migrate to the lymph nodes through the lymphatics, bearing antigens from the periphery and presenting these antigens to T cells in the lymph nodes, and lymphoid-tissue-resident DCs (thymic cDCs and splenic cDCs), which are restricted to one lymphoid organ and collect and present foreign and self-antigens there (Shortman and Naik, 2007). In mice, these DCs can be separated into $CD8^+$ cDCs that express high levels of CD8α on the cell surface, and $CD8^-$ cDCs that lack this marker (Vremec et al, 2000). Pre-DCs are cells without immediate dendritic form and DC function, but with a capacity to develop into DCs in response to an inflammatory or microbial stimulus. Different types of pre-DC give rise to different DC subtypes. Examples include interferon-producing plasmacytoid DCs (pDCs) and monocyte-derived inflammatory DCs (Shortman and Naik, 2007).

1.4.1 DC Maturation

The term DC maturation was first proposed by Steinman and colleagues (Schuler and Steinman, 1985) in a seminal study, which described that Langerhans cells (LCs)

extracted from the skin epidermis underwent dramatic phenotypic and functional changes during culture *in vitro*. They discovered that freshly purified LCs expressed low levels of MHC and T-cell costimulatory molecules, but after culture *in vitro* the expressions of these molecules were increased dramatically and their capacity to stimulate T cell proliferation was markedly enhanced. Compared with freshly isolated LCs, cultured LCs express lower levels of receptors involved in antigen uptake (such as FcγRs) but higher levels of molecules necessary for T-cell priming (including MHC molecules, the integrin lymphocyte function-associated antigen 1 (LFA1), and the costimulatory molecules CD80 and CD86) (Larsen et al, 1992; Inaba et al, 1994). These phenotypic distinctions have since been extended to other mouse and human DCs, and high levels of MHC, adhesion and costimulatory molecules are now widely considered to be markers of DC maturation (Reis e Sousa, 2006). Immature DCs are well-equipped with a series of receptors for PAMPs and for secondary inflammatory compounds, such as TLRs nucleotide-binding oligomerization domain (NOD) proteins, RIG-I-like receptors, C-type lectin receptors, cytokine receptors and chemokine receptors. Signaling through these receptors triggers DC migration towards the secondary lymphoid organs and results in maturation (Villadangos and Schnorre, 2007). For example, TLR signaling is linked to MyD88-dependent and TRIF-dependent signaling pathways that regulate the activation of different transcription factors, such as NF-κB. Activation of NF-κB in turn results in enhanced inflammatory cytokine responses and induction of DC maturation (CD80, CD83, CD86 up-regulation) (van Vliet et al, 2007). A variety of stimuli including CD40L, TNF-α, and calcium ionophores, can trigger these receptor-mediated signaling pathways and lead to DC maturation (Osada et al, 2006). Currently, most clinical trials utilize TNF-

α, or a cytokine cocktail that includes IL-1β, IL-6, TNF-α, and PGE2, as maturation reagents (de Vries et al, 2003; Schuler-Thurner et al, 2002). The cytokine cocktail was selected based on the observation that it enhanced HLA-DR, CD83, and CD86 expression by DCs and induced greater levels of allogeneic T-cell proliferation (Jonuleit et al, 1997). TLR agonists, such as LPS, double-stranded RNA, polyinosinic:polycytidylic acid, and CpG oligodeoxynucleotides, have the ability to promote DC maturation in the absence of other inflammatory agents and may thus warrant further investigation into their potential clinical application (Osada et al, 2006).

1.4.2 DC Immunogenicity Correlates with its Phenotypic Maturation

An immunogenic DC possesses the capacity to induce T-cell clonal expansion, differentiation into effector cells and a long-term increase in precursor frequency ('memory') (Reis e Sousa, 2006). To activate naïve T cells, signal 1, signal 2, and signal 3 are all needed to be delivered by an APC. Signal 1 is delivered through the TCR when it engages an appropriate peptide-MHC complex. Signal 1 alone is thought to promote naïve T-cell inactivation by anergy, deletion or co-option into a regulatory cell fate, thereby leading to 'tolerance' (Reis e Sousa, 2006). Signal 2 is referred to as 'co-stimulation' and is taken to mean an accessory signal(s) that, together with signal 1, induces 'immunity', which is often equated with signaling through CD28 when it engages CD80 and/or CD86 (Keir and Sharpe, 2005). Signal 3 refers to signals delivered from the APC to the T cell that determine its differentiation into an effector cell (Reis e Sousa, 2006). IL-12 is an example of a mediator that delivers a signal 3 that can promote Th1-cell or CTL development (Trinchieri, 2003). The signal 3 for Th2-cell development

could be a Notch ligand (Tu et al, 2005). Therefore, an immunogenic DC must be phenotypically mature, expressing high levels of MHC classs II and costimulatory molecules CD80 and CD86, and producing IL-12 or Notch ligand.

1.4.3 Phenotypically Mature DCs May Not Be Immunogenic

However, phenotypically mature DCs may not necessarily be immunogenic. Recently it was found that phenotypically mature DCs induce tolerance (Albert et al, 2001) or else do not induce immunity (Sporri and Reis e Sousa, 2005), even though they stimulated naïve T cells. DCs in the steady state, that is, in the absence of deliberate exposure to maturation signals, can tolerate peripheral $CD4^+$ and $CD8^+$ T cells by inducing deletion, anergy or regulation, depending on the model system studied (Reis e Sousa, 2006). Immature DCs in the steady state are thought to mature spontaneously and acquire the capacity to induce T cell tolerance (van Vliet et al, 2007). What is not yet known is the range of maturation signals available to immature DCs and whether the different lineages of maturing DCs contribute to functional diversity (van Vliet et al, 2007). There is accumulating evidence that antigen processing and tolerogenic cross-presentation of apoptotic material requires maturation of DCs (Niimi et al, 2001). Pulmonary DCs pulsed with antigen by intranasal ovalbumin (OVA) application induced tolerance and appeared as mature DCs after reaching the draining lymph nodes (Hayamizu et al, 1998). Self-antigen transport, processing and presentation for tolerance induction by steady-state migrating DCs require maturation, including the upregulation of MHC and costimulatory molecules (Groux et al, 2004). However, in the absence of microbial or inflammatory stimulation these DCs are not presumed to produce IL-12 or other pro-inflammatory

cytokines and their maturation process is obviously different from pro-inflammatory DCs (Groux et al, 2004).

1.4.4 Tolerogenic DC Subset?

It is currently unclear whether a distinct subset of DCs exists which is solely dedicated to the induction and maintenance of peripheral tolerance. It was found that a subset of DCs isolated from Peyer's patches, lungs, or the anterior chamber of the eye display a mature phenotype, secrete IL-10 but not IL-12, and drive the development of IL-10-producing regulatory T (Treg) cells (Hayamizu et al, 1998; Corcoran et al, 2003; Rutella et al, 2006). It was recently suggested that $CD11c^{low}CD45RB^{high}$ DCs represent a population of DCs that have matured to display a stable tolerogenic phenotype with a unique molecular design by the downregulation of T cell activation partners (Groux et al, 2004). Under steady-state conditions, these cells, loaded with self- and commonly encountered antigens, are driven to lymphoid organs by endogenous factors to induce the differentiation of Treg cells and tolerance (Groux et al, 2004). It has been shown that IL-10 is a key factor for the differentiation of TDC *in vitro* and *in vivo* (Groux et al, 2004). DCs from peripheral tissues are surrounded by stromal fibroblasts and epithelial cells that are sources of PGE2; TGF-β1 and IL-10 (Groux et al, 2004).

1.4.5 Process of Tolerogenic DC Induction of Tolerance

In the steady state, $CD8^-$ and $CD8\alpha^+$ DCs remain quiescent after capturing and processing exogenous antigen (through the internalization of apoptotic cells, vesicles and/or soluble molecules. These quiescent (semi-mature) DCs express low levels of costimulatory molecules and therefore induce deficient activation of naive T cells, and induce T-cell

apoptosis or anergy and probably the generation and/or expansion of regulatory T cells. The interaction of surface CD80/CD86 on both splenic DC subsets with cytotoxic T-lymphocyte antigen 4 (CTLA4) enhances the synthesis of functional indoleamine 2,3-dioxygenase (IDO), which is an enzyme that catalyzes the depletion of the essential amino acid tryptophan, resulting in the inhibition of T-cell proliferation, and produces tryptophan-derived metabolites that promote T-cell apoptosis (Morelli and Thomson, 2007).

1.5 Scope of Present Study

From the literature review, LBP appears to possess immunomodulatory properties. However, the mechanisms of immunomodulation have not been fully elucidated yet. For example, although LBP was found to stimulate lymphocyte proliferation and cytokines such as IL-2 secretion, indicating it can activate T cells, the complete cytokine profile and the mechanism of T cell activation stimulated by LBP, have not been reported. While previous studies have shown that LBP can activate macrophages, more insightful pieces of evidence to show its effects on innate immunity need to be demonstrated. Furthermore, the effects of LBP on DCs have seldom been addressed. In addition, although LBP has been successfully isolated from *L. barbarum* fruit and purified to several fractions, the structure, constituents, and molecular weights of LBP fractions obtained from different laboratories are not consistent. We hypothesize that LBP has effects on T cells, macrophages, and DCs.

The objectives of this study thus includes the following:

1. To isolate, purify and characterize LBP and its fractions;
2. To elucidate the mechanism of T cell activation by LBP;
3. To investigate the mechanism of macrophage activation by LBP;
4. To investigate whether LBP can induce immunogenic DCs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

The following reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA): RPMI-1640 medium, DMEM medium, fetal bovine serum (FBS), L-Glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate, penicillin, streptomycin, bovine serum albumin, Triton X-100, sodium azide, sodium chloride, ammonium chloride, potassium chloride, potassium dihydrgen orthophsosphate, propidium iodide, disodium hydrogen orthophosphate, Tris (hydroxy methyl) aminomethane, paraformaldehyde, glycine, bromophenol blue, Coomassie brilliant blue, 2-mercaptoethanol, chloroform, butanol, ethanol, sodium hydroxide, phenol, sulfuric acid, DEAE-cellulose, D-glucose, blue dextran, T-dextran standards, including T-25000, T-80000, T-270000, and T-670000, concavalin A (Con A), lipopolysaccharide (LPS), mitomycin C, phorbol myristate acetate (PMA), ionomycin, protease, E-TOXATE® kit, collagenase A, Histodenz, RNase, DNase I, ovalbumin (OVA), FITC-dextran, and FITC-*Staphylococcus aureus*.

The following reagents were purchased from BD Biosciences (San Diego, CA, USA): Mouse IL-2, IL-4, IFN-γ, and TNF-α, IL-1β, IL-12p40 and p70 OptiEIA™ sets, mouse IL-2, IL-4, and IFN-γ ELISPOT sets, luciferase reporter plasmids NFAT-luc, AP-1-luc, and NF-κB-luc, and the following antibodies, including rat anti-mouse CD3 (IgG2b, FITC, 17A2), rat anti-mouse CD19 (IgG2a, PE, 1D3), rat anti-mouse CD25 (IgG1, purified, PC61), goat anti-rat IgG (FITC, H + L), rat anti-mouse CD11b (IgG2b, APC, M1/70), American hamster anti-mouse CD11c (IgG1, APC, HL3), rat anti-mouse CD40

(IgG2a, FITC, 3/23), American hamster anti-mouse CD80 (IgG2, FITC, 16-10A1), rat anti-mouse CD86 (IgG2a, FITC, GL1), rat anti-mouse I-A/I-E (IgG2a, FITC, 2G9), isotype controls American hamster IgG1 (APC, G235-2356), American hamster IgG2 (FITC, B81-3), rat IgG1 (purified, R3-34), rat IgG2a (FITC, R35-95), rat IgG2a (PE, R35-95), rat IgG2b (FITC, A95-1), rat IgG2b (APC, A95-1).

The following reagents were purchased from companies as indicated:

Sodium dodecyl sulfate (SDS), bisacrylamide, acrylamide, ammonium persulfate, N, N, N', N'-tetramethylethylenediamine (TEMED), protein assay kit, protein standards (Bio-Rad, Hercules, CA, USA), PCR primers and fluorogenic probes for the genes TNF-α, IL-1β, IL-12p40, and β-actin and TaqMan® Gene Expression Assay kit (Applied Biosystems Incorporation, Foster, CA, USA); Dynal mouse T cell and B cell isolation kits, lipofectamine, Opti-MEM® I reduced serum medium, SuperScript™ first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA); XK column (2.6 × 40 cm), HiPrep 26/60 Sephacryl S-300 HR column, ^{3}H-thymidine (GE Healthcare, Buckinghamshire, UK), luciferase assay system (Promega, Madison, WI, USA); rmGM-CSF, rmIL-4, dialysis tubing (MWCO: 10,000 kDa) (Pierce, Rockford, IL, USA), RNeasy Mini Kit (QIAGEN, Hilden, Germany); Anti-mouse CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany); *L. barbarum* dried fruit (Eu Yan Sang Chinese medicine store, Singapore. The fruit was produced in Ningxia province, China).

2.1.2 Animals

Female C57BL/6 and BALB/c mice, 6-week old, were obtained from the Singapore Laboratory Animal Centre. All animals were housed at 22 ± 2°C on a 12-h light/dark cycle (lights on at 07:00 h) with 45-55% relative humidity in the Animal Holding Facility (AHU) at the National University of Singapore (NUS). Food and water were provided *ad libitum*. All procedures were performed in accordance with the Singapore Guidelines on the Care and Use of Animals for Scientific Purpose and were approved by the Institutional Animal Care and Use Committee (IACUC) of NUS.

2.1.3 Cell Lines

Jurkat T cell line and RAW264.7 murine macrophage cell line were purchased from American Type Culture Collection (ATCC, TIB-71 and TIB-152, respectively). Jurkat cells were cultured in RPMI-1640 medium and RAW264.7 cells were maintained in DMEM medium at 37°C in a 5% CO_2 humidified incubator. Both media were supplemented with 10% FBS, 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin).

2.2 Methods

2.2.1 Isolation of Crude LBP

500 g dried fruit of *L. barbarum* were homogenized and then soaked in 6 L of Milli-Q water at 4ºC overnight. The water extract was filtered by filter cloth followed by centrifugation at 10,000 ×*g* to remove tiny solid particles. The supernatant was concentrated to 800 ml under reduced pressure and then precipitated with 5 volumes of absolute ethanol. The precipitate was dissolved in 100 ml of Milli-Q water. One fifth volume of the Sevag reagent ($CHCl_3$:n-$BuOH_4$ = 4:1) was added 5× to remove free proteins. The aqueous phase was then dialyzed against water for 96 h and lyophilized by freeze dryer. For use in bioassays, LBP was dissolved in PBS or normal saline (for *in vivo* experiments), filtered through a 0.22-μm filter, and stored at 4ºC. The five fractions LBPF1-5 that were obtained were similarly treated.

2.2.2 DEAE-Cellulose Ion Exchange Chromatography

40 g of DEAE-cellulose was washed with 0.1 M NaOH/ 0.5 M NaCl, followed by 0.1 M HCl/ 0.5 M NaCl. The pH was adjusted to 7.8. Then the DEAE-cellulose was packed into XK column (2.6 × 40 cm). The column was equilibrated with Milli-Q water for 24 h. 0.5 g of crude LBP was dissolved in 20 ml of Milli-Q water and applied onto the column, which was sucessively eluted with 500 ml of water, followed by 500 ml of 0.05 M, 0.1 M, 0.2 M, and 0.5 M NaCl, respectively, at a flow rate of 0.8 ml/min. The fractions were collected at 10 ml/tube. The absorbance value at 280 nm was measured to monitor the protein level. The polysaccharide level was tested for in every other tube by phenol-sulfuric acid method, as described below. The fractions which produced a peak at 490 nm

were collected, concentrated under reduced pressure, dialyzed against water to remove salt, and lyophilized by freeze dryer.

2.2.3 Size Exclusion Chromatography

Fractions obtained from DEAE-cellulose ion exchange chromatography were dissolved in 5 ml of Milli-Q water and applied onto Sephacryl S-300 HR pre-packed column (2.6 × 60 cm). The column was eluted with 400 ml of water at a flow rate of 1.3 ml/min. The eluted solution was collected at 9 ml/tube. Absorbance values at 280 nm and 490 nm were monitored as above to test protein and polysaccharide levels, respectively. The fractions producing a peak at 490 nm were collected, concentrated under reduced pressure, and lyophilized by freeze dryer.

2.2.4 Carbohydrate Content Test

Carbohydrate content was determined by phenol-sulfuric acid method. Briefly, five LBP fractions, designated as LBPF1-5, were dissolved in Milli-Q water at 100 μg/ml. Glucose was dissolved in Milli-Q water at 1 mg/ml and further diluted to 320, 160, 80, 40, 20, and 10 μg/ml. 0.4 ml of standards or samples was added into a glass tube, followed by 0.2 ml of 5% phenol and 1 ml of concentrated sulfuric acid. The ratio of polysaccharide solution, phenol, sulfuric acid was 2:1:5 (V:V:V). The reaction system was heated at 100ºC for 10 min. The absorbance value was measured at 490 nm with a spectrometer (BioMate). The carbohydrate content was calculated by the linear regression equation deduced from the glucose standard curve.

2.2.5 Protein Content Test

Protein content was determined by the Bradford method. Briefly, BSA standards, including 25, 15, 10, 7.5, and 5 μg/ml, were prepared. LBPF1-5 was dissolved in Milli-Q water at 100 μg/ml. 800 μl of standards or samples were mixed with 200 μl of dye reagent concentrate and incubated at RT for 10 min. The absorbance value was read at 595 nm by a spectrometer (BioMate). The protein content was calculated by the linear regression equation deduced from the BSA standard curve.

2.2.6 Molecular Weight Measurement

The molecular weights of LBPF1-4 were determined by SDS-PAGE. Briefly, 2 mg of LPBF1-4 was dissolved in 200 μl of distilled water and mixed with 50 μl of 5× sample buffer (10% SDS, 10 mM β-mercaptoethanol, 20% v/v glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% bromophenol blue). The mixture was boiled for 10 min to denature protein. 25 μl of the mixture was loaded onto the well of the SDS-polyacrylamide gel consisting of stacking and running gel. The gel was run for 2 h and stained with Coomassie brilliant blue. The migration distances of protein standards, bromophenol blue, and sample on the gel were measured. Rf, which is the ratio of the distance migrated by the molecule to that migrated by bromophenol blue-front, was calculated. The molecular weights of LBPF1-4 were calculated by the linear regression equation (lg MW against Rf) deduced from protein standards.

The molecular weight of LBPF5 was determined by gel filtration. Sephacryl S-300 HR pre-packed column (2.6 × 60 cm) was equilibrated with 0.1 M KCl at 1.3 ml/min for 24 h.

LBPF5, T-dextran standards with different molecular masses, including 25000, 80000, 270000, and 670000 kDa, and blue dextran (MW: 2000000 kDa, used to measured the exclusion volume/void volume) were dissolved in 0.1 M KCl at 1 mg/ml and applied onto the Sephacryl S-300 HR pre-packed column, which was eluted with 0.1 M KCl at a flow rate of 1.3 ml/min. The eluted solution was collected at 5.2 ml/tube. The elution volume (Ve) and the void volume (Vo) were determined by measuring the peaks at 490 nm, which was monitored by phenol-sulfuric acid assay. The molecular weight of LBPF5 was calculated by the linear regression equation (lg MW against (1-Vo/Ve)) deduced from dextran standards.

2.2.7 Test of LPS Contamination

LPS contamination was tested by the *Limulus* amebocytes lysate (LAL) assay using E-TOXATE® kit and by B cell proliferation assay. For LAL assay, crude LBP and LBPF1-5 were dissolved in endotoxin-free water at 10 mg/ml. The endotoxin standard was reconstituted with 1 ml of endotoxin-free water to make 4000 EU/ml stock solution, which was serially further diluted to working standard solutions, including 0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 EU/ml. *Limulus* amebocyte lysate was reconstituted with 5 ml of endotoxin-free water. 100 µl of samples, standards, or endotoxin-free water (negative control) was mixed with 100 µl of LAL in a microcentrifuge tube. The mixed content was incubated at 37°C in water bath for 1 h and observed for gelation while inverting the tubes 180° slowly. 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 was described as below in the proliferation assay. 2×10^5 B cells were stimulated with 5 μg/ml of LPS, or 100 μg/ml of LBP, LBPF1-5 at 37ºC in a 5% CO_2 humidified incubator for 72 h.

2.2.8 *In vitro* Cytotoxicity Assay

Mouse splenocytes (prepared as below) were treated with LBP at serial concentrations, including 10, 20, 40, 80, 160, 320, 640, 1280, and 2560 μg/ml at 37ºC in a 5% CO_2 humidified incubator for 72 h and pulsed with ^{3}H-thymidine (0.5 μCi/well) for the last 18 h. The cells were harvested and the amount of ^{3}H-thymidine uptake by the cells was determined as described below in the proliferation assay.

2.2.9 Acute Toxicity Assay

BALB/c mice were administered p.o. or i.p. with a single dose of 20 mg of LBP (1 g/kg). Mice were observed for mortality, body weight, and clinical symptoms such as breathing, walking behavior, fur erection, feces, and urine, for 14 days.

2.2.10 Splenocyte Preparation

C57BL/6 or BALB/c mice were sacrificed by CO_2 inhalation. Spleen was aseptically removed from the abdominal cavity and minced through a 40-μm nylon cell strainer (BD Falcon) with a 5-ml syringe core in 10-ml of RPMI-1640 medium. Red blood cells were depleted with Tris-NH_4Cl lysis buffer (0.144 M NH_4Cl, 0.017 M Tris-HCl). Splenocytes were washed with PBS three times and maintained in RPMI-1640 medium, supplemented

with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator.

2.2.11 T and B Cell Purification

T and B cells were purified from mouse splenocytes using Dynal mouse T and B cell isolation kits (Invitrogen) according to the manufacturer's instructions. Briefly, splenocytes were washed twice with PBS and re-suspended in PBS/ 0.1% BSA to 1×10^8/ml. The desired number of splenocytes was transferred to a tube. 20 μl of FBS and 20 μl of Antibody Mix for T or B cell isolation were added to per 1×10^7 cells. After incubation at 4ºC for 20 min, the cells were washed with 2 ml of PBS/ 0.1% BSA and re-suspended in 800 μl of PBS/ 0.1% BSA. 200 μl of pre-washed Mouse Depletion Dynabeads were added. After incubation for 15 min at room temperature with gentle tilting and rotation, the cells were resuspended and the tube was placed in the magnet for approximately 2 min. The supernatant, which contains the negatively isolated mouse T or B cells, was transferred to a new tube. To determine the purities of T and B cells, 1×10^6 T, B cells, or splenocytes were suspended in 50 μl of PBS/ 0.1% NaN_3/ 1.0% FBS. The cells were treated with 50 μl of rat anti-mouse FITC-CD3 mAb (20 μg/ml) and rat anti-mouse PE-CD19 mAb (20 μg/ml) at 4ºC for 40 min in the dark. After incubation, the cells were washed twice with PBS/ 0.1% NaN_3/ 1.0% FBS. The cells were resuspended in 0.5 ml of PBS/ 2% paraformaldehyde and analyzed by flow cytometry (Beckman Coulter).

2.2.12 Proliferation Assay

2×10^5 splenocytes were stimulated with crude LBP or LBPF1-5 at serial concentrations, including 1, 3, 10, 30, 100, and 300 μg/ml. 2×10^5 T or B cells were stimulated with 100 μg/ml of LBP, LBPF4, or LBPF5. Con A (3 μg/ml) (Staska et al, 2005) and LPS (5 μg/ml) (Brian, 1988) were positive controls for T and B cell proliferation, respectively. The cells were cultured in RPMI 1640 medium, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator for 72 h and were pulsed with 0.5 μCi/well of ^{3}H-thymidine for the last 18 h. The cells were harvested on glass fiber filters using a Filtermate cell harvester (Packard). The amount of ^{3}H-thymidine incorporated into cells was measured using a β-scintillation counter (TopCount, Packard). The results are expressed as stimulation index (S.I.), as calculated by dividing cpm of stimulated cells with cpm of unstimulated cells.

2.2.13 Protease Digestion

Protein contained in LBP was destroyed by incubation with protease. Briefly, 20 mg of crude LBP, LBPF4, or LBPF5 was dissolved in 10 ml of 0.2% SDS/ 10 mM EDTA solution. Protease was added to 1 mg/ml. The reaction mixture was incubated at 37ºC in a water bath overnight, followed by dialysis against Milli-Q water for 3 days and lyophilization. Splenocytes were stimulated with 100 μg/ml of protease-digested LBP, LBPF4, or LBPF5 for 72 h. Cell proliferation was determined by ^{3}H-thymidine uptake assay.

2.2.14 Cell Cycle Profile Analysis

Cells were harvested and washed with cold PBS twice. The cells were then fixed in 1 ml of 70% ethanol at 4ºC overnight. The cells were washed twice with PBS and re-suspended in 0.5 ml of staining solution (100 μg/ml of RNase, 40 μg/ml of propidium iodide, 0.1% Triton X-100 in PBS) for 30 min at RT. Cell cycle profile was analyzed by flow cytometry (Beckman Coulter).

2.2.15 Flow Cytometric Analysis

Cells were washed with cold wash buffer (PBS/ 0.1% NaN_3/ 1.0% FBS). For analysis of CD25 expression, 10^6 splenocytes were treated with 0.5 μg of monoclonal rat anti-mouse CD25 (10 μg/ml) at 4ºC for 40 min, washed, followed by addition of 100 μl of 10 μg/ml FITC-conjugated goat anti-rat IgG and incubation at 4ºC for 40 min in the dark. For analysis of the expressions of MHC class II and costimulatory molecules on macrophages or dendritic cells (DCs), 10^6 cells were stained with 0.5 μg of APC-conjugated anti-mouse CD11b (for macrophages) or APC-conjugated anti-mouse CD11c (for DCs) and 0.5 μg of FITC-conjugated anti-mouse CD40, CD80, CD86, I-A/I-E, or isotype controls in 100 μl at 4ºC for 40 min. After incubation, the cells were washed, resuspended in 1 ml of PBS/ 2% paraformaldehyde, and analyzed by flow cytometry (Dako Cytomation).

2.2.16 RNA Extraction

Total RNA was extracted from splenocytes or RAW264.7 cells using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instruction. Briefly, cells were harvested, washed, and disrupted with 350 μl of Buffer RLT. 1 volume of 70% ethanol was added to

the homogenized lysate, which was mixed by pipetting, applied to an RNeasy mini column placed in a 2-ml collection tube, and centrifuged for 15 s at 10000 ×*g*. The flow-through was discarded. 700 µl of Buffer RW1 was added to the column, which was centrifuged for 15 s at 10000 ×*g*. The column was transferred to a new 2-ml collection tube and washed with Buffer RPE twice by centrifuging for 2 min at 10000 ×*g*. Finally, the RNA was eluted with 30 µl of RNase-free water by centrifuging for 1 min at 10000 ×*g*.

2.2.17 First-strand cDNA Synthesis

A total of 5 µg of total RNA was reverse transcribed to cDNA using SuperScrip™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instruction. In brief, total RNA (5 µg), oligo$(dT)_{20}$ (50 µM), and dNTP mix (10 mM) were mixed and the volume was brought to 10 µl in a 0.5-ml tube. The mixture was then incubated for 5 min at 65ºC and placed on ice for 1 min. cDNA Sythesis Mix (10 µl), comprising 10× RT buffer (2 µl), 25 mM $MgCl_2$ (4 µl), 0.1 M DTT (1 µl), RNaseOUT (1 µl), and SuperScript III RT (1 µl), were added and the tube was incubated for 50 min at 50ºC. The reaction was terminated at 85ºC for 5 min. RNA was removed by adding 1 µl of RNase H for 20 min at 37ºC.

2.2.18 Quantitative Real-time Reverse Transcription PCR

PCR primers and fluorogenic probes for all of the target genes (IL-2, IL-4, IFN-γ, TNF-α, IL-1β, IL-12p40) and endogenous control (β-actin) were purchased as TaqMan® Gene Expression Assays (ABI, Foster City, CA), which consists of 20× mixed PCR primers

and TaqMan minor groove binder 6-FAM dye-labeled probes with a nonfluorescent quencher at the 3′ end of the probe. The product numbers for the target genes and endogenous control (β-actin) were as follows: Mm00434256_m1 (IL-2), Mm00445259_m1 (IL-4), Mm00801778_m1 (IFN-γ), Mm99999068_m1 (TNF-α), Mm00434228_m1 (IL-1β), Mm00434174_m1 (IL-12p40), Mm00607939_s1 (β-actin). The PCR volume was 20 µl, composed of 1 µl of 20× mixed primers and probe, 10 µl of 2× TaqMan® Fast Universal PCR Master Mix, and 9 µl of cDNA template (500 ng diluted in RNase-free water). PCR was performed in an optical 96-well reaction plate on the ABI 7500 Fast Real-time PCR System. Each sample was run in triplicate. The thermal cycle conditions were 20-s hold at 95ºC, followed by 50 cycles of 1 s at 95ºC (denaturation) and 20 s at 60ºC (annealing/extension).

The relative quantification of the target gene expression was calculated by comparative C_T ($\Delta\Delta C_T$) method using the machine software (SDS 1.3.1). In this method, the threshold cycle (C_T) is the fractional cycle number at which the fluorescence passes the threshold, which level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The amount of target gene expressed is normalized to an endogenous reference (β-actin) and is relative to a calibrator (negative control, i.e. untreated cells). The target C_T and endogenous reference C_T is calculated for each sample. The C_T of the endogenous reference is then subtracted from the C_T of the target gene. This value is known as ΔC_T. The ΔC_T of each sample is then subtracted from the ΔC_T of the calibrator, and this value is known as $\Delta\Delta C_T$.

2.2.19 ELISA

Cytokines, including IL-2, IL-4, IFN-γ, TNF-α, IL-1β, IL-12p40, and IL-12p70 were quantified by sandwich ELISA using BD Biosciences OptiEIA^{TM} Set (San Diego, CA), according to the manufacturer's instructions. Briefly, Maxisorp plates (Nunc, Roskidle, Denmark) were coated with 100 µl of purified anti-mouse IL-2, IL-4, IFN-γ, TNF-α IL-1β, IL-12p40, or IL-12p70 monoclonal antibodies, sealed, and incubated at 4ºC overnight. The wells were aspirated and washed with 300 µl/well wash buffer (PBS with 0.05% Tween-20) for three times. The wells were blocked with 200 µl of assay diluent (PBS with 10% FBS) for 1 h at RT. The plates were washed 3 times with wash buffer. 100 µl of supernatant from cell culture, as well as serially diluted recombinant mouse IL-2, IL-4, IFN-γ, TNF-α, IL-1β, IL-12p40, or IL-12p70 standards, were added to the plates. The plates were sealed and incubated at RT for 2 h. After incubation, the contents of the wells were aspirated and washed five times with wash buffer. Then 100 µl of working detector (biotinylated anti-mouse IL-2, IL-4, IFN-γ, TNF-α, IL-1β, IL-12p40, or IL-12p70 + streptavidin-HRP conjugate) were added and the plates were incubated for 1 hour at room temperature with plate sealer. The plates were washed seven times with wash buffer. Finally, the plates were incubated with 100 µl of one step substrate reagent for 30 min at RT and the color development was stopped by adding 50 µl of stop solution. The absorbance was measured at 450 nm with a reference wavelength of 570 nm with a spectrometer (TeCan Sunrise).

2.2.20 Transfection

Jurkat cells or RAW264.7 cells were transiently transfected by using lipofectamine transfection reagent. Briefly, 8.0 μg of luciferase reporter plasmid NFAT-luc, AP-1-luc, or NF-κB-luc DNA was diluted in 0.5 ml of Opti-MEM® I reduced serum media (Invitrogen). 20 μl of lipofectamine (Invitrogen) was diluted in 0.5 ml of Opti-MEM® I reduced serum media. The DNA solution was then mixed gently with the lipofectamine solution and incubated at room temperature for 20 min. The mixed solution was added dropwise to 8×10^6 Jurkat cells or RAW264.7 cells in 5 ml of Opti-MEM® I reduced serum media in a 60-mm Petri dish. The cells were incubated at 37°C in a 5% CO_2 incubator for 48 h.

2.2.21 Luciferase Assay

At the end of the culture, cells were harvested, washed with PBS, and adjusted to 2×10^6/ml. 100 μl of cells were applied to each well of a 96-well tissue culture plate. The cells were stimulated with 100 μg/ml of LBP or LBPF1-5 for 6 h. PMA (20 ng/ml) plus ionomycin (0.5 μg/ml) was positive control for Jurkat cells. LPS (1 μg/ml) was positive control for RAW264.7 cells. At the end of the stimulation, the cells were harvested and lysed with 20 μl of lysis buffer (25 mM Tris-phosphate, 8 mM $MgCl_2$, 2 mM DTT, 1% Triton X-100, and 10% glycerol, 2 mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid). 100 μl of the luciferase assay reagent (Promega) was dispensed into a luminometer tube. 20 μl of cell lysate was added into the tube and mixed with the reagent. Luciferase activity was measured by a luminometer (Biotrace). The background obtained with the

lysis buffer was subtracted from each experimental value, and the specific trans-activation was expressed as the fold induction over untreated cells.

2.2.22 *In vivo* Activation of T Lymphocytes by LBP

LBP was given to BALB/c mice daily by i.p. injection at doses of 0.5, 1.5, 5, 15, or 50 mg/kg or by oral gavage at doses of 5, 15, or 50 mg/kg for 7 days. Each group consisted of 4 mice. Normal saline was negative control. Mice were sacrificed on day 8 by CO_2 inhalation. Spleen cells were harvested. Splenocyte proliferation was determined by ^{3}H-thymidine incorporation assay in the presence or absence of 1 μg/ml of Con A.

2.2.23 *In vivo* Endocytosis and Phagocytosis Assay

BALB/c mice were administered with LBP (50 mg/kg, i.p., × 7 d). 30 min prior to sacrifice, mice were i.p. injected with 1 ml of FITC-dextran (endocytosis assay) (1 mg/ml) or 1 ml of FITC-*Staphylococcus aureus* (phagocytosis assay) (1 mg/ml). Peritoneal cells were harvested, washed twice, and resuspended in DMEM medium supplemented with 10% FBS. The cells were applied to 24-well tissue culture plates with a microscope glass cover slip in each well at 37ºC in a 5% CO_2 humidified incubator for 6 h. The cover slip with adherent cells was picked up and mounted with DABCO-glycerol media. Cells were observed and photographed under a fluorescent microscope (Olympus BX-60, magnification ×40).

2.2.24 DC Culture and Activation

Bone marrow-derived DCs (BMDCs) were prepared as previously described (Inaba et al, 1992; Lutz et al, 1999), with some modifications. Briefly, bone marrow cells from the femurs and tibias of BALB/c mice were collected and filtered through a nylon mesh. After lysis of RBCs with the Tris-NH_4Cl lysis buffer, cells were seeded in 6-well plates at 10^6 cells/ml (3 ml/well) in RPMI-1640 medium supplemented with 5% FBS, 50 μM of 2-mercaptoethanol, 10 mM of HEPES, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. 10 ng/ml of rmGM-CSF and 10 ng/ml of rmIL-4 were added. On day 3, floating cells and loosely adherent cells were harvested and cultured in fresh medium containing GM-CSF and IL-4. On day 6, non-adherent cells were collected and cultured in 60-mm petri dishes at 10^6 cells/ml (5 ml/dish). The cells were stimulated with LPS (0.1-1 μg/ml), LBP (100 μg/ml), or LBPF1-5 (100 μg/ml) for 24 - 48 h and then analyzed.

2.2.25 Splenic DC Purification

BALB/c mouse spleens were removed aseptically and minced in Mg^{2+} and Ca^{2+} free HBSS supplemented with 5% FBS. The homogenate was incubated with 1 mg/ml of collagenase A and 0.2 mg/ml of DNase I for 35 min at 37ºC. The reaction was stopped by addition of EDTA (20 mM). The cells were incubated for 5 min at RT, passed through a 70-μm cell restrainer (BD Biosciences), layered over a Histodenz gradient (RPMI-1640/ 10% FBS/ 14.5%), and centrifuged for 20 min at 450 ×*g* at RT. Cells at the gradient interface were collected, washed, incubated with anti-CD11c microbeads for 15 min at 4ºC, and positively selected on a column using a MACS separator. The resulting DC preparations were routinely ≥ 85% positive for CD11c as determined by flow cytometry.

2.2.26 Mixed Leukocytes Reaction

Responder T cells were purified from C57BL/6 mice spleen cells using Dynal mouse T cell isolation kit according to the manufacturer's instruction. Day 6 BMDCs from BALB/c mice were stimulated with LBP or LBPF1-5 (100 μg/ml), or LPS (1 μg/ml) for 24 h, and then incubated with mitomycin C (50 μg/ml) for 1 h. After wash, DCs (5×10^4) were incubated with allogeneic T cells (2×10^5) in round-bottomed 96-well plates at 37°C in a 5% CO_2 humidified incubator. After 54 h, the cells were pulsed with ^{3}H-thymidine (0.5 μCi/well) for 18 h. The cells were harvested onto glass fiber filters using the Filtermate cell harvester (Packard) and cell incorporated-^{3}H-thymidine was measured using a β-scintillation counter (Packard TopCount).

2.2.27 *In vitro* Endocytosis Assay

Day 6 BMDCs (10^6 cells/ml) were incubated with 1 mg/ml of FITC-dextran (42 kDa) for 1 h at 37°C. The cells were washed, stained with APC-conjugated anti-mouse CD11c, and analyzed by flow cytometry. As controls, experiments were performed at 4°C.

2.2.28 DC Presentation of OVA Antigen *in vitro*

Day 6 BMDCs (10^6 cells/ml) were pulsed with OVA (100 μg/ml) for 2 h and then stimulated with 100 μg/ml of LBP for 24 h. The cells were washed and 5×10^4 DCs were incubated with 2×10^5 T cells isolated from BALB/c mouse splenocytes in ELISPOT plates which had been pre-coated with anti-IL-4 or anti-IFN-γ mAb. The cells were cultured for 48 h at 37°C in a 5% CO_2 humidified incubator. IL-4- and IFN-γ-producing cells were measured by the ELISPOT assay.

2.2.29 DC Presentation of OVA Antigen *in vivo*

Day 6 BMDCs (10^6 cells/ml) were pulsed with OVA (100 μg/ml) for 2 h and then activated with LBP (100 μg/ml) for 24 h. After wash, 5×10^5 cells were subcutaneously injected into each BALB/c mouse. Saline, BMDCs without stimulation or stimulated with OVA or LBP alone were controls. Each group consisted of 4 mice, which were sacrificed on day 8. Splenocytes were harvested and re-stimulated with OVA (10 μg/ml) in the ELISPOT assay measuring IL-4 and IFN-γ producing cells.

2.2.30 DC Stimulation with LBP *in vivo*

BALB/c mice were administered with a single dose of LBP (20 mg/kg, s.c., i.p., or p.o.). Naïve mice without LBP injection were used as control. Each group consisted of 4 mice. Mice were sacrificed after 24 h. Splenic DCs were purified and examined for the expression of CD40, CD80, CD86, and I-A/I-E by flow cytometry.

2.2.31 Helper T Cell Response to OVA Plus LBP *in vivo*

BALB/c mice were injected s.c. with OVA (100 μg) for 3 times at one week intervals and also given LBP (20 mg/kg) s.c., i.p., or p.o. daily. Naïve mice were used as control. Each group consisted of 4 mice. After 21 days, splenocytes were harvested and re-stimulated with OVA (10 μg/ml) in the ELISPOT plates pre-coated with anti-IL-4 or anti-IFN-γ antibodies. The cells were cultured at 37ºC in a 5% CO_2 humidified incubator for 48 h. IL-4- and IFN-γ-producing cells were determined by ELISPOT assay.

2.2.32 ELISPOT Assay

ELISPOT plates (Millipore) were coated with capture antibody (anti-IL-2, anti-IL-4, or anti-IFN-γ) at 4ºC overnight. The plates were washed and blocked with RPMI-1640/ 10% FBS. 2×10^5 splenocytes or T cells were seeded into each well of the plates and incubated at 37°C in a 5% CO_2 humidified incubator for 48 h in the presence of 10 μg/ml of OVA (or 5×10^4 DCs in some assays). Cells were decanted at the end of culture. The plates were washed and incubated with biotinylated detection antibody followed by streptavidin-HRP conjugate. IL-2, IL-4, and IFN-γ spots were developed by adding AEC substrate for 10 - 60 min. The development of spots were stopped by adding distilled water. After drying, the spots were counted using an automated ELISPOT plate reader (BIOREADER 2000, BioSys, Karben, Germany). Results are expressed as the number of cytokine-producing cells per 2×10^5 cells.

2.2.33 Statistical Analysis

Data are presented as mean ± SD, except for the RQ of cytokine mRNA, which was presented as 95% of confidence interval. Each experiment was repeated at least three times. Differences were analyzed for significance using the Student's unpaired, two-tailed *t*-test when LBP or LBPF1-5 were compared with negative control, or One Way ANOVA when the efficacy among LBP, LBPF1-5 were compared. A p value less than 0.05 was used as the threshold for significance.

CHAPTER 3

RESULTS AND SECTIONAL DISCUSSIONS

3.1 Isolation, Purification and Characterization of LBP

3.1.1 Aim of Study

It has been known that the bioactive component of *L. barbarum* is a polysaccharide-protein complex and to date five fractions have been isolated and purified from the raw fruit (Huang et al, 1998; Peng and Tian, 2001; Zhao et al, 1997). However, the structure, constituent, and molecular weight of the *L. barbarum* polysaccharide-protein complex fractions obtained from different laboratories are not consistent (Huang et al, 1998; Peng and Tian, 2001; Zhao et al, 1997; Wang et al, 2002). These variations made it necessary for us to isolate, purify and characterize *L. barbarum* polysaccharide-protein complex (LBP) used for further functional studies.

3.1.2 Results

3.1.2.1 Isolation of LBP

500 g of *L. barbarum* dried fruit produced 2 g of crude LBP after water extraction, ethanol precipitation, removal of free protein, and dialysis. The yield was 0.4%. The crude LBP was a dark-brown substance and is water-soluble. Five intermediary fractions, designated as LBP1, LBP2, LBP3, LBP4, and LBP5, were separated from crude LBP by DEAE-cellulose ion exchange chromatography after successive elution with water, 0.05, 0.1, 0.2, and 0.5 M NaCl. The elution profile is shown in Figure 2. The amounts of LBP1-5 from 0.5 g of crude LBP were 72 mg, 76 mg, 30 mg, 45 mg, and 40 mg, respectively.

3.1.2.2 Purification of LBP

To further purify LBP1-5 by size exclusion chromatography, LBP1-5 were applied onto Sephacryl S-300 HR column and eluted with water, respectively. The elution profiles are shown in Figure 3 (A-E). Five homogenous fractions, designated as LBPF1, LBPF2 LBPF3, LBPF4, and LBPF5, were obtained from LBP1-5, respectively. The yields were as follows: 20 mg of LBPF1 from LBP1; 30 mg of LBPF2 from LBP2; 19 mg of LBPF3 from LBP3; 29 mg of LBPF4 from LBP4, and 20 mg of LBPF5 from LBP5. LBPF1 was a white solid; LBPF2 and LBPF3 were light yellow solids; LBPF4 and LBPF5 were brown solids. All fractions were water-soluble.

3.1.2.3 Characterization of LBP on Carbohydrate and Protein Content and Molecular Weight

The carbohydrate and protein contents and molecular weights of LBPF1-5 were determined by the phenol-sulfuric acid method, the Bradford method, and SDS-PAGE, respectively. As shown in Figure 4 and Table 2, LBPF1 had the highest percentage of carbohydrate content (based on glucose), followed by LBPF3, LBPF2, LBPF5, and LBPF4. LBPF5 had the highest percentage in protein content, followed by LBPF4, LBPF2, LBPF3, and LBPF1. The molecular weights of LBPF1-4 were about 150 kDa. The molecular weight of LBPF5 was undetectable by SDS-PAGE. It was 290 kDa when determined by size exclusion chromatography (Figure 4E).

3.1.3 Discussion

During the last three decades, many polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants. The biological activities of these polysaccharides have attracted more attention recently in biomedical and medical fields because of their immunomodulatory and antitumor effects (Ooi and Liu, 2000). Based on the characteristic that polysaccharides are soluble in water but only slightly soluble in ethanol, they can be isolated by water extraction followed by ethanol precipitation. Currently there are mainly two approaches used in different laboratories for LBP isolation. One is to use organic chemicals such as acetone and petroleum to remove lipids and pigments, considering that polysaccharides are within plant cells that are covered by lipid membranes. Then oligosaccharides and saponins are removed by refluxing with 80% ethanol. Finally polysaccharides are extracted by hot water (Wang et al, 2002). Another is to directly extract LBP with water (Huang et al, 1998; Peng and Tian, 2001). Meng et al (1999) compared these two methods and found the LBP production rate was almost the same. As reflux by hot organic reagents may destroy the structure of the glycoconjugates, we prefer to directly isolate LBP with water.

Through water extraction, ethanol precipitation, ion exchange chromatography and gel filtration, five fractions of *L. barbarum* polysaccharide-protein complex (LBPF1-5) were obtained. All of them contain both carbohydrates and proteins, as determined by the phenol-sulfuric acid method and the Bradford method, respectively. Single peak was detected at absorbance 490 nm after passing LBP1-5 through Sephacryl S-300 column. This indicates that LBPF1-5 are homogenous. The polysaccharide component of LBP

consists of six monosaccharides, including rhamnose, xylose, arabinose, mannose, glucose, and galactose (Huang et al, 1998; Wang et al, 2002b). The protein part of LBP is composed of most of the amino acids (Huang et al, 1998; Zhao et al, 1997). The structure of LBP possesses a backbone consisting of (1→6)-β-galactosyl residues, about fifty percent of which are substituted at C-3 by galactosyl or arabinosyl groups and the major non-reducing end being made of arabinose (Peng et al, 1998).

Although it is well accepted that LBPs are polysaccharide and protein complex, their constituents, structures, and molecular weights vary from one laboratory to another (Huang et al, 1998; Peng and Tian, 2001; Zhao et al, 1997; Wang et al, 2002b). These variations could be due to the difference in the raw materials and the maturity status of fruit (Wang et al, 2002b). They could also be due to isolation approaches, and especially the different gels and eluents used in ion exchange chromoatography and size exclusion chromatography (Meng et al, 1999).

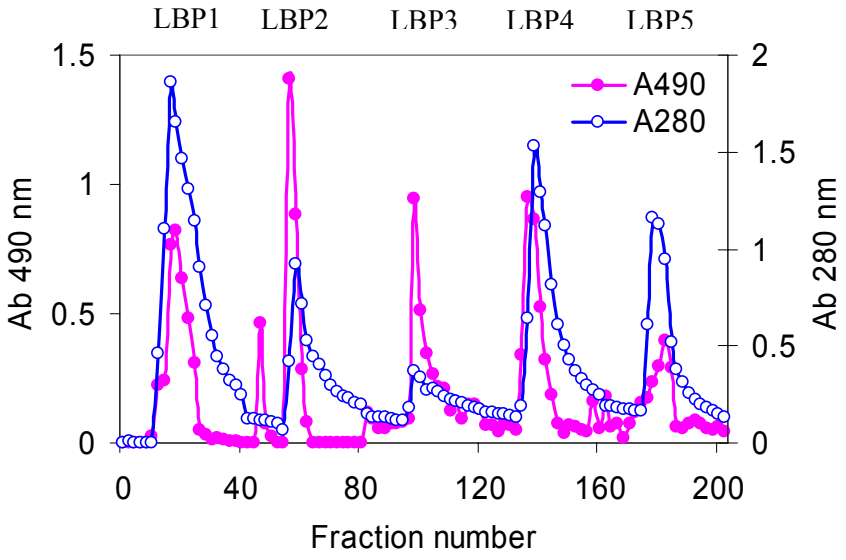

Figure 2. **Elution profile of LBP on DEAE-cellulose column (OH^-).** LBP (0.5 g) was loaded onto a DEAE-cellulose-packed column (2.6 × 40 cm), which was successively eluted with H_2O and 0.05, 0.1, 0.2, 0.5 M NaCl solution at a flow rate of 0.8 ml/min. Fractions were collected at 10 ml/tube. Absorbance values at 490 nm and 280 nm were monitored for evaluation of the carbohydrate and protein contents, respectively. Five fractions, designated as LBP1, LBP2, LBP3, LBP4, and LBP5, were eluted as indicated.

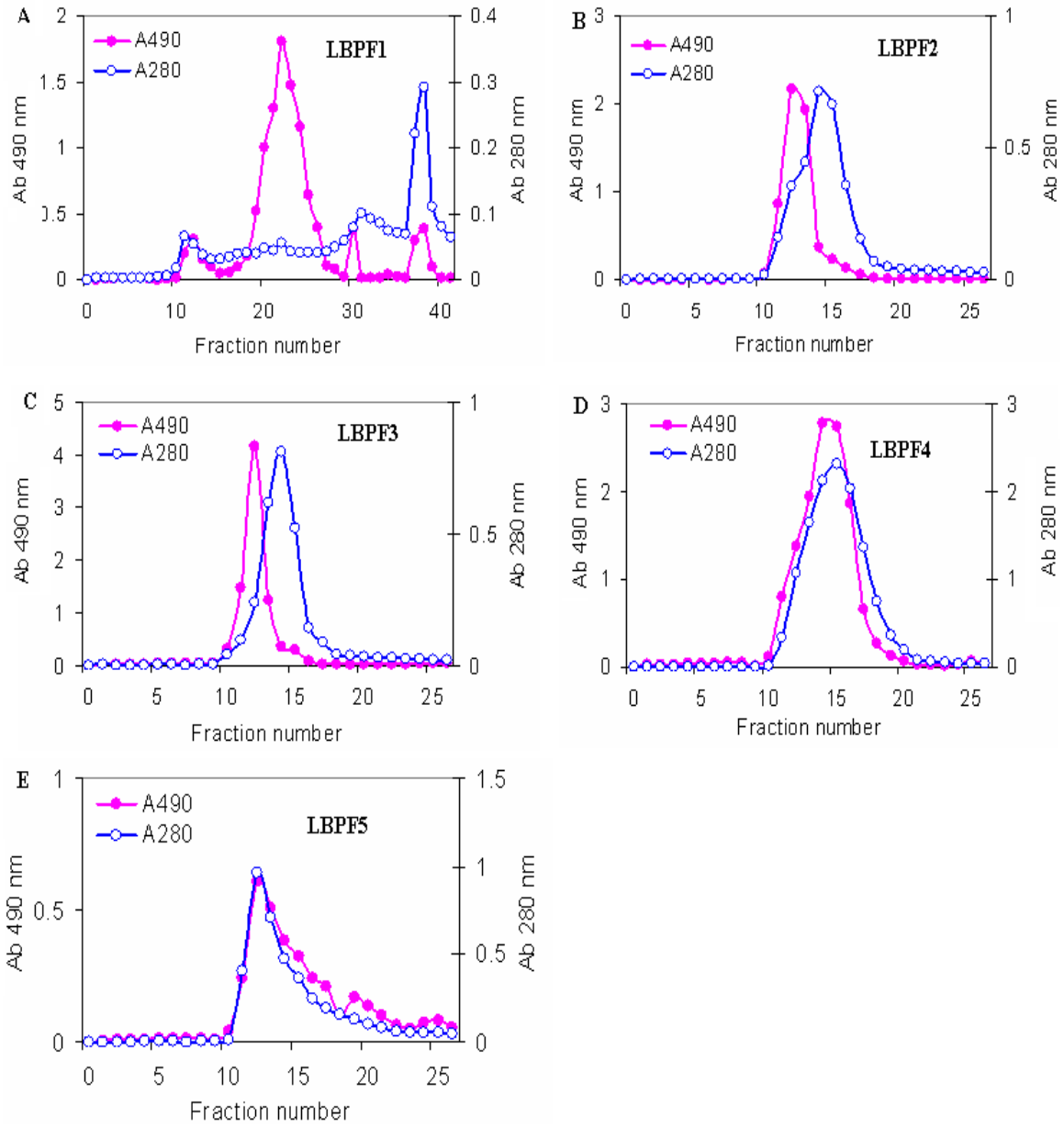

Figure 3. Elution profile of LBP1-5 on Sephacryl S-300 column. Samples were loaded onto Sephacryl S-300 column (2.6 × 60 cm), which was eluted with H_2O at a flow rate of 1.3 ml/min. Fractions were collected at 9 ml/tube. Absorbance values at 490 nm and 280 nm were monitored for evaluation of the carbohydrate and protein contents, respectively. Five fractions were eluted, designated as: **A.** LBPF1, **B.** LBPF2, **C.** LBPF3, **D.** LBPF4, and **E.** LBPF5.

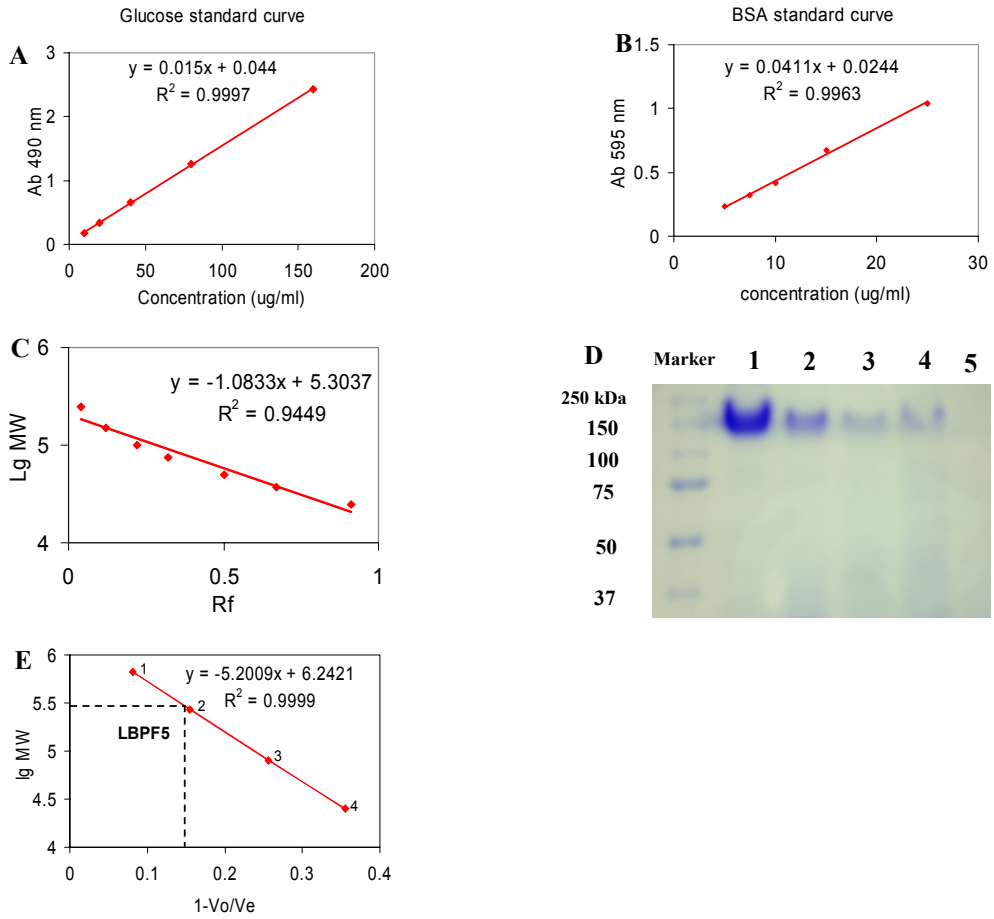

Figure 4. Characterization of LBPF1-5 on carbohydrate and protein contents and molecular weights.

A. Standard curve of glucose. Glucose was used as standard to determine the carbohydrate content of LBPF1-5 by phenol-sulfuric acid method. Absorbance value was measured at 490 nm. **B.** Standard curve of BSA. BSA was used as reference to determine the protein content of LBPF1-5 by the Bradford method. Absorbance value was measured at 595 nm. **C.** Plot of lg MW of protein standards against their Rf, the ratio of the distance migrated by the molecule to that migrated by bromophenol blue-front on SDS-PAGE. **D.** SDS-PAGE of LBPF1-5. Samples were loaded onto 10% SDS-PAGE and run for 2 h. The gel was stained with Coomassie brilliant blue. Lane 1: LBPF1; 2. LBPF2; 3. LBPF3; 4. LBPF4; 5. LBPF5. **E.** Plot of (1-Vo/Ve) of dextrans and LBPF5 on Sephacryl S-300 column against their lgMW. T-dextran series, blue dextran, and LBPF5 were loaded onto Sephacryl S-300 column (2.6 × 60 cm), which was eluted with 0.1 M KCl at a flow rate of 1.3 ml/min. Fractions were collected at 5.2 ml/tube. The elution volume (Ve) and the void volume (Vo) were determined by the absorbance peak at 490 nm. 1. T-670 (670 kDa); 2. T-270 (270 kDa); 3. T-80 (80 kDa); 4. T-25 (25 kDa). LBPF5 position was indicated.

Table 2. Carbohydrate and protein contents and molecular weights of LBPF1-5

	LBPF1	LBPF2	LBPF3	LBPF4	LBPF5
Carbohydrate (%)	48.2	30.5	34.5	20.3	23.2
Protein content (%)	1.2	4.8	4.1	13.7	17.3
Molecular weight (KDa)	151	147	146	150	290

3.2 Test of LPS Contamination and Evaluation of Toxicity

3.2.1 Aim of Study

Endotoxin LPS, which is found in the outer membrane of various Gram-negative bacteria, is a potent simulator of immune cells, such as macrophages, DCs and B cells. LPS can bind to LPS binding protein, which transfers it to bind to CD14 and associates with adaptor MD2. This complex then binds to TLR and subsequently triggers the signaling cascades and leads to cell activation. During the process of isolation and purification, it is possible that LBP is contaminated by Gram-negative bacteria with cell walls that consist of LPS. In addition, compounds isolated from plants may be toxic. Therefore, this section was to rule out LPS contamination and to evaluate the toxicity before biological studies were conducted to investigate the immunomodulatory properties of LBP.

3.2.2 Results

3.2.2.1 LBP is Free of LPS Contamination

LPS contamination was tested by *Limulus* amoebocyte lysate (LAL) assay. The principle is LAL will form hard gel in the presence of a moiety of LPS. Table 3 shows that LAL did not form hard gel in the presence of LBP or LBPF1-5. In contrast, it did when incubated with ≥0.03 EU/mg LPS. The result indicates that the quantity of endotoxin in crude LBP and LBPF1-5 was less than 0.015 EU/mg (negative) (Table 3). The result was further confirmed by B cell proliferation assay, as LPS is a strong stimulator of B cell proliferation. B cells were isolated from mouse spleen and stimulated with LPS, LBP, or LBPF1-5 and the cell proliferation was measured by ^{3}H-thymidine uptake assay. As

shown in Figure 5, LPS stimulated strong B cell proliferation, whereas LBP and LBPF1-5 did not. The result suggests there was no LPS in LBP and LBPF1-5.

3.2.2.2 *In vitro* Cytotoxicity

To evaluate the cytotoxicity of LBP *in vitro*, mouse splenocytes were incubated with LBP at serial concentrations, including 10, 20, 40, 80, 160, 320, 640, 1280, 2560 μg/ml, for 72 h. The cell viability was determined by ^{3}H-thymidine incorporation assay. The result showed that LBP promoted splenoctye growth at certain concentrations (≤ 320 μg/ml), whereas at concentrations higher than 320 μg/ml, it inhibited splenocyte growth. At 1260 and 2560 μg/ml of LBP, the growth of splenocytes was almost completely inhibited (Figure 6).

3.2.2.3 Acute Toxicity

To evaluate the acute toxicity of LBP, BALB/c mice were administered with a single dose of LBP (1 g/kg, p.o. or i.p.). Mice were observed for mortality, body weight, and clinical symptoms such as breathing, walking behavior, fur erection, feces, and urine, for 14 days. The results showed that none of the mice died after p.o. or i.p. administration with LBP. The body weight was not reduced and no abnormal clinical signs were observed in the mice administered p.o. with LBP. In the mice that were administered i.p. 20 mg of LBP, abnormal clinical symptoms were observed, including depression, reluctance to move, fur erection, shallow rapid breathing, reduced feed, diarrhea, and bright yellow urine. These clinical signs appeared 4 h after LBP injection but subsided three days after the injection. The average body weight was also reduced 1.5 g (7.5% of

the body weight) on the first day after LBP injection but became normal by the fourth day after the injection (Figure 7).

3.2.3 Discussion

Endotoxin (LPS) is a known immunomodulator and is often a contaminant in biological preparations. Thus, one of the principal concerns is that the immune stimulating properties of botanical polysaccharides might be due to contamination from bacterial endotoxin (Schepetkin and Quinn, 2006). Probably, the most common approach to detecting LPS contamination involves the use of LAL assay (Hase et al, 1997; Sanzen et al, 2001). Therefore, we tested whether LBP and its five fractions were contaminated with LPS by LAL assay. The result showed that the samples were not contaminated with LPS, as was further supported by the fact that crude LBP and LBPF1-5 could not stimulate B cell proliferation. If the samples were contaminated with LPS, they would stimulate B cell proliferation.

LBP was non-toxic *in vitro* at certain concentrations (≤ 320 μg/ml). At extremely high concentrations (i.e. 1 mg/ml), it caused cell death. This could be because LBP is a substance with high molecular weight. At high concentrations, LBP may have a higher osmotic pressure that the cell interior, and consequently its hypertonicity causes cells to shrink, and subsequently death. The negative charge of LBP may aslo cause cytotoxic stress and affect its viability.

The data showed that LBP was non-toxic at a high dose of 1 g/kg by p.o. administration. Although it showed some acute toxicities at this dosage by i.p. adminitration, no mortality was detected. These results indicate that LBP is safe for *in vivo* studies.

Table 3. Test of LPS contamination of LBP by *Limulus* assay

Standards/Samples	Hard Gel Formation
Endotoxin standard (EU/ml)	
0.5	+
0.25	+
0.125	+
0.06	+
0.03	+
0.015	-
Sample (10 mg/ml)	
Crude LBP	-
LBPF1	-
LBPF2	-
LBPF3	-
LBPF4	-
LBPF5	-

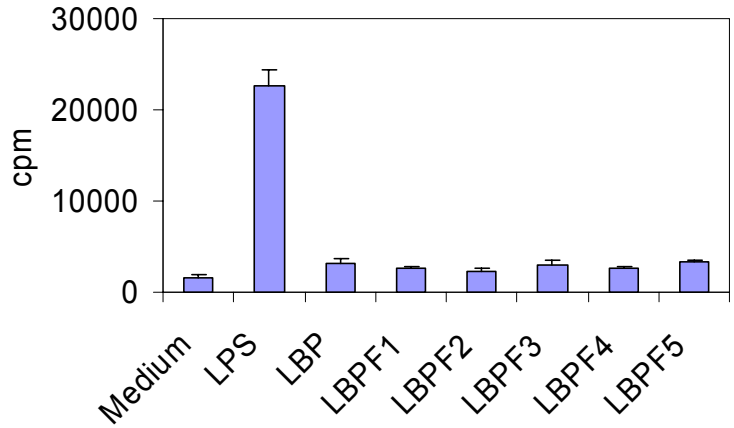

Figure 5. Test of LPS contamination by B cell proliferation assay. B cells were purified from BALB/c mice by magnetic bead negative selection. The purified B cells were stimulated with LBP (100 μg/ml), LBPF1-5 (100 μg/ml), or LPS (5 μg/ml) for 72 h. B cell proliferation was measured by ^{3}H-thymidine incorporation assay. Values are represented as mean (CPM) ± SD of four replicates.

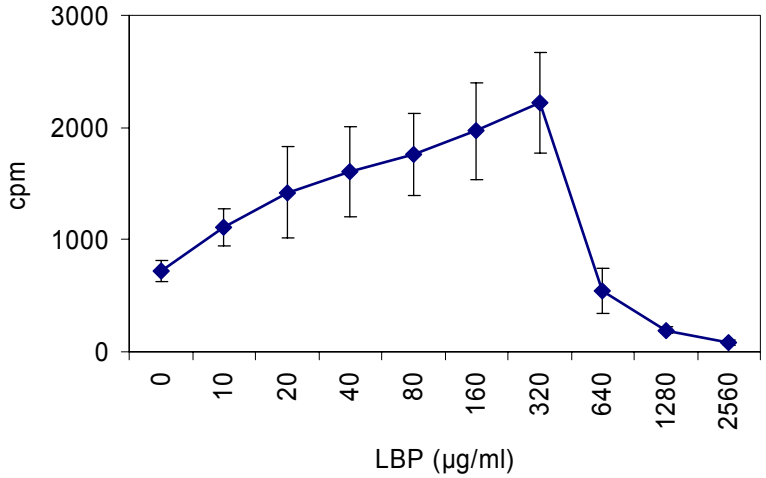

Figure 6. *In vitro* cytotoxicity of LBP. Mouse splenocytes were treated with LBP (10 - 2560 μg/ml) for 72 h. ^{3}H-thymidine (0.5 μCi/well) was added for the last 18 h. The cells were harvested and the incorporated ^{3}H-thymidine was measured. Values are represented as mean (CPM) ± SD of four replicates.

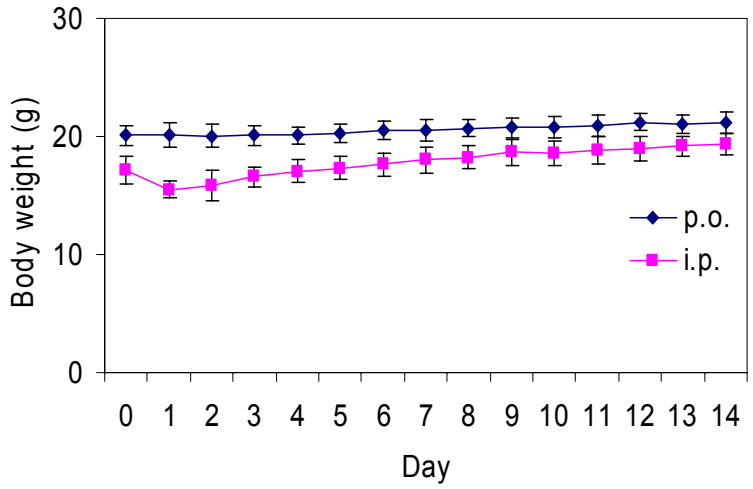

Figure 7. Mouse body weight changes after LBP administration. Mice were administered p.o. or i.p. with a single dose of 20 mg of LBP (1 g/kg) at day 0. The body weight was measured before LBP administration and daily for 14 days after LBP administration. Values are mean ± SD of 6 mice.

3.3 Activation of T Cells by LBP

3.3.1 Aim of Study

T lymphocytes are thymus-derived lymphocytes that play a central role in the generation and regulation of immune response to protein antigens. Although LBP was found to stimulate lymphocyte proliferation and cytokines such as IL-2 secretion (Qian et al, 1988; Hu et al, 1995; Ma et al, 1996; Qi et al, 1999; Liu et al, 2000; Wang et al, 1990; Gan et al, 2003; Gan et al, 2004), indicating it can activate T cells, the complete cytokine profile and the mechanism of T cell activation by LBP, have not been reported yet. In this section, we investigated whether LBP was able to stimulate T cell proliferation, activate CD25, activate NFAT and AP-1 transcription factors, as well as induce IL-2 and IFN-γ production at mRNA and protein level.

3.3.2 Results

3.3.2.1 Effects of LBP on Splenocyte, T and B Cell Proliferation

To screen the immunomodulatory function of LBP and its five fractions, mouse splenocytes were stimulated with LBP or LBPF1-5 at serial concentrations from 1 to 300 μg/ml for 72 h. Cell proliferation was measured by ^{3}H-thymidine uptake assay. As shown in Figure 9A, crude LBP, LBPF4, and LBPF5 could induce splenocyte proliferation in a dose-dependent manner. 300 μg/ml of LBP significantly enhanced splenocyte proliferation by about 4.5-fold. In contrast, LBPF1, LBPF2, and LBPF3 could not stimulate splenocyte proliferation. The proliferative effects of LBPF4 and LBPF5 were not higher than that of crude LBP. As splenocytes contain both T and B lymphocytes, to study which subset is stimulated by LBP, T and B cells were purified from splenocytes

by negative selection technique using magnetic beads. More than 85% and 60% of the remaining cells were T and B cells, respectively, as determined by flow cytometry (Figure 8). The purified T or B cells were stimulated with 100 μg/ml of LBP, LBPF4, or LBPF5 for 72 h and cell proliferation was measured. The results showed that LBP, LBPF4, and LBPF5 activated T cells but not B cells (Figure 9B). As positive control, Con A (3 μg/ml) stimulated T cell proliferation by 80-fold while of LPS (5 μg/ml) stimulated B cell proliferation by 13-fold (data not shown). To further investigate whether the protein part of LBP contributes such effects, LBP, LBPF4, and LBPF5 were digested with protease to destroy protein. The lymphocyte proliferative activities of LBP, LBPF4, and LBPF5 were significantly reduced after treatment with protease (Figure 9C).

3.3.2.2 Effects of LBP on Cell Cycle Progression

As LBP could stimulate T cell proliferation, it might promote cells to enter S and G_2/M phases of the cell cycle. To test this hypothesis, splenocytes were stained with propidium iodide after LBP stimulation and cell cycle profile was analyzed by flow cytometry. The result showed that the percentage of cells in S and G_2/M phases did not increase after treatment with LBP and LBPF1-5 (Figure 10). However, crude LBP, LBPF4, and LBPF5 could reduce the percentage of apoptotic cells. Up to 67% of untreated primary splenocytes underwent apoptosis after *in vitro* culture for 48 h. In contrast, the percentage of apoptotic cells was lowered to 38%, 47%, and 44% in the presence of crude LBP, LBPF4, and LBPF5, respectively. LBPF1, LBPF2, and LBPF3 again did not have such an effect.

3.3.2.3 Activation of CD25 by LBP

To investigate whether LBP could enhance the expression of T cell activation marker CD25, mouse splenocytes were stimulated with 100 μg/ml of LBP or LBPF1-5 for 48 h. CD25 expression was determined by flow cytometry (Figure 11). 4.3% of resting T cells expressed CD25. After stimulation with crude LBP, LBPF4, and LBPF5, CD25 expression was increased to 12.5%, 18.7%, and 10.9% of the cells, respectively. LBPF1, LBPF2, and LBPF3 failed to induce CD25 expression. The positive control, Con A enhanced the expression of CD25 in up to 42.5% of the cells. The result demonstrated that LBP, LBPF4 and LBPF5 can induce CD25 expression.

3.3.2.4 Induction of Cytokine mRNA Expression by LBP

To investigate whether crude LBP and its five fractions could induce cytokine gene expression, mouse splenocytes were stimulated with 100 μg/ml of crude LBP, LBPF1, LBPF2, LBPF3, LBPF4, or LBPF5 for 48 h. Cytokine gene expression, including IL-2, IL-4, IFN-γ, and TNF-α gene, were quantified by real-time PCR. As shown in Figures 12A and 13A, IL-2 mRNA expression was enhanced 2-, 9-, and 1.5-fold after stimulation with LBP, LBPF4, and LBPF5, respectively. IL-4 mRNA expression was enhanced 5-, 4-, and 24-fold after simulation with LBP, LBPF4, and LBPF5, respectively (Figures 12B and 13B). IFN-γ mRNA expression was enhanced 200000-, 120000-, and 1700-fold after stimulation with LBP, LBPF4, and LBPF5, respectively (Figures 12C and 13C). It is noted that the high levels of IFN-γ mRNA relative expression after stimulation with LBP, LBPF4, and LBPF5 was due to its extremely low expression in the untreated cells. TNF-α mRNA expression was enhanced 6-, 5-, and 2-fold after stimulation with LBP, LBPF4,

and LBPF5, respectively (Figures 12D and 13D). In contrast, LBPF1, LBPF2, and LBPF3 could not induce the mRNA expression of these cytokine genes. As positive control, Con A could enhance IL-2, IL-4, IFN-γ, and TNF-α mRNA expression 21-, 636-, 188000-, and 8-fold, respectively.

3.3.2.5 Induction of Cytokine Production by LBP

To study whether LBP could induce cytokine production, mouse splenocytes were treated with LBP or LBPF1-5 at serial dosages and different time points. Secreted cytokines, including IL-2, IL-4, IFN-γ, and TNF-α, were measured by ELISA. As shown in Figure 14, crude LBP, LBPF4, and LBPF5 significantly induced IL-2 and IFN-γ production in dose-dependent and time-dependent manners, while LBPF1, LBPF2, and LBPF3 did not have such functions. 30 μg/ml to 100 μg/ml of crude LBP, LBPF4, or LBPF5 significantly induced IL-2 and IFN-γ production (Figures 14A and 14C, $P < 0.01$-0.001). Both IL-2 and IFN-γ productions peaked at 48 h after stimulation (Figures 14B and 14D). This was consistent with the proliferation result. However, IL-4 and TNF-α were undetectable by ELISA after stimulation with LBP, LBPF4, and LBPF5.

3.3.2.6 Activation of NFAT and AP-1, but not NF-κB by LBP

Transcription factors NFAT, AP-1, and NF-κB play important roles in T cell activation. Once activated, they can enter the nucleus and bind to the promoter or enhancer of cytokine genes, triggering their mRNA transcription. To investigate whether induction of cytokine mRNA expression and cytokine production by LBP was due to the activation of transcription factors, Jurkat T cells were transiently transfected with luciferase reporter plasmid NFAT-luc, AP-1-luc, or NF-κB-luc. The transfected cells were then stimulated with 100 μg/ml of LBP or LBPF1-5 for 6 h. Luciferase activity was measured by luciferase assay. As shown in Figure 15, crude LBP, LBPF4 and LBPF5 could activate NFAT 4- to 6-fold, and AP-1 2- to 3-fold compared to that of untreated cells. In contrast, NF-κB was not activated by LBP. As positive control, PMA in combination with ionomycin activated NFAT and AP-1 50- and 80-fold, respectively (data not shown).

3.3.2.7 Activation of T Lymphocytes *in vivo* by LBP

To verify whether LBP could activate T cells *in vivo*, we first administered LBP to BALB/c mice by i.p. injection. Splenocytes were harvested and cell proliferation was measured in the presence or absence of Con A. The result showed that splenocytes harvested from the mice injected with LBP had stronger proliferative activity than those from the mice injected with normal saline after culture *in vitro* for 72 h in the presence or absence of Con A (Figure 16). The effect was visible at a low dose of 0.5 mg/kg of LBP and was significantly different at 15 and 50 mg/kg of LBP. Similar effects were found when LBP was administered p.o. to mice (Figure 17). The results were consistent with the *in vitro* data.

3.3.3 Discussion

In this section, investigation of the biological functions of LBP was launched by proliferation assay using mouse spleen lymphocytes. We found that crude LBP, LBPF4 and LBPF5 could stimulate splenocyte proliferation, but LPBF1, LBPF2, and LBPF3 could not. The proliferation effect was specific to T cells, but not B cells, as confirmed by the finding that crude LBP, LBPF4, and LBPF5 stimulated T cell proliferation and activated T cell activation marker CD25. Polysaccharides represent a structurally diverse class of macromolecules and this structural variability can profoundly affect their cell-type specificity, especially with respect to B and T lymphocytes (Han et al, 2003). For example, β (1→3)-glucans were found to be T cell stimulators, but did not accelerate B cell antibody formation (Borchers et al, 1999), whereas polysaccharides from *Phellinus. linteus* activated both B and T cells (Kim et al, 1996). Poylsaccharides from *Acanthopanax koreanum* actiavted B cells (Han et al, 2003). As T lymphocytes play central roles in adaptive immunity, activation of T lymphocytes by LBP suggests that LBP may be involved in augmenting the response on adaptive immunity to eradicate endogenous infection. In the previous phase I clinical trial investigating the effect of *L. barbarum* on Epstein-Barr virus (EBV), it was showed that *L. barbarum* reduced serum EBV DNA copies in subjects with elevated serum IgA anti-EBV (data not shown). It could be because LBP helps to activate T cells such as CTLs to control EBV replication. Digestion of LBP with protease resulted in a decrease in the immunoactivity. This is not surprising as protein antigen is generally needed to activate T cells. Similar findings were observed previously (Kralovec et al, 2006). Interestingly, materials with higher purity such as LBPF4 and LBPF5 did not display markedly higher immunostimulating activity

than the crude parental LBP (Figure 9C). Crude LBP may contain other active components but these may have been removed during the processes of ion exchange chromatography and size exclusion chromatography.

Cytokines play pivotal roles in immune cell differentiation and proliferation. IL-2 is the essential cytokine for T lymphocyte growth. IL-2-induced proliferation occurs via pro-proliferative signals through the proto-oncogenes *c-myc* and *c-fos*, in combination with anti-apoptotic signals through Bcl-2 family members (Miyazaki et al, 1995). IFN-γ promotes the differentiation of Th1 cells and therefore a predominantly cell-based immune response. It functions predominantly on macrophages to enhance antimicrobial properties (Decker et al, 2005). IL-4 is crucial for the differentiation of naïve T helper cells into the Th2 cells that promote humoral immunity and provide protection against intestinal helminthes. It also has a central role in the pathogenesis of allergic inflammation (Weber and Krammer, 2003). Upon stimulation with antigen, $CD4^+$ T cells can differentiate in a highly polarized manner into Th1 or Th2 subsets (Jankovic et al, 2001). ELISA and real-time PCR data showed that crude LBP, LBPF4, and LBPF5 could activate T cells to produce IL-2 and IFN-γ at both protein and mRNA levels. However, IL-4 and TNF-α were undetectable by ELISA, although their genes were transcribed after stimulation with LBP, LBPF4 and LBPF5. This could be because IL-4 mRNA expression was low and the protein amount translated from the mRNA might not be enough to be measured by ELISA, or the IL-4 secreted in the supernatant had been fully utilized. The reason for the absence of TNF-α secretion could be that it is mainly produced by

macrophages, with T cells producing only some. There may not be sufficient macrophages to secrete TNF-α measurable by ELISA in the stimulated 2×10^6 splenocytes. Cytokine gene transcription needs to be initiated with the participation of transcription factors, of which NFAT, AP-1, and NF-κB are three key factors. NFAT was initially identified as an inducible nuclear factor that could bind the IL-2 promoter in activated T cells (Shaw et al, 1988). AP-1 proteins are the main transcriptional partners of NFAT during T-cell activation (Shaw et al, 2001; Jain et al, 1992). NF-κB is triggered by pro-inflammatory stimuli and genotoxic stress, including the following: cytokines, such as TNF and IL-1; bacterial cell-wall components, such as LPS; viruses; and DNA-damaging agents (Karin and Greten, 2005). Our data showed that LBP, LBPF4, and LBPF5 activated NFAT and AP-1 transcription factors using Jurkat T cells, but not NF-κB. This is understandable as activation of NF-κB needs TNF and IL-1β, both of which are mainly produced by macrophages.

In animal models and clinical situations, wolfberry is used as supportive treatment for a number of conditions such as cancer, hepatitis, tuberculosis, and aging (He et al, 1993; Bian et al, 1996; Song et al, 2002; Luo et al, 1997; Luo et al, 2004; Liu et al, 1996a; Liu et al, 1996b; Cao et al, 1994). One of the common characteristics of these diseases is that the immunity may be low or suppressed. Our data showed that administration of LBP to mice either by i.p. injection or by oral gavage for 7 days induced T lymphocyte proliferation significantly after *in vitro* culture for 72 h in the presence or absence of Con A, indicating that LBP could activate T lymphocytes *in vivo*. Although such activation is

non-specific, it may enhance the immunity and therefore is beneficial to patients with such conditions.

In summary, the results presented in this section suggest that LBP enhances immunity by activating T cells. The active fractions appear to be LBPF4 and LBPF5. Crude LBP, LBPF4 and LBPF5 could activate transcription factors NFAT and AP-1, inducing IL-2 and IFN-γ gene transcription and protein production. We conclude that the activation of T lymphocytes by LBP may contribute to its known immuno-enhancement function.

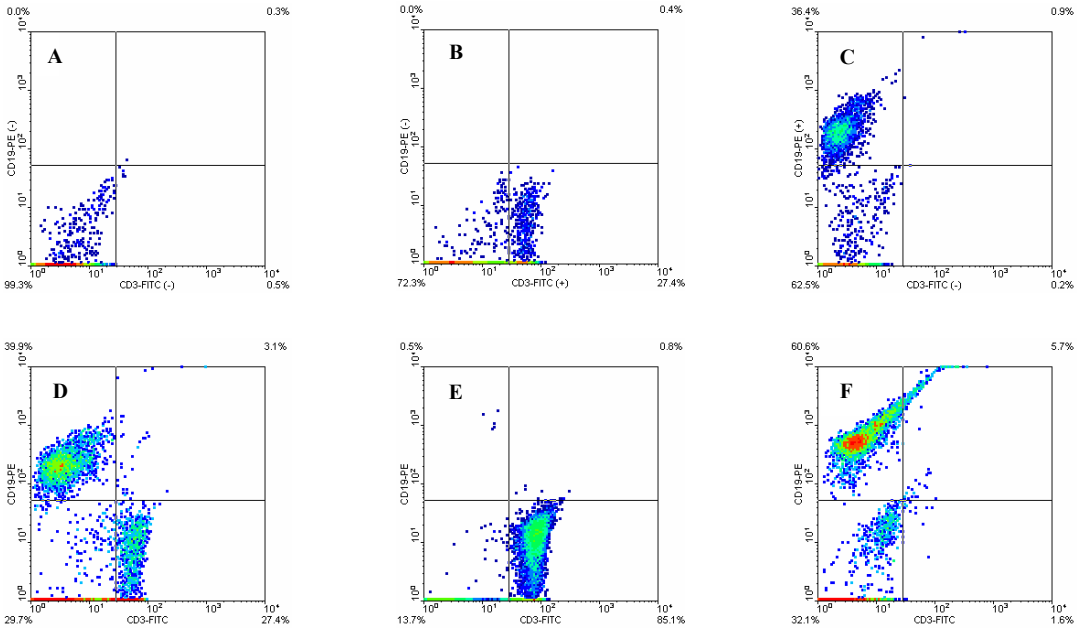

Figure 8. Purification of T and B cells from mouse splenocytes.

T and B cells were purified from mouse splenocytes by negative selection using magnetic bead. Cells were stained with anti-mouse CD3-FITC and CD19-PE and analyzed by flow cytometry. **A.** Double negative control; **B.** CD3-FITC control; **C.** CD19-PE control; **D.** Before purification; **E.** Purified T cells; **F.** Purified B cells.

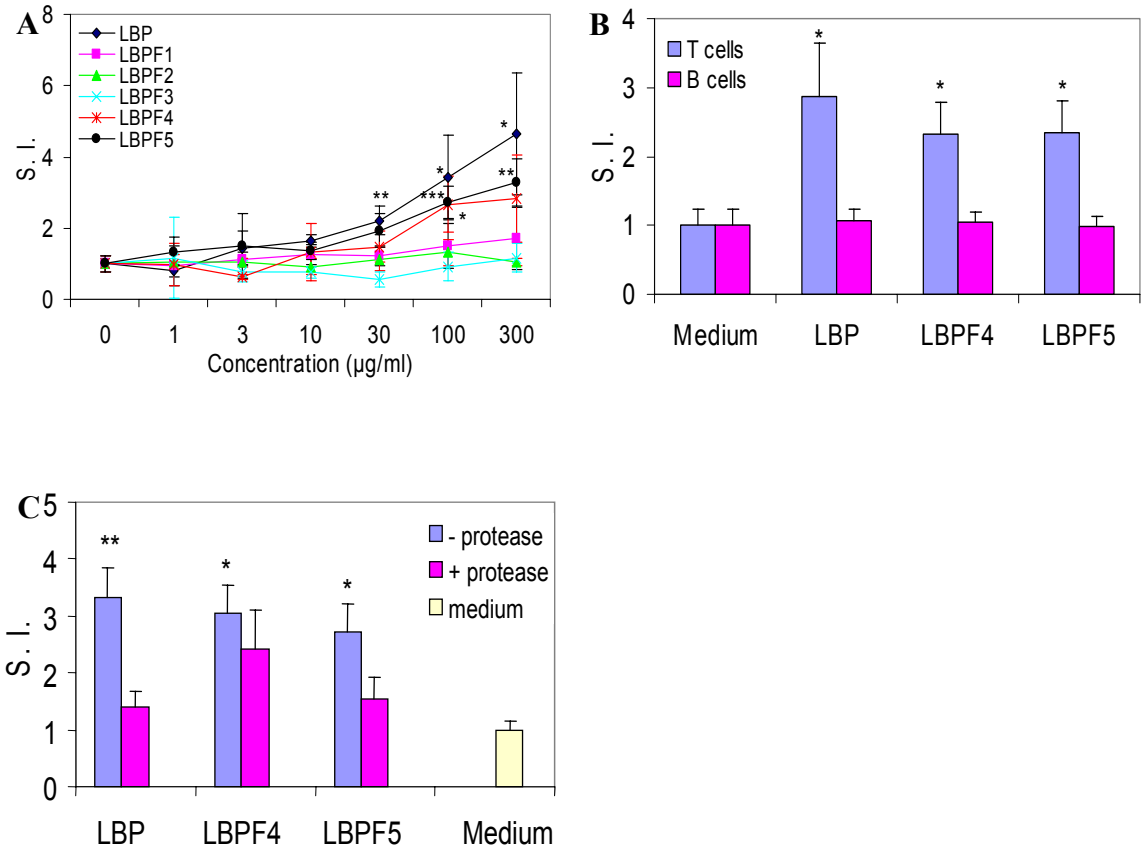

Figure 9. Effects of LBP and LBPF1-5 on splenocyte, T, and B cell proliferation.

A. 2×10^5 splenocytes in 200 μl were stimulated with 1, 3, 10, 30, 100, or 300 μg/ml of crude LBP or LBPF1-5 for 72 h. **B.** 2×10^5 T or B cells purified from mouse splenocytes in 200 μl were treated with 100 μg/ml of LBP, LBPF4, or LBPF5 for 72 h. **C.** 2×10^5 splenocytes in 200 μl were treated with 100 μg/ml of protease digested-LBP, LBPF4, or LBPF5 for 72 h. Cells were pulsed with ^{3}H-thymidine (0.5 μCi/well) for the last 18 h. Cell proliferation was measured by ^{3}H-thymidine incorporation assay. Results are expressed as stimulation index (S.I.), which is calculated by dividing the cpm of stimulated cells with the cpm of unstimulated cells. Values are mean ± SD of four replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to medium (0), or protease digestion.

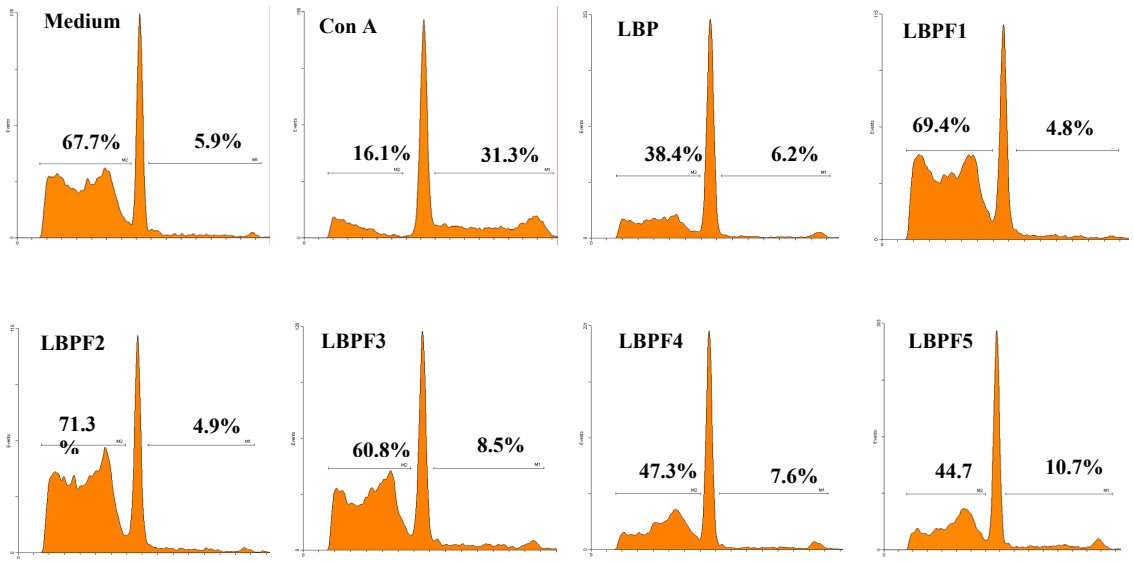

Figure 10. Effects of LBP and LBPF1-5 on cell cycle progression.

Mouse splenocytes were stimulated with LBP (100 μg/ml) or LBPF1-5 (100 μg/ml) for 48 h. Con A (3 μg/ml) was positive control. The cells were stained by propidium iodide and the cell cycle profile was analyzed by flow cytometry. The percentage of apoptotic cells and cells entering S and G_2/M phases are indicated. Results are representative of three independent experiments.

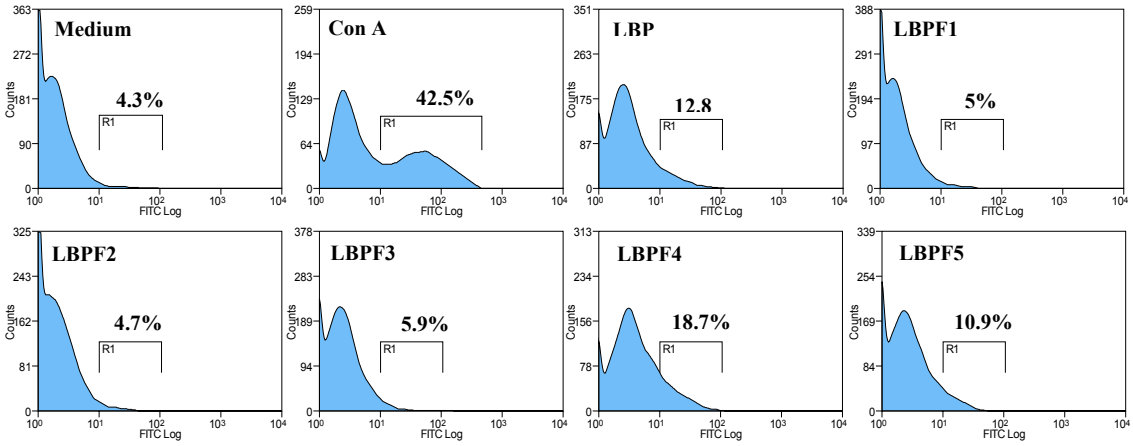

Figure 11. Effects of LBP and LBPF1-5 on CD25 expression.

Mouse splenocytes were stimulated with LBP (100 μg/ml) or LBPF1-5 (100 μg/ml) for 48 h. Con A (3 μg/ml) was positive control. CD25 expression was determined by flow cytometry. Results are representative of three independent experiments.

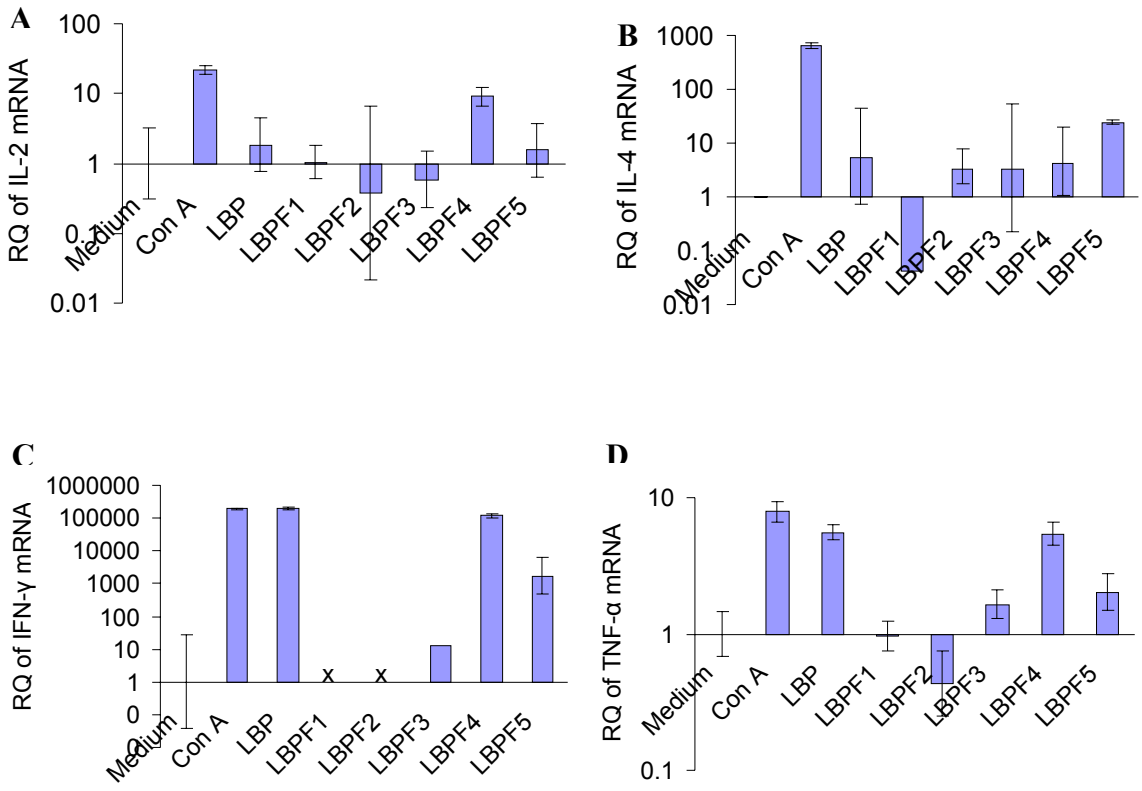

Figure 12. Relative quantification of cytokine mRNA upon treatment of LBP or LBPF1-5.

2×10^6 splenocytes were stimulated with 100 μg/ml LBP, LBPF1, LBPF2, LBPF3, LBPF4, or LBPF5 for 48 h. Con A (3 μg/ml) was positive control. RNA was extracted and reverse transcribed to cDNA. Gene expression was measured by real-time RT-PCR. The relative mRNA expression was normalized to the endogenous control gene β-actin and calibrated by untreated cells. Results are represented as 95% confidence interval (CI) of triplicate as determined by the ABI RQ software. **A.** IL-2; **B.** IL-4; **C.** IFN-γ; **D.** TNF-α.

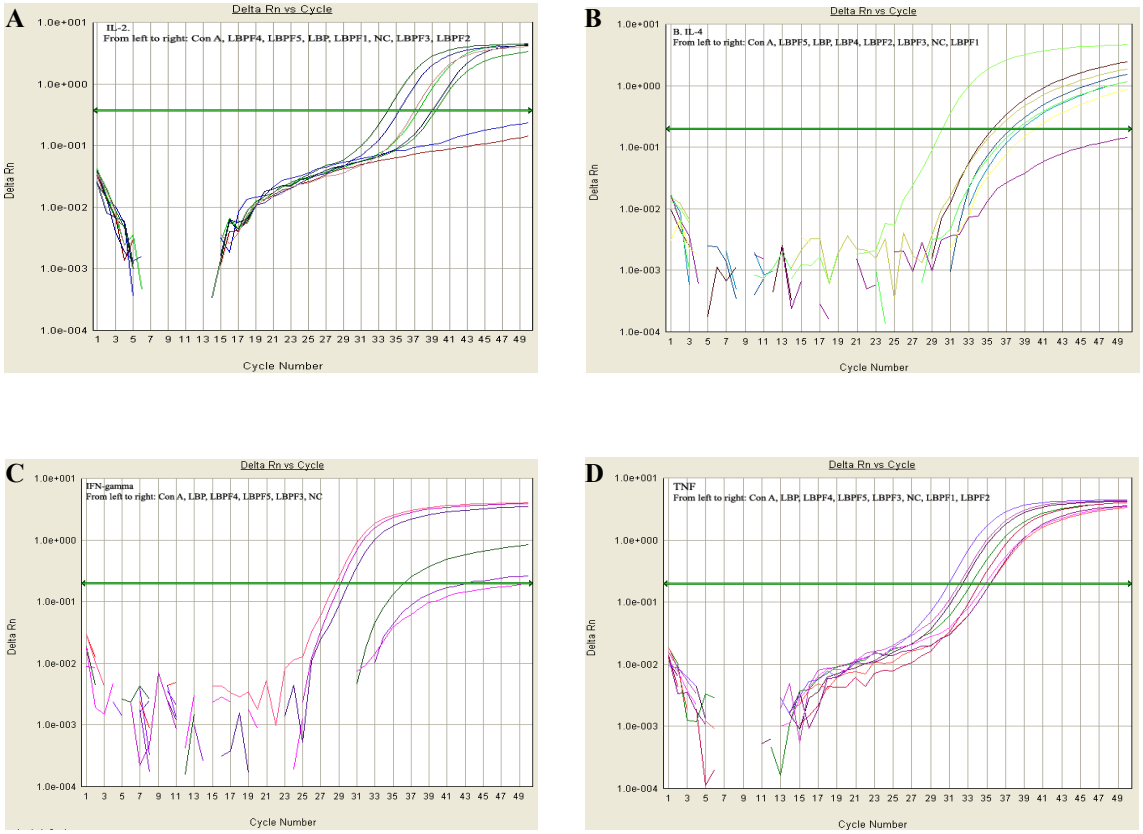

Figure 13. Amplification plot of cytokine mRNA by real-time PCR.

A. IL-2; **B.** IL-4; **C.** IFN-γ; **D.** TNF-α.

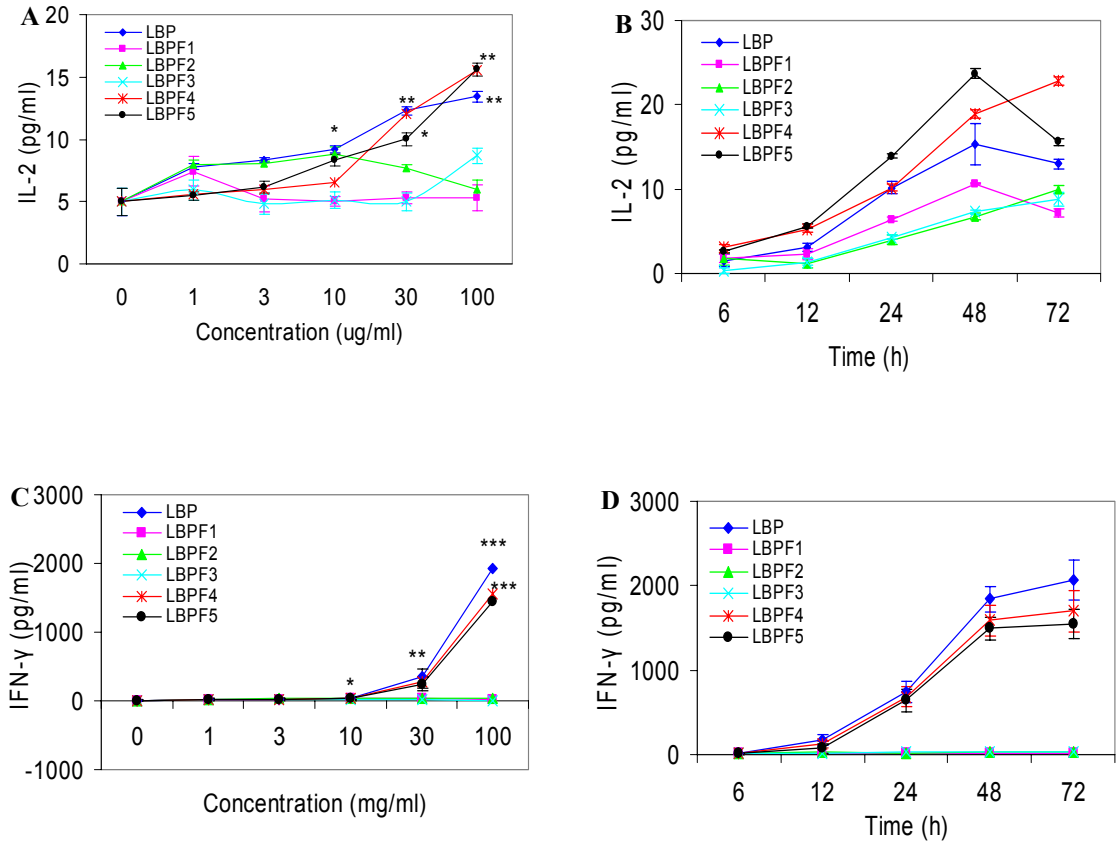

Figure 14. Dose-dependence and kinetics of cytokine production upon treatment with LBP or LBPF1-5.

2×10^6 mouse splenocytes were stimulated with 1, 3, 10, 30, 100 μg/ml of LBP, or LBPF1-5 for 48 h in dose response assays, or with 100 μg/ml of LBP, LBPF1-5 for 6, 12, 24, 48, 72 h in kinetic response assays. Cytokines secreted into the culture supernatant were measured by ELISA. Values are represented as mean ± SD of four replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to untreated cells. **A.** Dose-dependence of IL-2 production; **B.** Kinetics of IL-2 production; **C.** Dose-dependence of IFN-γ production; **D.** Kinetics of IFN-γ production.

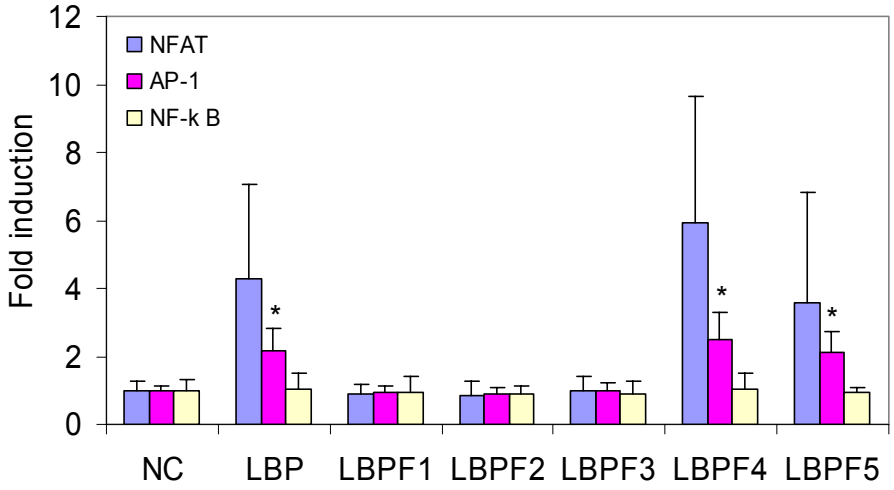

Figure 15. Activation of transcription factors by LBP and LBPF1-5.

Jurkat T cells were transiently transfected with luciferase reportor plasmid NFAT-luc, AP-1-luc, or NF-κB-luc for 48 h. Transfected cells were stimulated with 100 μg/ml of LBP or LBPF1-5 for 6 h. Luciferase activity was measured by luciferase assay. Results are expressed as fold induction of relative light units (RLU) of treated cells over that of untreated cells (NC). Values are mean ± SD of four replicates. * $P < 0.05$, compared to untreated cells.

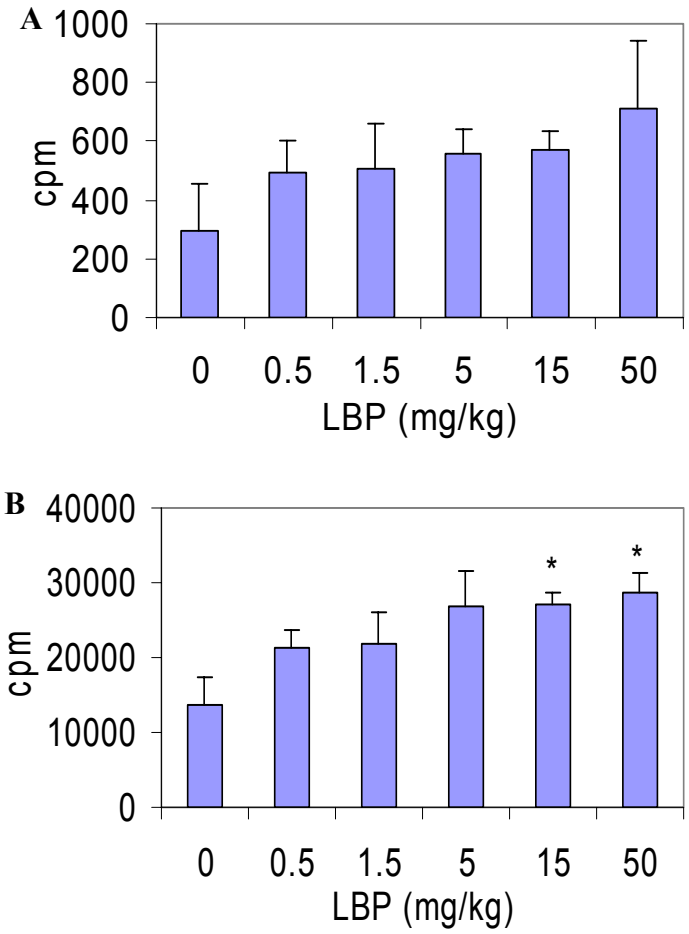

Figure 16. LBP activates T lymphocytes *in vivo* by i.p. injection.

BALB/c mice were administered i.p.with LBP at 0.5, 1.5, 5, 15, or 50 mg/kg daily for 7 days. Normal saline was used as negative control (0). Mice were sacrificed on day 8. Splenocytes were harvested and cultured in 5% CO_2 at 37ºC for 72 h in the presence or absence of 1 μg/ml of Con A. Cell proliferation was determined by ^{3}H-thymidine incorporation assay. Values are mean ± SD of 4 mice. * $P < 0.05$, compared to normal saline. **A.** Without Con A; **B.** With Con A.

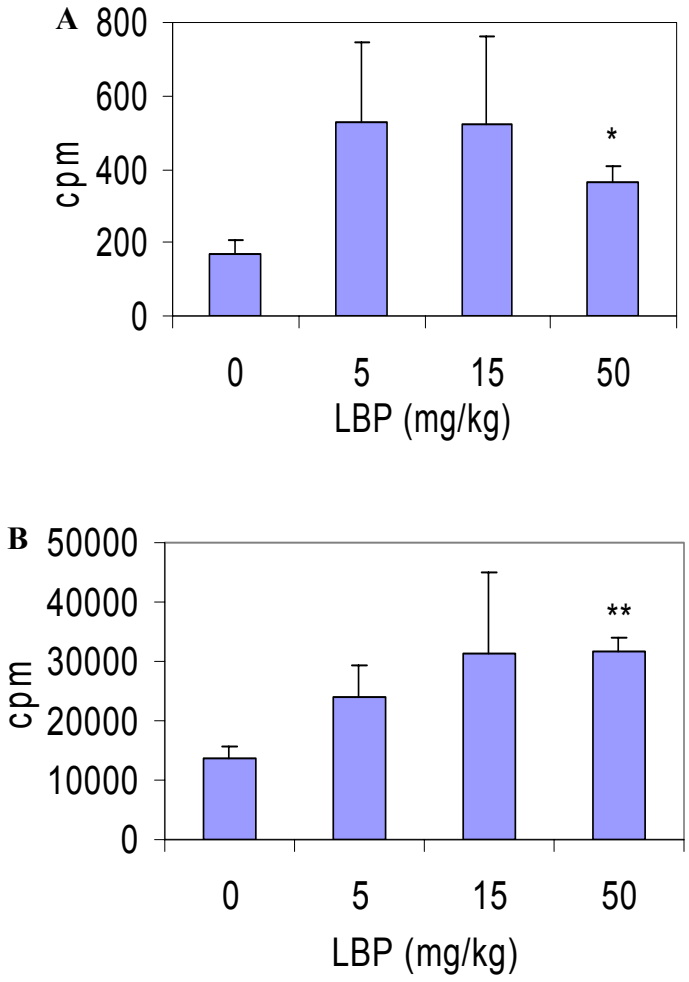

Figure 17. LBP activates T lymphocytes *in vivo* by oral gavage.

BALB/c mice were administered p.o. with LBP at 5, 15, or 50 mg/kg daily for 7 days. Normal saline was used as negative control (0). Mice were sacrificed on day 8. Splenocytes were harvested and cultured in 5% CO_2 at 37ºC for 72 h in the presence or absence of 1 μg/ml of Con A. Cell proliferation was determined by ^{3}H-thymidine incorporation assay. Values are mean ± SD of 4 mice. * $P < 0.05$, ** $P < 0.01$, compared to normal saline. **A.** Without Con A; **B.** With Con A.

3.4 Activation of Macrophages by LBP

3.4.1 Aim of Study

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

Previous studies have demonstrated that LBP targets C3b and Fc receptors on peritoneal macrophages (Li et al, 1990), whereas more insightful evidences to show its effects on innate immunity have not been reported yet. In this section, we investigated whether LBP and its five fractions LBPF1-5 could upregulate the expression of costimulatory molecules on macrophages, induce cytokine production, and enhance the capacities in endocytosis, and phagocytosis.

3.4.2 Results

3.4.2.1 Effects of LBP on the Expressions of CD40, CD80, CD86, and MHC Class II Molecules on Macrophages.

Macrophages are types of antigen presenting cells which enhance their antigen presenting ability by up-regulating the expressions of MHC class II molecules and co-stimulators such as CD40, CD80, and CD86 (Hancock et al, 1996). To investigate whether LBP upregulates the expressions of such molecules on macrophages, we injected LBP i.p. to mice and harvested the peritoneal macrophages 7 days later. Expressions of CD40, CD80, CD86, and MHC Class II molecules were analyzed by flow cytometry. The result showed that LBP remarkably upregulated the expressions of CD40, CD80, CD86, and MHC class II molecules on peritoneal macrophages by i.p. injection. As shown in Figure 18, 3.9% of peritoneal cells in the mice injected with saline expressed CD40. In contrast, the expression was increased to 74.1% in the mice with LBP. Similarly, the expressions of CD80, CD86, and MHC class II after LBP treatment were upregulated from 18.3%, 14.5%, and 64.5% to 94.1%, 90.4%, and 96.9%, respectively.

3.4.2.2 Effects of LBP and LBPF1-5 on the Activation of Transcription Factors

Transcription factors are critical for macrophage activation. Transcriptional signaling is necessary for inducible expressions of a suite of genes required to initiate inflammation and eliminate pathogens (Guha and Mackman, 2001). For example, NF-κB and AP-1 are necessary for inflammatory cytokines TNF and IL-1β mRNA transcription. Therefore, we studied the effects of LBP on transcription factors NFAT, AP-1, and NF-κB on macrophages. We transfected RAW264.7 cells with the three corresponding plasmids

containing the luciferase reporter gene, stimulated the transfected cells with LBP and LBPF1-5, and tested the luciferase activities by luciferase assay. As shown in Figure 19, LBP and LBP1-5 significantly activated AP-1 and NF-κB ($P < 0.01$ to $P < 0.05$, compared to those of untreated cells). The luciferase activities were increased 1.5- to 2.5-folds. In contrast, NFAT was almost completely suppressed by LBP, LBPF1-5, and LPS (0.01-0.03 compared to medium control).

3.4.2.3 LBP and LBPF1-5 Enhance TNF-α, IL-1-β, and IL-12p40 mRNA Expression

As activation of transcription factors initiate gene transcription, we next investigated whether LBP induces TNF-α, IL-1-β, and IL-12p40 mRNA expression. TNF-α and IL-1-β are inflammatory cytokines which can attract neutrophils and monocytes to sites of infection. Production of IL-12 can enhance macrophage antigen presentation. The result shows that fter RAW264.7 cells were treated with LBP or LBPF1-5 for 48 h, TNF-α, IL-1β, and IL-12p40 mRNA expressions were strikingly increased, of which TNF-α mRNA was increased 1.5- to 2.7-fold (Figure 20A), IL-1β mRNA was increased 1600- to 4700-fold (Figure 20B), and IL-12p40 was increased 1360000- to 6810000-fold (Figure 20C). The extremely high levels of relative expression of IL-12p40 and IL-1β mRNA after LBP stimulation were because of the very low level of those untreated cells', whose CT values was 50 and 35, respectively (data not shown).

3.4.2.4 LBP and LBPF1-5 Enhance TNF-α Production

We then conducted a dose-response assay to test the effects of LBP and LBPF1-5 on cytokine production, as measured by ELISA. RAW264.7 cells were stimulated with LBP

or LBPF1-5 at various concentrations, including 1, 10, 100, and 500 μg/ml for 48 h. We found that LBP and LBPF-5 enhanced TNF-α production by RAW264.7 cells in a dose-dependent manner (Figure 21). The effects were visible at 1 μg/ml, and 100 μg/ml of LBP and LBP1-5 induced 7.6 to 19.3 ng/ml of TNF-α by RAW264.7 cells. In contrast, RAW264.7 cells did not produce any IL-1β and IL-12p40 detectable by ELISA after LBP and LBPF1-5 stimulation.

3.4.2.5 LBP Enhances Endocytosis and Phagocytosis *in vivo*

Endocytosis and phagocytosis are the main functions of macrophages. Macrophages engulf large molecules by endocytosis while destroying microbes by phagocytosis. We then set up an animal model to investigate whether LBP enhanced such macrophage functions. Mice were injected with LBP (50 mg/kg, i.p., × 7 d) and 30 min prior to sacrifice were injected with FITC-dextran for endocytosis assay or FITC-*Staphylococcus aureus* for phagocytosis assay. The result showed that the FITC-dextran endocytosed by the peritoneal macrophages harvested from the LBP-treated mice (Figure 22B) was much more intensive than that endocytosed by the peritoneal macrophages from the saline control mice (Figure 22A). Peritoneal macrophages of LBP-treated mice became bigger, irregular, spreading, and strongly adherent, whereas peritoneal macrophages of saline control mice were round, small, and loosely adherent. Similarly, phagocytosis assay showed that fluorescence in the peritoneal macrophages harvested from LBP-treated mice (Figure 22D) was much brighter and more intensive than that in the peritoneal macrophages from saline control mice (Figure 22C), indicating much more bacteria were phagocytozed by macrophages after LBP administration. After phagocytozing bacteria,

macrophage subsequently underwent apoptosis characterized as cell shrinking, blebbing, and fragmentation (Figures 22C and 22D).

3.4.3 Discussion

In this section, we demonstrated that LBP is capable of activating macrophages. LBP enhanced the expressions of CD40, CD80, CD86, and MHC class II molecules on mouse peritoneal macrophages. LBP and LBPF1-5 activated transcription factors NF-κB and AP-1, induced TNF-α, IL-1β, and IL-12p40 mRNA expression, and enhanced TNF-α production by RAW264.7 macrophage cells. Peritoneal macrophages of mice treated with LBP were very active in endocytosis and phagocytosis.

LBP markedly upregulated the expressions of CD40, CD80, CD86, and MHC class II molecules on primary macrophages. For an APC, upregulation of the expression of such molecules means enhanced capacity in antigen presentation. Therefore, our results indicate that LBP enhances the capacity of primary macrophages to present antigens to T cells and subsequently augments immune response.

LBP and LBPF1-5 manifestly activated transcription factors AP-1 and NF-κB in the RAW264.7 macrophage cell line. In contrast, NFAT was completely inhibited. The reason that NFAT induction is supressed after LBP treatment is not known, probably because it mainly participates in the regulation of T-cell function and development (Macian, 2005). Unlike NFAT, NF-κB plays crucial roles in macrophage activation. NF-κB induction is essential for the expression of a wide variety of immune-response genes,

including pro-inflammatory cytokines, chemokines, and adhesion molecules (Beinke and Ley, 2004). Stimulation of the respective receptors of the pro-inflammatory cytokines TNF-α and IL-1, which in turn strongly activates NF-κB, amplifying and extending the duration of the innate immune response (O'Neill and Dinarello, 2000; Wallach et al, 1999). AP-1 proteins have been implicated in invasive cell growth and matrix metalloprotease production and in cell line models, have been suggested to mediate induction of inflammatory genes such as TNF-α. But evidence for an inflammatory role of AP-1 proteins in the responses of primary cells to physiological or microbial factors is sparse (Hu et al, 2007). The upstream of NF-κB and AP-1 signaling pathway is initiated by binding ligands to respective TLRs and involved in activation of some intracellular adapter proteins such as MyD88. The success of TLRs functioning as major sensors of invading pathogens is attributed to their ability to identify a range of conserved microbial motifs termed 'pathogen-associated molecular patterns' (PAMPs) (McCoy and O'Neill, 2008). To date, 11 human TLRs and 13 mouse TLRs have been identified. TLR1, -2, and -6 recognize microbial lipopeptides, TLR3 recognizes double-stranded (ds) RNA produced from many viruses during replication, while TLR4 recognizes LPS from Gram-negative bacteria and TLR9 recognizes bacterial and viral CpG DNA motifs (Miggin and O'Neill, 2006). It can be hypothesized that LBP activates NF-κB and AP-1 signaling pathway by binding to TLRs; and it will be interesting to identify the LBP corresponding receptors in the future.

As expected, activation of NF-κB and AP-1 induced TNF-α, IL-1β, and IL-12p40 mRNA expression. However, only TNF-α production was detected by ELISA, and RAW264.7

cells constitutively secreted substantial levels of TNF-α. Lack of IL-1β and IL-12p40 production could be because their absolute mRNA expression was low. As the principal physiological function of TNF-α is to stimulate the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes, LBP enhancement of TNF-α production is beneficial to host defense. Activated macrophages also convert molecular oxygen into ROIs and produce NO to destroy microbes (Aderrm and Underhill, 1999; Taylor et al, 2005). It is intriguing to investigate whether LBP enhances the activities of phagocyte oxidase and nitric oxide synthase and subsequently upregulates ROIs and NO production.

Macrophages recognize pathogens through PRRs, which include scavenger receptors (SRs), mannose receptor, TLRs, Dectin-1, and complement receptor type 3 (CR3) (Underhill and Ozinsky, 2002). SRs are originally defined as molecules that bind and internalize modified lipoproteins such as oxidized or acetylated LDL particles that can no longer interact with the conventional LDL receptor (Plüddemann et al, 2007). The mannose receptor is a macrophage lectin that recognizes a range of carbohydrates with mannose and fucose residues present on the surface and cell walls of micro-organisms (Apostolopoulos and McKenzie, 2001). Dectin-1 is the beta-glucan receptor involved in innate immune responses to fungal pathogens (Brown, 2006; Dennehy and Brown, 2007). Macrophage activation by plant polysaccharides is thought to be mediated primarily through the recognition of polysaccharide polymers by specific receptors, which are known to be pattern recognition molecules like those for microbes, such as SRs, mannose receptor, TLRs, and Dectin-1 (Schepetkin and Quinn, 2006). Our results clearly

demonstrated that i.p. injection of LBP extraordinarily enhanced the endocytosis and phagocytosis of primary macrophages, indicating that those macrophages have been activated. Besides TLRs as discussed above, the receptors for LBP may include mannose receptor and Dectin-1, as LBP is composed of mannose, galactose, rhamnose, xylose, arabinose, and glucose and is a glycan that possesses a backbone consisting of (1→6)-β-galactosyl residues (Huang et al, 1998; Peng and Tian, 2001). However, clarification of the structure-function relationship of polysaccharide BRMs is still challenging due to the complexity of monosaccharide compositions and glycosidic linkages (Leung et al, 2006).

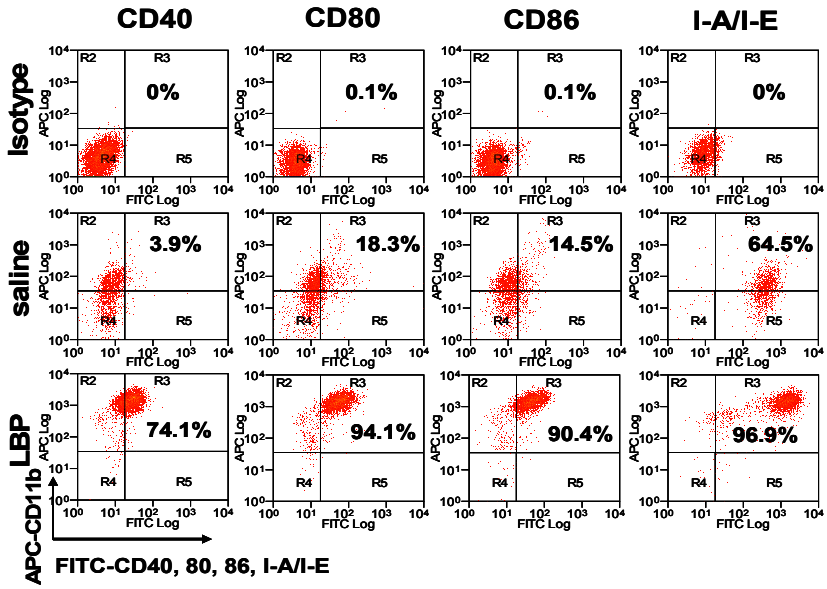

Figure 18. Effects of LBP on the expressions of CD40, CD80, CD86, and MHC class II molecules on macrophages. BALB/c mice were administered with LBP (50 mg/kg, i.p., × 7 d). Peritoneal macrophages were harvested, stained with APC-conjugated anti-CD11b and FITC-conjugated anti-CD40, CD80, CD86, or I-A/I-E, and analyzed by flow cytometry. Results are representative of three independent experiments.

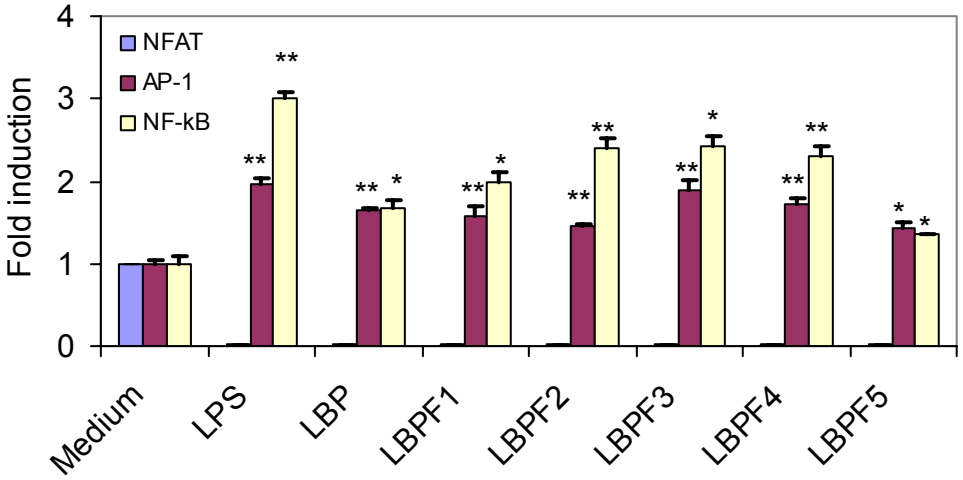

Figure 19. Effects of LBP and LBPF1-5 on the activation of transcription factors. RAW264.7 cells were transiently transfected with luciferase reporter plasmid NFAT-luc, AP-1-luc, or NF-κB-luc for 48 h. Transfected cells were stimulated with 100 μg/ml of LBP or LBPF1-5 for 6 h. Luciferase activity was measured by luciferase assay. Results are expressed as fold induction of relative light units (RLU) of treated cells over that of untreated cells. Values are mean ± SD of triplicate. * $P < 0.05$, ** $P < 0.01$, compared to untreated cells.

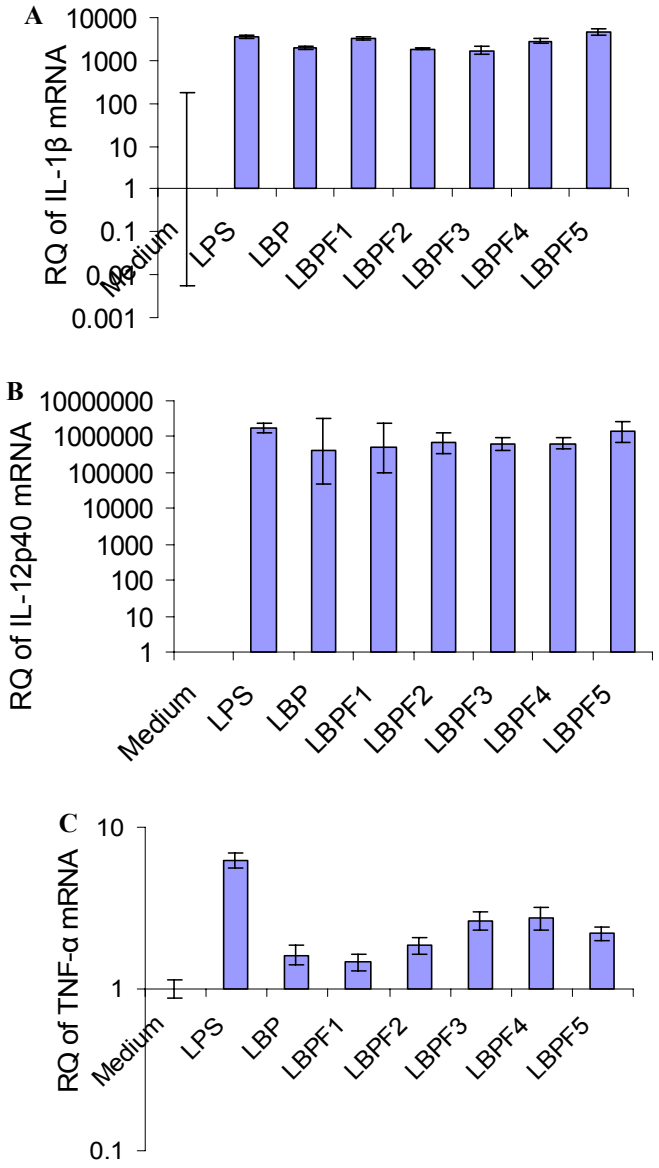

Figure 20. LBP and LBPF1-5 enhance TNF-α, IL-1β, IL-12p40 mRNA expressions. 5×10^5 RAW264.7 cells were stimulated with 100 μg/ml of LBP, LBPF1-5, or 1 μg/ml of LPS for 48 h. Cytokine mRNA expression was quantified by RT-PCR relative to that of untreated cells using β-actin gene as endogenous control gene. Results are represented as 95% confidence interval of triplicate, compared to medium control (untreated cells). **A.** TNF-α; **B.** IL-1β; **C.** IL-12p40.

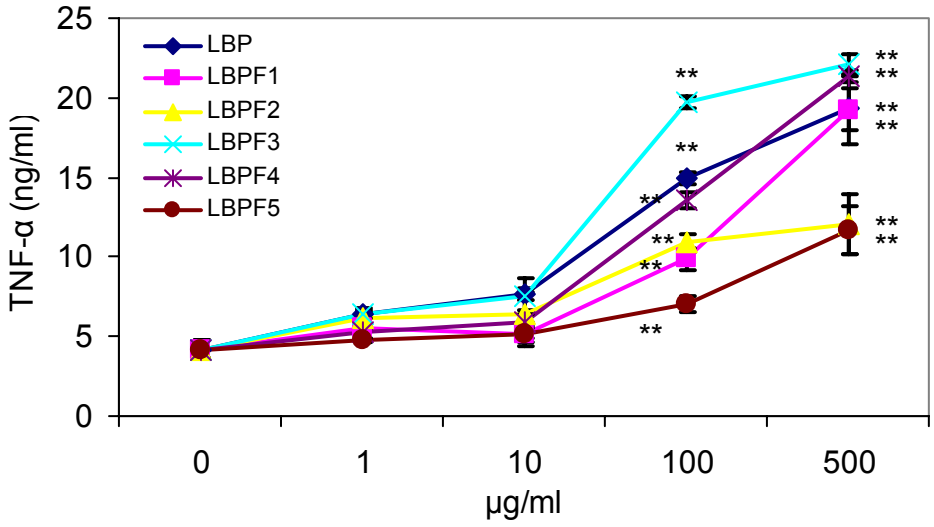

Figure 21. LBP and LBPF1-5 enhance TNF-α production.

5×10^5 RAW264.7 cells were stimulated with 100 μg/ml of LBP, or LBPF1-5, or 1 μg/ml of LPS for 48 h. TNF-α concentration in the supernatant was measured by ELISA. Values are mean ± SD of triplicate. ** $P < 0.01$, compared to medium control (untreated cells).

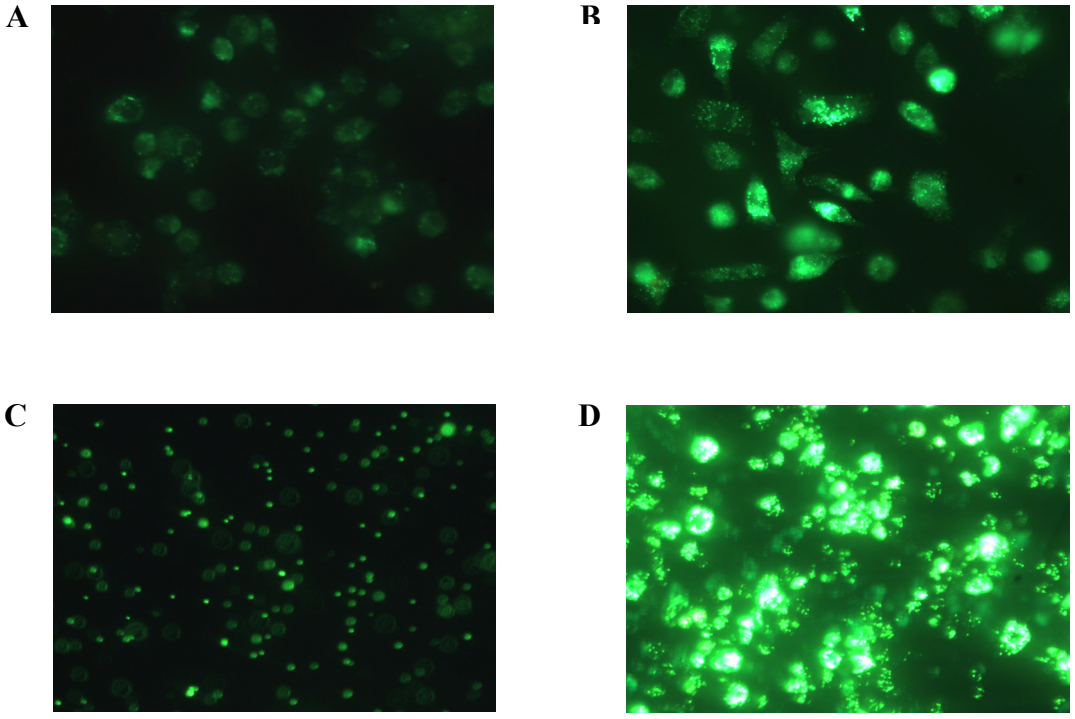

Figure 22. LBP enhances endocytosis and phagocytosis *in vivo*.

BALB/c mice were injected with LBP (50 mg/kg, i.p., × 7 d). Normal saline was negative control. Mice were sacrificed on day 8. 30 min prior to sacrifice, mice were i.p. injected with 1 ml of 1 mg/ml FITC-dextran (A, B) or 1 ml of 1 mg/ml FITC-*Staphylococcus aureus* (C, D). Peritoneal cells were harvested, washed, and incubated in 24-well tissue culture plates with a microscope glass cover slip in each well for 6 h. The cover slips with adherent cells were observed and photographed under a fluorescent microscope (Olympus BX-60, magnification × 40). **A.** Saline + dextran; **B.** LBP + dextran; **C.** Saline + *Staphylococcus aureus*; **D.** LBP + *Staphylococcus aureus*.

3.5 LBP Is a Novel Stimulus of Dendritic Cell Immunogenicity

3.5.1 Aim of Study

DCs represent a heterogeneous population of professional antigen-presenting cells that initiate primary immune response (Banchereau et al, 2000). DCs take up antigen in peripheral tissues and migrate to secondary lymphoid organs where they become mature and competent to present antigens to T lymphocytes, thus initiating antigen-specific immune responses, or immunological tolerance (Guermonprez et al, 2002). DC immunogenicity correlates with its functionally mature state, which is characterized by high levels of expression of MHC and T-cell costimulatory molecules, acute decrease 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) (Sallusto and Lanzavecchia, 1994; Winzler et al, 1997; Roake et al, 1995). While these mediators are potent stimuli for DC maturation, they are toxic and have limited applications. In this regard, non-toxic vehicles that are able to induce DC maturation and immunogenetics are useful.

While the immunostimulating activities of LBP have been generally attributed to its induction of lymphocyte proliferation (Du et al, 2004; Gan et al, 2004) and cytokine production (Gan et al, 2003; Du et al, 2004), little is known about its effects on dendritic cells (DCs). In this section, we investigated whether LBP could induce DC maturation and enhance DC immunogenicity. We studied the effects of LBP and its fractions LBPF1-5 on the expressions of CD40, CD80, CD86, and MHC class II molecules, DC

allostimulatory activity, DC productions of IL-12p40 and p70, and DC presentation of antigen to Th1 and Th2 cells.

3.5.2 Results

3.5.2.1 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 the BMDCs with LBP or its five fractions LBPF1-5. The cells were analyzed for the expression of CD40, CD80, CD86, and MHC class II molecules. As shown in Figure 23A, after treatment with LBP or LBPF1-5 for 24 h, the expression of all these molecules was upregulated. The most pronounced upregulation was seen for CD40 and CD86, with more than 10% increase on average after stimulation. Immature DCs express high level of MHC class II (30.3%) and this was further increased by LBP and its fractions. None of the LBP fractions showed greater activities than crude LBP. These results indicate that LBP promotes DCs to the phenotypically mature stage *in vitro*. We next tested whether these results were applicable *in vivo*. LBP was s.c., i.p., or p.o. given to BALB/c mice, and after 24 h, splenic DCs were isolated. As shown in Figure 23B, the expression of CD40, CD80, CD86, and MHC class II on splenic DCs was increased in LBP-treated mice, as compared to that of naïve mice. LBP administration p.o. seemed to be the best route, followed by s.c. and i.p. administration. These results demonstrate that LBP is capable of inducing DC maturation *in vivo*.

3.5.2.2 LBP Strengthens DC Allostimulatory Activity

One of the characteristics of DCs is that they are potent stimulators of allogeneic T cells in mixed leukocytes reaction (MLR) (Steinman and Witmer, 1978). We next performed the LBP-activated DCs MLR using spleen T cells. We used 5×10^4 BALB/c mouse DCs to stimulate 2×10^5 C57BL/6 mouse splenic T cells. Fresh bone marrow cells were used as control which induced a 10-fold increase in allogeneic T cell proliferation. Immature DCs induced a 26-fold increase (Figure 24). After activation with LBP or LBPF1-5, DCs induced allogeneic T cell proliferation by 32- to 45-fold, which was significantly higher than that increased by immature DCs ($P < 0.01$ to $P < 0.05$). This was consistent with the increased CD40, CD80, CD86, and MHC class II expressions, suggesting that mature DCs induce stronger mixed leukocytes reaction than immature DCs.

3.5.2.3 LBP Downregulates DC Endocytosis

Efficient antigen uptake is a specific attribute of immature DCs, which is reduced upon DC maturation (Sallusto and Lanzavecchia, 1994). In this experiment, LBP- and LBPF1-5-activated DCs were incubated with soluble FITC-dextran for 1 h at 37ºC. As controls, immature DCs were used. LBP- and LBPF1-5-treated DCs also showed decrease in soluble dextran uptake (Figure 25). Parallel experiments were also performed at 4ºC to determine non-specific FITC-dextran binding to the cells, which detected a small percentage of DCs (5-8%) engulfing FITC-dextran at 4ºC (Figure 25).

3.5.2.4 LBP Induces IL-12 Production from DCs

IL-12 is a disulfide-linked heterodimer of p35 and p40 subunits, and the bioactive IL-12 has an apparent molecular mass of 70 kDa (p70) (Kobayashi et al, 1989). 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 heterodimeric IL-12 (Kubin et al, 1994; Macatonia et al, 1995). To examine whether LBP induces IL-12 from DCs, BMDCs were stimulated with LBP or LBPF1-5 for 48 h. IL-12p40 mRNA expression was determined by quantitative RT-PCR. The result shows that LBP induced IL-12p40 mRNA expression in BMDCs in a dose-dependent manner (Figure 26A). At 100 μg/ml, LBP increased p40 mRNA expression 2.6-fold. At 1 mg/ml, it increased 10-fold. Consistently with the RT-PCR results, LBP also induced IL-12p40 and p70 secretion from BMDCs in a dose-dependent manner (Figures 27A and 27B, respectively). At 100 μg/ml, LBP stimulated BMDCs to produce 8.6 ng/ml of IL-12p40 and 355.6 pg/ml of IL-12p70. This was highly significant compared with immature BMDCs ($P < 0.01$). All five LBP fractions induced these cytokines. At 100 μg/ml, LBPF1-5 significantly enhanced IL-12p40 mRNA expression, and also IL-12p40 and p70 production ($P < 0.001$ to $P < 0.05$, Figures 26B, 27C, 27D, respectively). LBPF3 was most potent whereas LBPF5 was the least potent fraction.

3.5.2.5 LBP Promotes Th1 and Th2 Response *in vitro*

To investigate whether LBP-activated DCs exhibit augmented capacity in antigen presentation, BMDCs were pulsed with OVA antigen and then activated with LBP and LBPF1-5. These cells were then co-cultured with $CD3^+$ splenic T cells and examined for

Th1 and Th2 stimulation as measured by the production of IFN-γ and IL-4, respectively, using ELISPOT assay. As shown in Figure 28, more IFN-γ- and IL-4-producing cells were detected in wells where T cells were co-cultured with LBP- or LBPF1-5-stimulated DCs compared with wells in which T cells were co-cultured with immature DCs. However, there was no difference in the number of IL-2-producing cells among these wells. IFN-γ and IL-4 are characteristic of Th1 and Th2 cells, respectively. Therefore, DCs activation by LBP *in vitro* augmented T cell differentiation to both Th1 and Th2 cells.

3.5.2.6 DCs Activated by LBP *in vitro* Enhance Th1 and Th2 Response *in vivo*

We next used an *ex vivo* approach to investigate the immunogenicity of the LBP stimulated-DCs. BMDCs were pulsed with OVA and 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 by ELISPOT assay. As shown in Figure 29, 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 normal saline, OVA, DCs, LBP-stimulated DCs, or OVA-pulsed DCs. Splenocytes from mice injected with saline and OVA did not produce IFN-γ and IL-4 spots (less than 10). 2×10^5 splenocytes from mice injected with DCs alone, LBP-stimulated DCs, or OVA-pulsed DCs induced 250 to 400 IFN-γ and IL-4 spots, which were increased to about 500 in the mice injected with OVA-pulsed and LBP-stimulated DCs.

3.5.2.7 LBP Primes Th1 Response *in vivo*

Since 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, we administered LBP s.c., i.p., or p.o. to mice. After 24 h, splenic DCs were purified and co-cultured with primary $CD3^+$ T cells from naïve mice. As shown in Figures 30 A and 30B, primary T cells from naïve mice co-cultured with DCs from mice administered with LBP produced significantly more IFN-γ spots than those co-cultured with DCs from naïve mice ($P < 0.001$). Few IL-4-producing cells were detected. Second, we injected OVA s.c. into mice for three times at one week intervals and gave the mice LBP daily via s.c., i.p., or p.o. routes. Splenic cells were examined by ELISPOT assay. The result shows that splenocytes from mice administered with LBP produced more IFN-γ spots than those from the OVA control. 2×10^5 splenocytes from mice injected with OVA produced about 120 IFN-γ spots, which were increased to about 170 in the mice administered with OVA plus LBP. Again, few IL-4-producing cells were detected (Figures 30C and 30D).

3.5.3 Discussion

DCs are heterogeneous and can be mainly categorized into conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (Villadangos and Schnorrer, 2007). The cDCs can be further divided into migratory cDCs and lymphoid-tissue-resident cDCs (Shortman and Naik, 2007). These DCs are usually in the steady state (Shortman and Naik, 2007). They enter a developmental program called maturation when receiving signals from pathogens (Guermonprez et al, 2002). Mature DCs undergo phenotypic change, expressing high cell-surface levels of MHC molecules, CD40, CD80, CD83, and CD86 (Wilson and

Villadangos, 2005). In this study, we demonstrated that LBP and its five homogeneous fractions LBPF1-5 upregulated the expressions 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*. S.c., i.p. or p.o. administration of LBP induced splenic DC phenotypic maturation. DC pheonotypic maturation is related to its immunogenicity. Priming of T-cells needs peptide-MHC complex to bind TCR and CD80 and CD86 to bind CD28 on T cells (Kapsenberg, 2003). CD40 ligation is necessary and sufficient to drive the maturation of DCs and confer on them the ability to prime $CD8^+$ T cells (Guermonprez, 2002). LBP substantially upregulated CD40 expression on DCs, indicating it may mimic the function of CD40L to prime cytotoxic T lymphocytes mediated by DCs.

However, while immunogenic DCs must be phenotypically mature, phenotypically mature DCs are not necessarily immunogenic. Instead, they may induce tolerance rather than immunity (Spörri and Reis e Sousa, 2005). Expressions of CD80 and CD86 by APCs are crucial for delivering the costimulatory signals through CD28 that promote T-cell survival, metabolic competence, cell-cycle progression and IL-2 mRNA stabilization. The same molecules engage cytotoxic T-lymphocyte antigen 4 (CTLA4), a negative regulator of T-cell activation (Reis e Sousa, 2006). In these cases, DCs are tolerogenic. Therefore, other experimental models need to be set up to evaluate whether LBP-induced DCs are immunogenic. It is noted that functionally mature DCs have an acute decrease in antigen uptake (Winzler et al, 1997). We found that BMDCs dramatically reduced dextran uptake after LBP and LBPF1-5 treatment, suggesting that LBP induces

functionally mature DCs. Receptor-mediated endocytosis allows the uptake of macromolecules through specialized regions of the plasma membrane termed coated pits. Immature DCs selectively express a large number of endocytic receptors, such as Fc, complement, heat shock proteins, and scavenger receptors, most of which decrease during DC maturation (Guermonprez, 2002). It is possible that LBP reduces DC endocytosis through downregulating the expressions of these antigen receptors.

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 (Gubler et al, 1991). Mature DCs are marked with high levels of IL-12 production (Langenkamp et al, 2000). We found that LBP and LBPF1-5 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 levels of p40 mRNA. It is noted that the concentration of IL-12p70 was about 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 in accordance with the previous findings (Segura et al, 2007). Cells that secrete bioactive IL-12p70 usually also secrete free p40 chains in approximately 10-fold excess, though secretion of significant amounts of p35 in the absence of p40 has not been found (Robertson and Ritz, 1996). IL-12 production is regulated through multiple signal transductions, such as NF-κB, p38MAPK, cAMP-modulating molecules, cell membrane ion channels and pumps,

NO, and receptors (Kang et al, 2005). LBP may target these signaling pathways on DCs to induce IL-12 production.

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 (Reis e Sousa, 2006). Our data indicate that LBP primes Th1-cell response *in vivo*. Primary T cells co-cultured with LBP-matured splenic DCs and the OVA-restimulated splenocytes harvested from mice administered with LBP and OVA differentiated to a large quantity of IFN-γ-producing cells. This is consistent with the IL-12 data. IL-12 is the most potent known stimulus that induces IFN-γ production by resting and activated T cells and NK cells, favoring the polarization of Th1 cells or CTL development (Trinchieri, 2003). However, our *in vitro* data showed that DCs treated with LBP enhanced both Th1 and Th2 responses. Primary T cells co-cultured with OVA-pulsed and LBP-treated BMDCs differentiated to both IL-4- and IFN-γ-producing cells. The results were further confirmed by s.c. injection of these BMDCs into mice, whose splenocytes also differentiated to both IL-4- and IFN-γ-producing cells after *in vitro* re-stimulation with OVA. One possibility is that BMDCs contain both $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets. It has been found that s.c. injection of antigen-loaded $CD8\alpha^+$ DCs primed Th1 responses, whereas $CD8\alpha^-$ DCs favored Th2 responses (Maldonado-López et al, 1999).

In conclusion, the data presented in this study show that LBP induced phenotypic and functional maturation of DCs with strong immunogenicity. Our study provides scientific

support and rationale for using LBP in various clinical conditions with poor immunity, especially for the design of DC-based vaccines in the future.

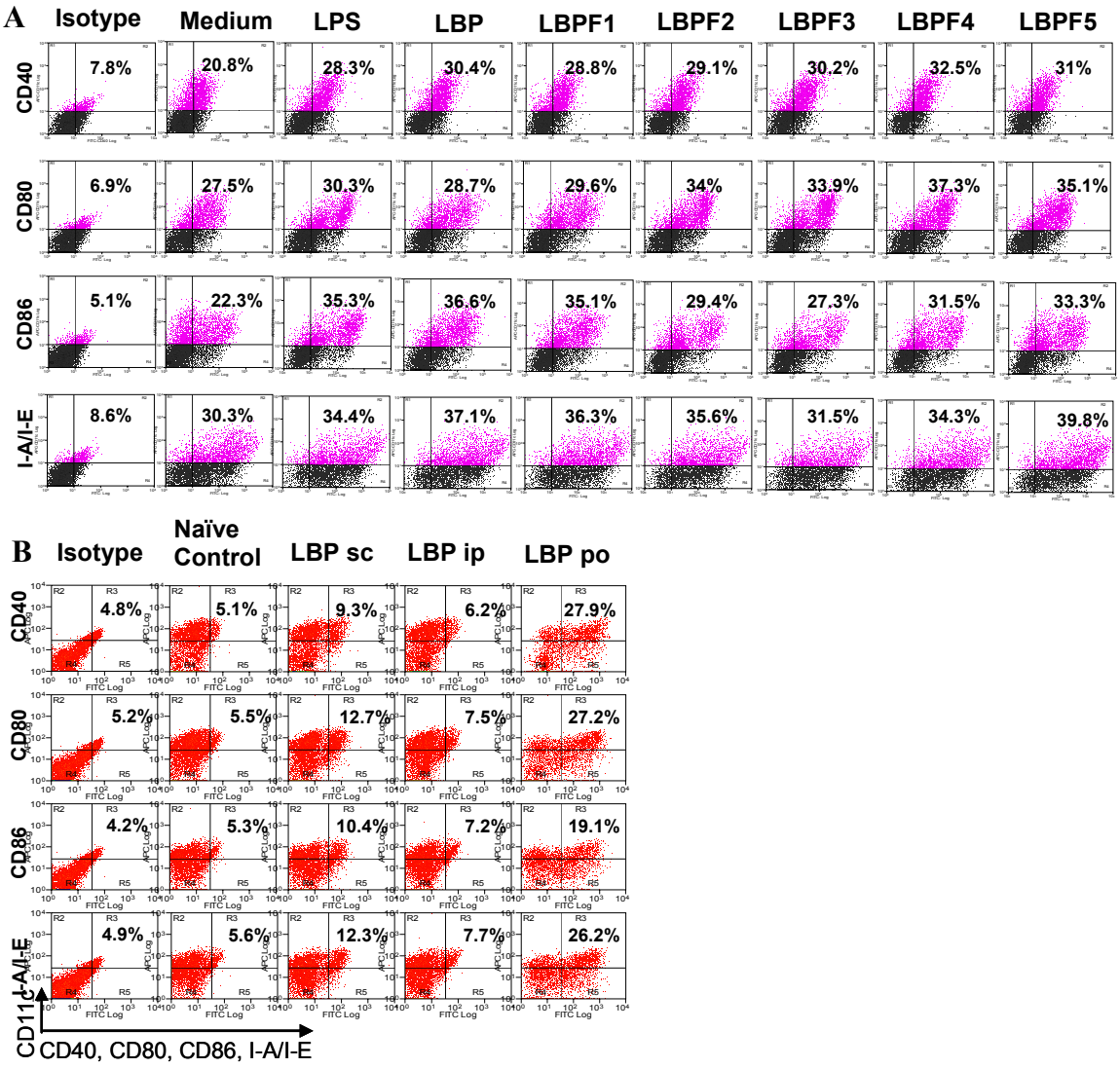

Figure 23. LBP induces DC maturation both *in vitro* and *in vivo*.

A. LBP induces DC maturation *in vitro.* Day 6 BMDCs were stimulated with 100 μg /ml LBP, LBPF1-5, or 0.1 μg /ml LPS for 24 h. **B.** LBP induces DC maturation *in vivo.* BALB/c mice were s.c., i.p., or p.o. administered with 20 mg/kg of LBP for 24 h. Splenic DCs were purified. BMDCs and splenic DCs were stained with APC-conjugated anti-CD11c and FITC-conjugated anti-CD40, CD80, CD86, or I-A/I-E, and analyzed by flow cytometry. Results are representative of three independent experiments.

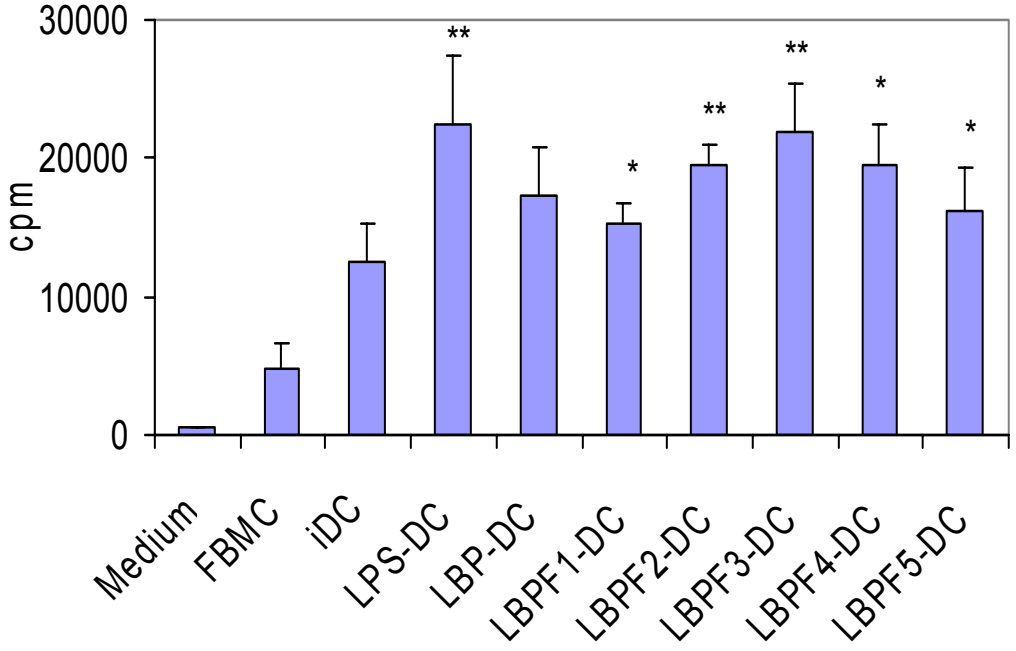

Figure 24. LBP strengthens DC allostimulatory activity.

Day 6 BMDCs from BALB/c mice were stimulated with 100 μg/ml of LBP, LBPF1-5, or 1 μg/ml of LPS for 24 h. Cells were harvested, washed, and 5×10^4 DCs were incubated with 2×10^5 T cells isolated from C57BL/6 mice for 72 h. T cell proliferation was measured by ^{3}H-thymidine incorporation assay. Values are mean ± SD of four replicates. FBMC: fresh bone marrow cells from BALB/c mice; iDC: immature DC. * $P < 0.05$; ** $P < 0.01$, compared to iDCs.

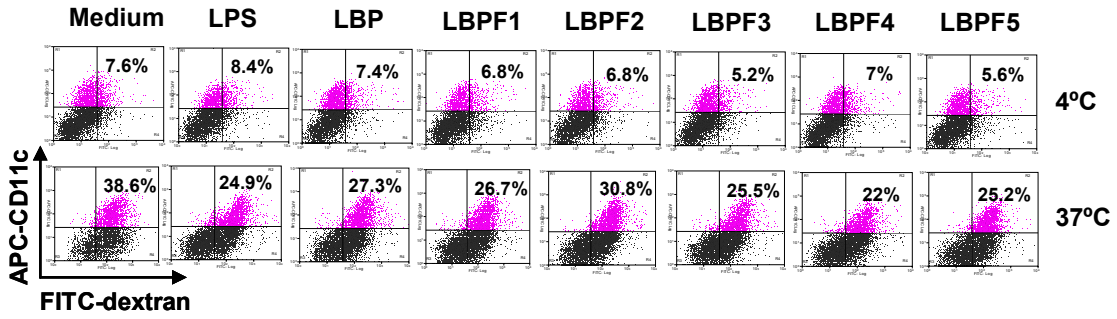

Figure 25. LBP reduces DC endocytosis.

Day 6 BMDCs were stimulated with 100 μg/ml of LBP, LBPF1-5, or 1 μg/ml of LPS for 24 h followed by incubation with 1 mg/ml of FITC-dextran at 4ºC or 37ºC for 1 h. Cells that expressed CD11c and endocytosed FITC-dextran were analyzed by flow cytometry. Results are representative of three independent experiments.

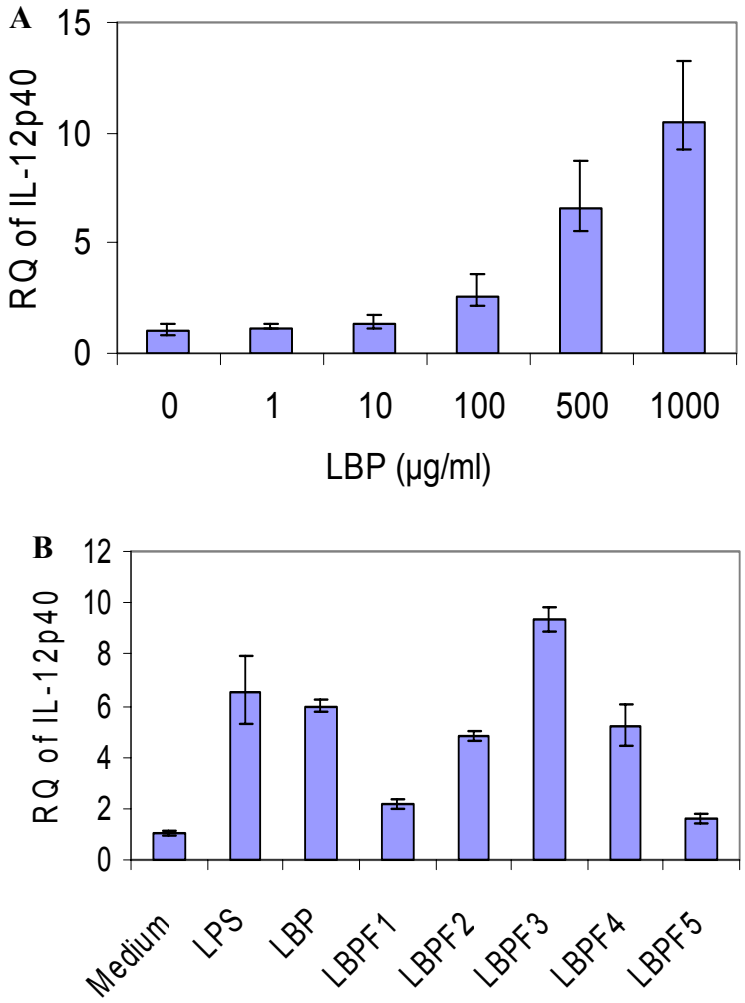

Figure 26. LBP enhances IL-12p40 mRNA expression by DCs.

A. Dose response of LBP on IL-12p40 mRNA expression. Day 6 BMDCs were stimulated with LBP at serial concentrations as indicated for 48 h. **B.** Effects of LBP and LBPF1-5 on IL-12p40 mRNA expression. Day 6 BMDCs were stimulated with 100 μg/ml of LBP or LBPF1-5, or 1 μg/ml of LPS for 48 h. IL-12p40 mRNA expression expression was quantified by real-time RT-PCR relative to iDCs (untreated DCs) using β-actin as endogenous control. Values are represented as 95% confidence level of triplicate.

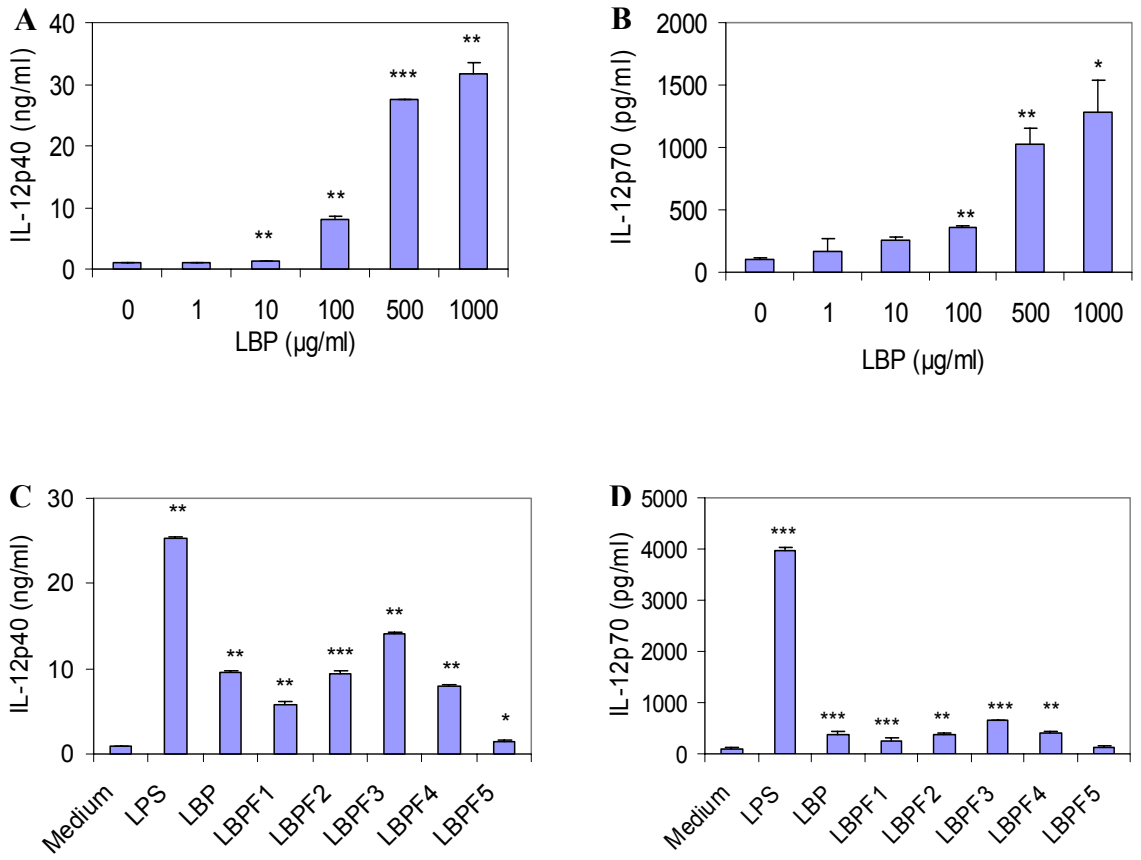

Figure 27. LBP enhances IL-12p40 and p70 productions by DCs.

Day 6 BMDCs were stimulated with LBP at serial concentrations as indicated, or 100 μg/ml of LBPF1-5, or 1 μg/ml of LPS for 48 h. **(A, B).** Dose response of LBP on IL-12p40 and p70 productions, respectively. **(C, D).** Effects of LBP and LBPF1-5 on IL-12p40 and p70 productions, respectively. IL-12p40 and p70 productions were measured by ELISA. Values are mean ± SD of triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to medium control (untreated DCs).

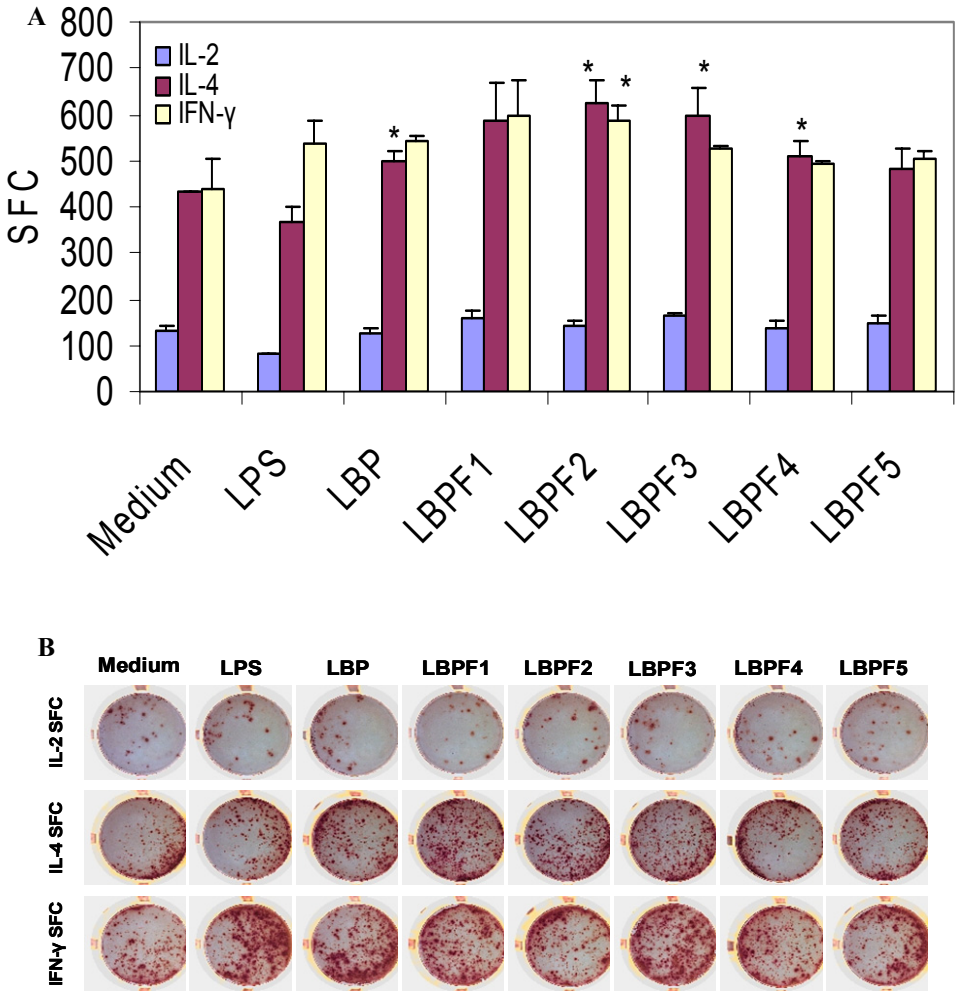

Figure 28. LBP and LBPF1-5 enhance Th1 and Th2 response *in vitro*.

Day 6 BMDCs were pulsed with 100 μg/ml of OVA for 2 h followed by further stimulation with 100 μg/ml of LBP, LBPF1-5, or 1 μg/ml of LPS for 48 h. Cells were harvested, washed, and 5×10^4 DCs were incubated with 2×10^5 T cells purified from BALB/c mouse splenocytes for 48 h in capture antibody (anti-IL-2, IL-4, IFN-γ)-coated PVDF-backed micro-well plates. IL-2, IL-4, and IFN-γ spot forming cells (SFC) were determined by ELISPOT assay. Values are mean ± SD of triplicate. * $P < 0.05$, compared to medium control. **A.** Spot number. **B.** Spot image.

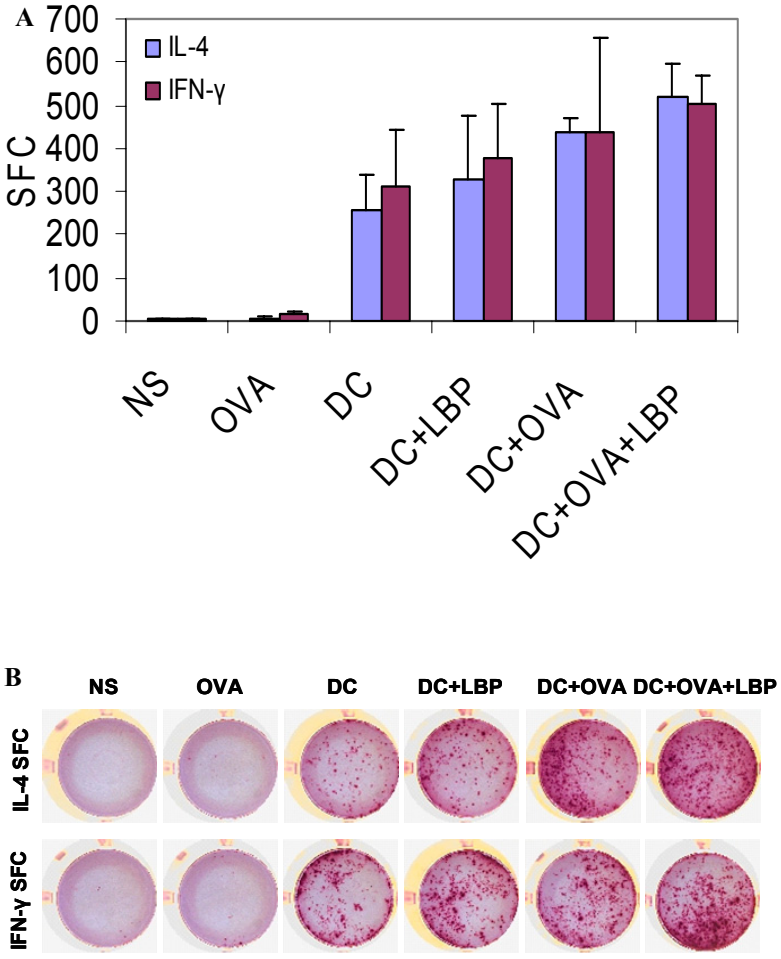

Figure 29. DCs matured by LBP *in vitro* enhance Th1 and Th2 response *in vivo.*

Day 6 BMDCs were pulsed with 100 μg/ml of OVA for 2 h followed by stimulation with 100 μg/ml of LBP for 24 h. Cells were harvested, washed, and 1×10^6 cells were s.c. injected into each BALB/c mouse. Normal saline, OVA (100 μg/mouse), DCs without OVA pulse and/or LBP stimulation were controls. 7 days later, mice were sacrificed and splenocytes were harvested. $2x10^5$ splenocytes were re-stimulated with 10 μg/ml of OVA in ELISPOT plates for 48 h. IL-4 and IFN-γ SFCs were determined by ELISPOT assay. Values are mean ± SD of 4 mice. **A.** Spot number. **B.** Spot image.

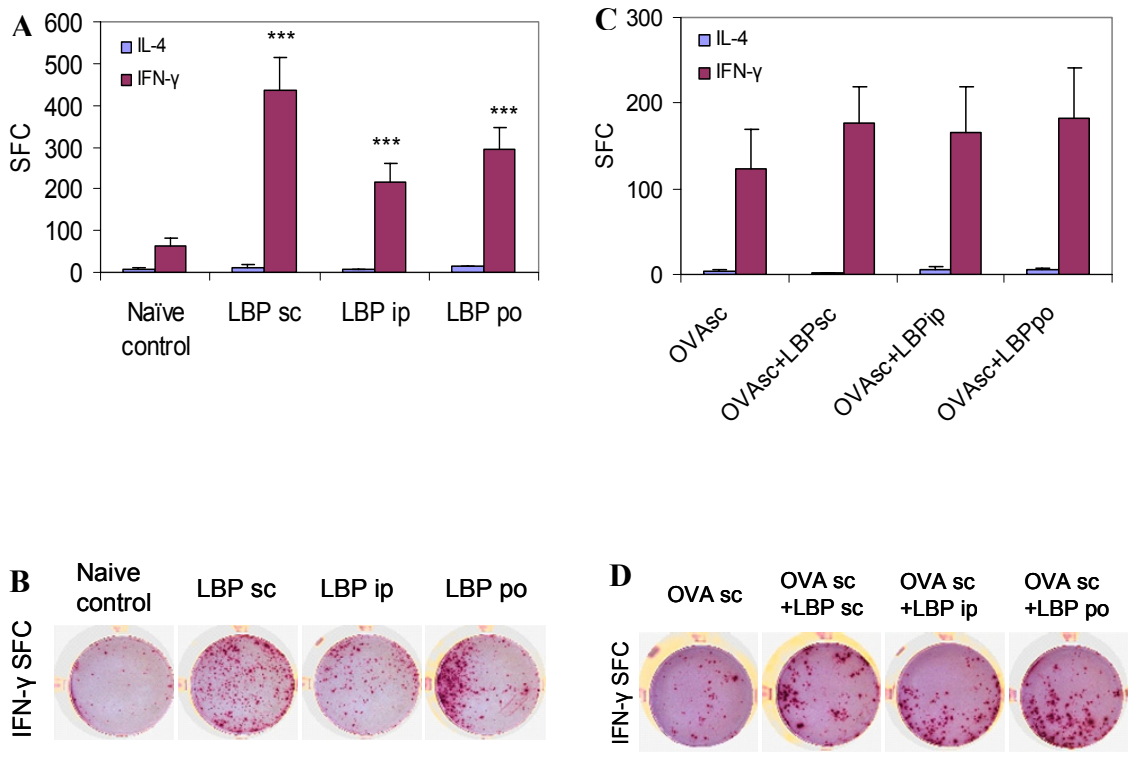

Figure 30. LBP primes Th1 response *in vivo*.

(**A, B**) DCs matured by LBP *in vivo* prime Th1 response. BALB/c mice were s.c., i.p., or p.o. administered with 20 mg/kg of LBP for 24 h. Splenic DCs were purified. T cells were purified from naïve BALB/c mice 5×10^4 DCs were incubated with 2×10^5 T cells in ELISPOT plates for 48 h. IL-4 and IFN-γ spot forming cells (SFC) were determined by ELISPOT assay. Values are mean ± SD of five replicates. *** $P < 0.001$, compared to naïve control. (**C, D**) LBP enhances Th1 response either by s.c., i.p., or p.o. administration. BALB/c mice were s.c. injected with 100 μg of OVA at weekly intervals 3 weeks. Meanwhile, mice were given 20 mg/kg of LBP (s.c., i.p., or p.o.) daily. Naïve mice were control. Mice were sacrificed on day 21. Splenocytes were harvested and restimulated with 10 μg/ml of OVA in ELISPOT plates for 48 h. Values are mean SFC ± SD of 4 mice. **A, C.** Spot number. **B, D.** Spot image.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

4.1 General Discussion

L. barbarum, commonly known as wolfberry, is a well-known Chinese herbal medicine with various biological activities, such as hematopoiesis promotion, liver protection, and immunity improvement. The latter has been attributed to the polysaccharides, the major component of *Lycium* fruit. In this study, we have demonstrated that polysaccharide-protein complex isolated from *L. barbarum* fruit was able to enhance immunity in various aspects, including activation of macrophages, DCs, and T cells. Our results provide a clear profile of LBP as an active stimulus that can improve both innate and adaptive immunities.

Keeping in mind that there are variances in structure, constituents, and molecular weights of LBP fractions obtained from different laboratories, we isolated and purified LBP from *L. barbarum* fruit on our own. The fruit we used for LBP isolation was produced in Zhongning county, Ningxia province, China, which is the best region for *L. barbarum* growth (Gross et al, 2006). The fruit produced in this area is always bigger and contains a higher percentage of sugar than those of other places (Gross et al, 2006). By using standard techniques, such as water extraction, ethanol precipitation, DEAE-cellulose chromatography, and size exclusion chromatography, five fractions of LBP (LBPF1-5) were obtained. The molecular weights of LBPF1-4 we purified were around 150 kDa, which was similar to those obtained by Gan et al (2003), but differed from other groups. These variations could be due to the difference in the raw materials and the state of maturity of the fruit (Wang et al, 2002). They could also be due to the difference in

isolation procedures, and especially the difference in gels and eluents used in ion exchange chromatography and size exclusion chromatography (Meng et al, 1999).

We ruled out the possibility of LPS contamination during the isolation and purification processes, clearly demonstrating that there was no LPS existence in LBP by LAL assay and B cell proliferation assay. This is very important in that LPS is a potent stimulus of immune cells such as macrophages, B cells, and DCs. We also proved that LBP was non-toxic or of low toxicity both *in vitro* and *in vivo*.

It was found that LBP could activate T cells, but not B cells. The active fractions were LBPF4 and LBPF5, but not LBPF1, LBPF2, and LBPF3. This could be because LBPF4 and LBPF5 contained a higher percentage of protein than the other three fractions. It is known that protein antigen is needed to activate T cells. This was further supported by the finding that T cell proliferation stimulated by crude LBP, LBPF4, and LBPF5 was affected when the protein was digested by protease. The mechanism of T cell activation by LBP, LBPF4, and LBP5 may invovle activation of CD25 and transcription factors NFAT and AP-1, which may in turn initiate IL-2 gene transcription and subsequently result in IL-2 protein production. IL-2 can bind to its receptor and cause T cell clonal expansion. The expanded T cells continue to secrete IL-2 and produce a positive feedback. LBP, LBPF4, and LBPF5 seemed to induce Th1 differentiation, as supported by the fact that they induced IFN-γ production instead of IL-4, although IL-4 mRNA was transcribed. It has been known that IFN-γ is produced by Th1 cells and that IL-4 is produced from Th2 cells (Murphy and Reiner, 2002). It is remarkable that the *in vitro*

results were further supported by *in vivo* experiments. LBP (p.o. or i.p.) was also able to stimulate T cell proliferation.

The current study also demonstrated that LBP was capable of activating macrophages. Administration of LBP i.p. significantly upregulated the expressions of CD40, CD80, CD86 and MHC class II molecules on mouse peritoneal macrophages. As these molecules are very important in antigen presentation and T cell activation, this result indicates that LBP can enhance the macrophage ability of presenting antigens to T cells. By using RAW264.7 cells, it was showed that LBP and LBPF1-5 could activate transcription factors NF-κB and AP-1 and induce TNF-α gene transcription and subsequently protein production in a dose-dependent manner. Although IL-1β and IL-12 mRNA were expressed after LBP and LBPF1-5 stimulation, there was no protein production. It could be that the mRNA amount was too low to translate enough protein detectable by ELISA. It is also possible that RAW264.7 cells have lost the capacity to produce such cytokines. TNF-α is principally responsible for attracting neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes (Taylor et al, 2005). Therefore, LBP is good for the host to defend invading pathogens. More amazingly, LBP could activate macrophages *in vivo*. LBP (i.p.) significantly improved the capacity of mouse peritoneal macrophages to kill bacteria.

DC immunogenicity correlates with its maturation. However, while immunogenic DCs must be phenotypically mature, DCs with phenotypic maturation may not necessarily be immunogenic. They can induce T cell tolerance when the maturation markers CD80 and

CD86 bind to CTLA4 on T cells (Spörri and Reis e Sousa, 2005; Reis e Sousa, 2006). Nevertheless, to investigate whether LBP induces immunogenic DCs, we must first look at whether it can induce DC maturation. LBP and LBPF1-5 could upregulate the expressions of CD40, CD80, CD86 and MHC class II molecules on BMDCs. Furthermore, we found that crude LBP (p.o., i.p., or s.c.) upregulated the expressions of these molecules on mouse splenic DCs. These results indicate that LBP is capable of inducing DC phenotypic maturation. In addition, DCs matured by LBP were accompanied by increase of allostimulatory activity and downregulation of antigen uptake. More strikingly, LBP and LBPF1-5 induced IL-12 production by BMDCs at gene and protein levels in a dose-dependent manner, indicating that they can activate the third signal necessary for activating T cells. Secondly, to evaluate whether the LBP-induced phenotypically mature DCs are immunogenic, we tested their antigen presentation capacity. The result shows that LBP-stimulated DCs had enhanced antigen presenting ability, as characterized by increases in IL-4 and IFN-γ production by ELISPOT assay. These results were reproducible by using *in vivo* animal models, indicating that LBP can induce immunogenic DCs.

While LBP was able to activate T cells, macrophages, and DCs, indicating it has multiple targets on the immune system, the activation profiles of different types of cells were different. Only LBPF4 and LBPF5 could activate T cells, whereas all of the fractions were able to activate macrophages and DCs, although LBPF5 was weaker than other fractions in macrophage and DC activation. We have known that LBPF1, LBPF2, and LBPF3 contained more carbohydrate but less protein than LBPF4 and LBPF5. It is

possible that the carbohydrate component is responsible for macrophage and DC activation while the protein part is mainly involved in T cell activation. In comparison, LBP showed stronger activities on DCs and macrophages than on T cells. T cell activation by LBP was relatively low. IL-2 production from mouse splenocytes induced by LBP was only around 15 pg/ml. It appears that LBP is more inclined to target DCs and macrophages than T cells. In addition, various experiments showed that the pure fractions of LBP did not show significantly stronger activities than the crude one. It could be that crude LBP contained other bioactive components which were removed during the process of purification. If so, it would be adequate to use the crude extract in future studies. This study provides profiles on transcription factor activation and downstream cytokine induction by LBP. In the future, it would be interesting to identify the LBP receptors on DCs, macrophages, and T cells and elucidate the early stages that occur upon LBP binding to immune cells.

All in all, LBP is able to activate DCs, macrophages, and T cells, indicating it can enhance both innate and adaptive immunity. Unlike the strong immune cell stimuli, such as LPS, Con A, and PHA, the immuno-enhancement ability of LBP is generally mild, but on the other hand, this property may be good for the host, as those strong stimuli may cause inflammation and are harmful. Our study provides sceintific evidence on using it as valuable supplement to immprove immunity. This will extremely benefit those people under poor immune conditions such as cancer, hepatitis, tuberculosis, and aging. Indeed, wolfberry supplement is becoming more and more popular nowadays.

4.2 Conclusion

From the present study, the following conclusions can be drawn:

1. LBP can activate T cells. The mechanism may involve activation of CD25 and transcription factors NFAT and AP-1. The active fractions are LBPF4 and LBPF5. T cells activated by LBP produce IL-2 and IFN-γ.

2. LBP and LBPF1-5 are able to activate macrophages. The mechanism may involve activation of transcripion factors NF-κB and AP-1 and induction of TNF-α gene transcription and protein production, Macrophages activated by LBP upregulate the expression of costimulatory and MHC class II molecules and improve phagocytic and endocytic capacities.

3. LBP and LBPF1-5 are able to induce immunogenic DCs characterized by phenotypic maturation and enhancement in antigen presentation ability.

4.3 Future Directions

Although this study showed that LBP can activate T cells, macrophages, and DCs, its corresponding receptors on these cells have not been addressed yet. Therefore, it will interesting to indentify LBP binding receptors on these cells in the future.

CHAPTER 5

REFERENCES

Aderem A, Underhill DM. 1999. Mechanisms of phagocytosis in macrophages. ***Annu Rev Immunol.*** 17:593-623.

Aguado E, Martínez-Florensa M, Aparicio P. 2006. Activation of T lymphocytes and the role of the adapter LAT. ***Transpl Immunol.*** 17:23-6.

Albert ML, Jegathesan M, Darnell RB. 2001. Dendritic cell maturation is required for the cross-tolerization of $CD8^+$ T cells. ***Nature Immunol.*** 2:1010-7.

Anderson CF, Mosser DM. 2002a. A novel phenotype for an activated macrophage: the type 2 activated macrophage. ***J Leukoc Biol.*** 72:101-6.

Anderson CF, Mosser DM. 2002b. Cutting edge: biasing immune responses by directing antigen to macrophage Fc gamma receptors. ***J Immunol.*** 168:3697-701.

Apostolopoulos V, McKenzie IF. 2001. Role of the mannose receptor in the immune response. ***Curr Mol Med.*** 1:469-74.

Ashok BT, Ali R. 1999. The aging paradox: free radical theory of aging. ***Exp Gerontol.*** 34:293-303.

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ et al. 2000. Immunobiology of dendritic cells. ***Annu Rev Immunol.*** 18:767-811.

Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. ***Nature.*** 392:245-52.

Beinke S, Ley SC. 2004. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. ***Biochem J.*** 382:393-409.

Beutler B. 2004. Innate immunity: an overview. ***Mol Immunol.*** 40:845-59.

Bhatia S, Edidin M, Almo SC, Nathenson SG. 2006. B7-1 and B7-2: similar costimulatory ligands with different biochemical, oligomeric and signaling properties. ***Immunol Lett.*** 104:70-5.

Bian L, She X, Wang Y. 1996. Morphological study of CCL_4 induced liver damage of mouse and its treatment with *Lycium barbarum* polysaccharides. ***J Ningxia Med*** 18:196-198.

Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. 1999. Mushrooms, tumors, and immunity. ***Proc Soc Exp Biol Med***. 221:281-93.

Bowie A, O'Neill LA. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. ***J Leukoc Biol.*** 67:508-14.

Brian AA. 1988. Stimulation of B-cell proliferation by membrane-associated molecules from activated T cells. ***Proc Natl Acad Sci U.S.A.*** 85:564-568.

Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. 2001.The immunological synapse. ***Annu Rev Immunol.*** 19:375-96.

Brown GD. 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. ***Nat Rev Immunol.*** 6:33-43.

Bryant P, Ploegh H. 2004. Class II MHC peptide loading by the professionals. ***Curr Opin Immunol.*** 16:96-102.

Caamano J, Hunter CA. 2002. NF-κB family of transcription factors: central regulators of innate and adaptive immune functions. ***Clin Microbiol Rev.*** 15:414-29.

Cao G, Du P. 1992. Effects of *Lycium barbarum* polysaccharides and IL-2 on LAK cell anticancer activities in 2-month-old and 12-month-old mice. ***Chin J Microbiol Immunol.*** 12:390-2.

Cao G, Du P. 1993. Priming effect of intraperitoneally injected *Astragalus*, *Acanthopanax senticosus* and *Lycium barbarum* polysaccharides on the induction of lymphokine-activated killer cells *in vitro*. ***Acad J Sec Mil Med Univ.*** 14:10-13.

Cao G, Yang W, Du P. 1994. Observation of the effects of LAK/IL-2 therapy combined with *Lycium barbarum* polysaccharides in the treatment of 75 cancer patients. ***Chin J Oncol*** 16:428-431.

Cao G, Yang W, Du P, Wang X. 1995. Observation of the effect of LAK/IL-2 therapy combined with *Lycium barbarum* polysaccharides in the treatment of patients with advanced cancer (a report of 75 cases). ***Chin J Can Res.*** 7:293-7.

Cemerski S, Shaw A. 2006. Immune synapses in T-cell activation. ***Curr Opin Immunol.*** 18:298-304.

Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA et al. 1992. Molecular cloning of the interleukin-1 beta converting enzyme. ***Science.*** 256:97-100.

Chan HC, Chang RC, Ip KC, Chiu K, Yuen WH, Zee SY et al. 2007. Neuroprotective effects of *Lycium barbarum* Lynn on protecting retinal ganglion cells in an ocular hypertension model of glaucoma. ***Exp Neurol.*** 203:269-73.

Chao JC, Chiang SW, Wang CC, Tsai YH, Wu MS. 2006. Hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa* inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells. ***World J Gastroenterol.*** 12:4478-84.

Chen L, Glover JN, Hogan PG, Rao A, Harrison SC. 1998. Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. ***Nature.*** 392:42-8.

Chen W. 2004. The late stage of T cell development within mouse thymus. ***Cell Mol Immunol.*** 1:3-11.

Chen Z, Wu Z, Wang L. 2001. Effects of LBP on IL-2 and IL-2R expression in senile mice. ***Chin J Immunol.*** 17:312-3.

Chinenov Y, Kerppola TK. 2001. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. ***Oncogene.*** 20:2438-52.

Chizzolini C, Rezzonico R, De Luca C, Burger D, Dayer JM. 2000. Th2 cell membrane factors in association with IL-4 enhance matrix metalloproteinase-1 (MMP-1) while decreasing MMP-9 production by granulocyte-macrophage colony-stimulating factor-differentiated human monocytes. ***J Immunol.*** 164:5952-60.

Clements JL. 2003. Known and potential functions for the SLP-76 adapter protein in regulating T-cell activation and development. ***Immunol Rev.*** 191:211-9.

Corcoran L, Ferrero I, Vremec D, Lucas K, Waithman J, O'Keeffe M, Wu L, Wilson A, Shortman K. 2003. The lymphoid past of mouse plasmacytoid cells and thymic dendritic cells. ***J Immunol.*** 170:4926-32.

Cui T, Li M, Zhao Y. 2006. *Lycium barbarum* polysaccharide induces apoptosis in chronic myeloid leukemia K562 Cells. ***J Jinzhou Med Coll***. 27:30-33.

Cui X, Luo Q, Yang M, Wang L, Li D, Liang C, Yan J, Li Z. 2006. Effect of *Lycium barbarum* polysaccharides on growth of human cervical carcinoma Hela cell and cell apoptosis. ***Chin J Pub Health.*** 22:1141-2.

Cui X, Luo Q, Yang M, Wang L, Yan J, Li Z. 2006. Effect of *Lycium barbarum* polysaccharides on apoptosis of human prostatic cancer PC-3 cell line. ***J Toxicol.*** 20:221-4.

Dai S, Li X, Du C, Yan J, Wang M, Zhao X et al. 1987. Effects of *Lycium barbarum* fruit on some blood parameters in aged people. ***J Gerontol.*** 7:45-7.

Dai S, Wen R, Li W, Ma W, Pei X, Zhao X et al. 1994. The values of *Lycium barbarum* L and its polysaccharides in anti-aging and prolonging lifespan. ***J Gerontol.*** 14:33-6;64.

Davis MM, Boniface JJ, Reich Z, Lyons D, Hampl J, Arden B et al. 1998. Ligand recognition by alpha beta T cell receptors. ***Annu Rev Immunol.*** 16:523-44.

Davis RJ. 2000. Signal transduction by the JNK group of MAP kinases. ***Cell.*** 103:239-52.

De Vries IJ, Krooshoop DJ, Scharenborg NM, Lesterhuis WJ, Diepstra JH, Van Muijen GN et al. 2003. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. ***Cancer Res.*** 63:12-7.

Decker T, Kovarik P, Meinke A. 1997. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. ***J. Interferon Cytokine Res.*** 17:121-34.

Decker T, Müller M, Stockinger S. 2005.The *yin* and *yang* of type 1 interferon activity in bacterial infection. ***Nat Rev Immunol.*** 19:1-13.

Deng HB, Cui DP, Jiang JM, Feng YC, Cai NS, Li DD. 2003. Inhibiting effects of *Achyranthes bidentata* polysaccharide and *Lycium barbarum* polysaccharide on nonenzyme glycation in D-galactose induced mouse aging model. ***Biomed Environ Sci.*** 16:267-75.

Dennehy KM, Brown GD. 2007. The role of the beta-glucan receptor Dectin-1 in control of fungal infection. ***J Leukoc Biol.*** 82:253-8.

Diegelmann RF, Evans MC. 2004. Wound healing: an overview of acute, fibrotic and delayed healing. ***Front Biosci.*** 9:283-9.

Dong C. 2008. TH17 cells in development: an updated view of their molecular identity and genetic programming. ***Nat Rev Immunol.*** 8:337-48.

Dong J, Gao T, Hu Q. 1997. Experimental study of the anticancer activities of *Lycium barbarum* polysaccharides and allicin. ***Chin J Basic Tra Chin Med.*** 3(6):32-5.

Du G. 2005. Immunomodulatory mechanism of *Lycium barbarum* polysaccharide and its clinical application. ***J Hubei Med.*** 27:54-5.

Du S, Zhang X, Lou L, Qian Y. 1994. Effects of *Lycium barbarum* L. water extract on IL-6 and TNF production. ***Chin J Immunol.*** 10:356-8.

Ehrchen J, Steinmuller L, Barczyk K, Tenbrock K, Nacken W, Eisenacher M et al. 2007. Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. ***Blood.*** 109:1265-74.

Farrar JD, Asnagli H, Murphy KM. 2002. T helper subset development: roles of instruction, selection, and transcription. ***J Clin Invest.*** 109:431-5.

Feske S, Draeger R, Peter HH, Eichmann K, Rao A. 2000. The duration of nuclear residence of NFAT determines the pattern of cytokine expression in human SCID T cells. ***J Immunol.*** 165:297-305.

Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B et al. 2006. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. ***Nature.*** 441:179-85

Feske S. 2007. Calcium signalling in lymphocyte activation and disease. ***Nat Rev Immunol.*** 7:690-702.

Foulds KE, Wu CY, Seder RA. 2006. Th1 memory: implications for vaccine development. ***Immunol Rev.*** 211:58-66.

Fu G, Li B, Zhou Z. 2007. Effect of compound LBP on the immune adjustment and anti-lipid peroxidation of fluoride-exposed workers. ***Occup Health.*** 23:324-5.

Gaffen SL, Liu KD. 2004. Overview of interleukin-2 function, production and clinical applications. ***Cytokine.*** 28(3):109-23.

Gan L, Wang J, Luo Q, Zhang S. 2001. Effect of *Lycium barbarum* polysaccharide on apoptosis of human leukemia cells. ***Acta Nutr Sin.*** 23:220-4.

Gan L, Wang J, Zhang S. 2001. Inhibition the growth of human leukemia cells by *Lycium barbarum* polysaccharides. ***J Hyg Res.*** 30:333-7.

Gan L, Zhang S. 2002. Effects of *Lycium barbarum* polysaccharide on cytokine expression in human monocytes. ***Acta Nutr Sin.*** 24:67-9.

Gan L, Zhang S. 2003. Effect of *Lycium barbarum* polysaccharide on antitumor activity and immune function. ***Acta Nutr Sin.*** 25:200-02, 214.

Gan L, Zhang S, Liu Q, Xu H. 2003. A polysaccharide-protein complex from *Lycium barbarum* upregulates cytokine expression in human peripheral blood mononuclear cells. ***Eur J Pharmacol.*** 471:217-22.

Gan L, Zhang S, Yang X, Xu H. 2004. Immunomodulation and antitumor activity by a polysaccharide-protein complex from *Lycium barbarum*. ***Int Immunopharmacol.*** 4:563-9.

Geng C, Ding Y, Wang G. 1987. Immuno-enhancing effect of *Lycium barbarum* crude extract on mouse T lymphocytes. ***Chin J Milit Med Coll.*** 11:476.

Gong H, Shen P, Jin L, Xing C, Tang F. 2005. Therapeutic effects of *Lycium barbarum* polysaccharide (LBP) on irradiation or chemotherapy-induced myelosuppressive mice. ***Cancer Biother Radiopharm***. 20:155-62.

Gordon S. 2003. Alternative activation of macrophages. ***Nat Rev Immunol.*** 3:23-35.

Greenwald RJ, Freeman GJ, Sharpe AH. 2005.The B7 family revisited. ***Annu Rev Immunol*** 23:515-48.

Gross PM, Zhang X, Zhang R. Wolfberry phytochemicals and disease research: implied health benefits, chapter 6 and Wolfberries and Traditional Chinese Medicine, chapter 7 in *Wolfberry: Nature's Bounty of Nutrition and Health*. ***Booksurge Publishing***. 2006.

Groux H, Fournier N, Cottrez F. 2004. Role of dendritic cells in the generation of regulatory T cells. ***Semin Immunol.*** 16:99-106.

Gu S. 1990. Analysis of *Lycium barbarum* polysaccharides in treatment of 20 cases of primary liver cancer. ***Pharmacol Clin Chin Materia Medica.*** 6(6):38.

Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R et al. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. ***Proc Natl Acad Sci U S A.*** 88:4143-7.

Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. 2002. Antigen presentation and T cell stimulation by dendritic cells. ***Annu Rev Immunol.*** 20:621-67.

Guha M, Mackman N. 2001. LPS induction of gene expression in human monocytes. ***Cell Signal.*** 13:85-94.

Ha KT, Yoon SJ, Choi DY, Kim DW, Kim JK, Kim CH. 2005. Protective effect of *Lycium chinense* fruit on carbon tetrachloride-induced hepatotoxicity. ***J Ethnopharmacol.*** 96:529-35.

Hai YG, Ping S, Li JI, Chang HX, Fu T. 2004. Therapeutic effects of *Lycium barbarum* polysaccharide (LBP) on mitomycin C (MMC)-induced myelosuppressive mice. ***J Exp Ther Oncol.*** 4:181-7.

Han SB, Part SK, Ahn HJ, Yoon YD, Kim YH, Lee JJ et al. 2003. Characterization of B cell membrane receptors of polysaccharide isolated from the root of *Acanthopanax koreanum*. ***Int Immunopharmaocol***. 3:683-691.

Hancock WW, Sayegh MH, Zheng XG, Peach R, Linsley PS, Turka LA. 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. ***Proc Natl Acad Sci U S A.*** 93:13967-72.

Hara H, Bakal C, Wada T, Bouchard D, Rottapel R, Saito T, Penninger JM. 2004. The molecular adapter Carma1 controls entry of IκB kinase into the central immune synapse. ***J Exp Med.*** 200:1167-7.

Harman D. 1956. Aging: a theory based on free radical and radiation chemistry. ***J Gerontol.*** 11:298-300.

Harman D. 1972. The biologic clock: the mitochondria? ***J Am Geriatr Soc.*** 20:145-147.

Hart PH, Burgess DR, Vitti GF, Hamilton JA. 1989. Interleukin-4 stimulates human monocytes to produce tissue-type plasminogen activator. ***Blood.*** 74:1222-5.

Hase K, Basnet P, Kadota S, Namba T. 1997. Immunostimulating activity of Celosian, an antihepatotoxic polysaccharide isolated from *Celosia argentea*. ***Planta Med.*** 63:216-9.

Hayamizu K, Huie P, Sibley RK, Strober S. 1998. Monocyte-derived dendritic cell precursors facilitate tolerance to heart allografts after total lymphoid irradiation. ***Transplantation***. 66:1285-91.

Hayashi K, Altman A. 2007. Protein kinase C theta (PKCθ): A key player in T cell life and death. ***Pharmacol Res.*** 55:537-44.

He J, Liang Y, Zhang S. 1996. Isolation, purification and property identification of polysaccharide-I from *Lycium barbarum* L. ***J Plant Resour Environ.*** 5:61-2.

He J, Pan L, Guo FX. 1993. Protective effect of *Lycium barbarum* polysaccharide on experimental mouse liver damage model. ***Chin J Pharmacol Toxicol*** 7:293-295.

He L, Liu P. 2006. Effect of *Lycium barbarum* polysaeeharides on oxidative stress of diabetic nephropathy in type 2 diabetic rats. ***Chin J Hospital Pharm.*** 26:1475-8.

He Y, Ying Y, Su N, Ma W. 2006. Antitumor activity of *Lycium barbarum* polysaccharides in hepatoma H22-bearing mice and its effect on VEGF secretion. ***Guangdong Med J.*** 27:950-1.

He Y, Ying Y, Wang B, Su N, Su J, Luo H, Zhong Z. 2005. The effect of *Lycium barbarum* polysaccharides on immunosuppressive factors VEGF and TGF-β in H22 bearing mice. ***Pharmacol Clin Chin Materia Medica.*** 21(5):28-9.

Herman EM, Lamb CJ. 1992. Arabinogalactan rich glycoproteins are localized on the cell surface and in intravacuolar multivesicular bodies. ***Plant Physiol.*** 98:264-272.

Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW et al. 2001. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. ***J Immunol.*** 167:6533-44.

Ho SC, Liu JH, Wu RY. 2003. Establishment of the mimetic aging effect in mice caused by D-galactose. ***Biogerontology.*** 4:15-8.

Ho YS, Yu MS, Lai CS, So KF, Yuen WH, Chang RC. 2007. Characterizing the neuroprotective effects of alkaline extract of *Lycium barbarum* on beta-amyloid peptide neurotoxicity. ***Brain Res.*** 1158:123-34.

Hu GJ, Bai HQ, Du SY. 1995. Regulation of T, B lymphocytes and T cell subsets by *Lycium barbarum*. ***Chin J Immunol.*** 11:163-165.

Hu X, Chen J, Wang L, Ivashkiv LB. 2007. Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. ***J Leukoc Biol.*** 82:237-43.

Huang L, Lin Y, Tian G, Ji G. 1998. Isolation, purification and physico-chemical properties of immunoactive constituents from the fruit of *Lycium barbarum* L. ***Acta Pharmaceutica Sinica.*** 33:512-16.

Huang X, Yang M, Wu X, Yan J. 2003. Study on protective action of *Lycium barbarum* polysaccharides on DNA impairments of testicle cells in mice. ***J Hyg Res.*** 32:599-601.

Huang Y, Wange RL. 2004. T cell receptor signaling: beyond complex complexes. ***J Biol Chem.*** 279:28827-30.

Huppa JB, Davis MM. 2003. T-cell-antigen recognition and the immunological synapse. ***Nat Rev Immunol.*** 3:973-83.

Inaba K, Inaba M, Romani N, and Aya H. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. ***J Exp Med.*** 176:1693-702.

Inaba K, Witmer-Pack M, Inaba M, Hathcock KS, Sakuta H, Azuma M et al. 1994. The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells *in situ* and during maturation *in vitro*. ***J. Exp. Med.*** 180:1849-60.

Irvine DJ, Doh J. 2007. Synthetic surfaces as artificial antigen presenting cells in the study of T cell receptor triggering and immunological synapse formation. ***Semin Immunol.*** 19:245-54.

Jain J, McCaffrey PG, Valge-Archer VE, Rao A. 1992. Nuclear factor of activated T cells contains Fos and Jun. ***Nature.*** 356:801-804.

Jankovic D, Sher A, Yap G. 2001. Th1/Th2 effector choice parasitic infection: decision making by committee. ***Curr Opin Immunol.*** 13:403-409.

Jia YX, Dong JW, Wu XX, Ma TM, Shi AY. 1998. The effect of *Lycium barbarum* polysaccharide on vascular tension in two-kidney, one clip model of hypertension. ***Acta Physiol Sinica.*** 50:309-14.

Jonuleit H, Kühn U, Müller G, Steinbrink K, Paragnik L, Schmitt E et al. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. ***Eur J Immunol.*** 27:3135-42.

Jun JE, Wilson LE, Vinuesa CG, Lesage S, Blery M, Miosge LA et al. 2003. Identifying the MAGUK protein Carma-1 as a central regulator of humoral immune responses and atopy by genome-wide mouse mutagenesis. ***Immunity.*** 18:751–62.

Kane LP, Lin J, Weiss A. 2000. Signal transduction by the TCR for antigen. ***Curr Opin Immunol.*** 12:242-9.

Kang BY, Kim E, Kim TS. 2005. Regulatory mechanisms and their therapeutic implications of interleukin-12 production in immune cells. ***Cell Signal.*** 17:665-73.

Kapsenberg ML. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. ***Nat Rev Immunol.*** 3:984-93.

Karin M, Greten FR. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. ***Nat Rev Immuno***l 5:749-59.

Keir ME, Sharpe AH. 2005. The B7/CD28 costimulatory family in autoimmunity. ***Immunol Rev.*** 204:128-43.

Khanolkar A, Badovinac VP, Harty JT. 2007. CD8 T cell memory development: CD4 T cell help is appreciated. ***Immunol Res.*** 39:94-104.

Khawli LA, Hu P, Epstein AL. 2008. Cytokine, chemokine, and costimulatory fusion proteins for the immunotherapy of solid tumors. ***Handb Exp Pharmacol.*** 181:291-328.

Kim HM, Han SB, Oh GT, Kim YH, Hong DH Hong ND et al. 1996. Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom *Phellinus linteus*. ***Int J Immunopharmacol***. 18:295-303.

Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S et al. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. ***J Exp Med.***170:827-45.

Kralovec JA, Metera KL, Kumar JR, Watson LV, Girouard GS, Guan Y et al. 2006. Immunostimulatory principles from *Chlorella pyrenoidosa*---part 1: isolation and biological assessment *in vitro*. ***Phytomed.*** 14:57-64.

Krishnan J, Selvarajoo K, Tsuchiya M, Lee G, Choi S. 2007. Toll-like receptor signal transduction. ***Exp Mol Med.*** 39:421-38.

Krogsgaard M, Davis MM. 2005. How T cells 'see' antigen. ***Nat Immunol.*** 6:239-45.

Krysko DV, D'Herde K, Vandenabeele P. 2006. Clearance of apoptotic and necrotic cells and its immunological consequences. ***Apoptosis.*** 11:1709-26.

Kubin M, Chow JM, Trinchieri G. 1994. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor alpha, and IL-1 beta production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. ***Blood.*** 83:1847-55.

Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. ***Nat Immunol.*** 1:311-6.

Larsen CP, Ritchie SC, Pearson TC, Linsley PS, Lowry RP. 1992. Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. ***J. Exp. Med.*** 176:1215-20.

Lee KH, Dinner AR, Tu C, Campi G, Raychaudhuri S, Varma R et al. 2003. The immunological synapse balances T cell receptor signaling and degradation. ***Science.*** 302:1218-22.

Lefrançois L. 2006. Development, trafficking, and function of memory T-cell subsets. ***Immunol Rev.*** 211:93-103.

Leung MY, Liu C, Koon JC, Fung KP. 2006. Polysaccharide biological response modifiers. ***Immunol Lett.*** 105:101-14.

Lewis RS. 2001. Calcium signaling mechanisms in T lymphocytes. ***Annu Rev Immunol.*** 19:497-521.

Li B, Tournier C, Davis RJ, Flavell RA. 1999. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. ***Embo J.*** 18:420-32.

Li G, Yang J, Ren B, Wang Z. 2002. Effect of *Lycium barbarum* L on defending free radicals of mice caused by hypoxia. ***J Hyg Res.*** 31:30-1.

Li Q, Verma IM. 2002. NF-kappaB regulation in the immune system. ***Nat Rev Immunol.*** 2:725-33.

Li X, Wu W, Zhou Y. 1990. Effect of *Lycium barbarum* polysaccharides on the expression of C3b and Fc receptors on mouse peritoneal macrophages. ***Chin J Exp Clin Immunol.*** 5:29.

Li XM, Ma YL, Liu XJ. 2007. Effect of the *Lycium barbarum* polysaccharides on age-related oxidative stress in aged mice. ***J Ethnopharmacol.*** 111:504-11.

Li XM. 2007. Protective effect of *Lycium barbarum* polysaccharides on streptozotocin-induced oxidative stress in rats. ***Int J Biol Macromol.*** 40:461-5.

Li XR, Wu WX, Zhou Y. 1990. Effect of *Lycium barbarum* polysaccharide on C3b and Fc receptors on murine peritoneal macrophages. ***Chin J Exp Clin Immunol***. 5:29.

Li Y, Qi C, Zhao X, Cheng J, Wei C, Zhou W et al. 2005. Effects of glycoconjugate and its glycan isolated from *Lycium barbarum* L on macrophage function. ***Chin Pharmacol Bulletin.*** 21:1304-8.

Li Z, Luo Q, Yang M, Yan J, Pang Y, Jiang M. 2005. Effect of *Lycium barbarum* polysaccharides on human prostatic cancer cell line independent of androgen apoptosis and apoptosis-related gene expression. ***J Chin Med Res.*** 5:847-9.

Li Z, Luo Q, Yang M. 2006. Effect of *Lycium barbarum* polysaccharides on the growth of human prostatic cancer cells. ***Chin J Pub Health.*** 22:311-2.

Liang G, Zhang C. 2007. Experimental research on the skin aging function of *Lycium barbarum* polysaccharides. ***Chin J Aesth Med.*** 16:734-6.

Lin RD, Hou WC, Yen KY, Lee MH. 2003. Inhibition of monoamine oxidase B (MAO-B) by Chinese herbal medicines. ***Phytomedicine.*** 10:650-6.

Lin X, O'Mahony A, Mu Y, Geleziunas R, Greene WC. 2000. Protein kinase C-θ participates in NF-κB activation induced by CD3-CD28 costimulation through selective activation of IκB kinase β. ***Mol Cell Biol.*** 20:2933-40.

Lin X, Wang D. 2004. The roles of CARMA1, Bcl10, and MALT1 in antigen receptor signaling. ***Semin Immunol.*** 16:429-35.

Liu J, Cheng B, Zhang J. 1996a. Effect of *Lycium barbarum* polysaccharides on the immune function of malignant cancer patients with radiotherapy. ***Chin J Radiol Med Prot.*** 16:18-20.

Liu J, Zhang L, Qian Y. 1996b. Anticancer and immunomodulatory effects of *Lycium barbarum* polysaccharides on S180 mice. ***Chin J Immunol.*** 12:115-7.

Liu Y, Mao H, Li P. 2000. The effects of *Lycium barbarum* polysaccharide on T lymphocyte subset and lymphocyte transformation in mice. ***J Qinghai Med Coll.*** 21:4-6.

Lopez-Rodriguez C, Aramburu J, Rakeman AS, Rao A. 1999. NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. ***Proc Natl Acad Sci U S A.*** 96:7214-9

Lu CX, Cheng BQ. 1991. Radiosensitizing effects of *Lycium barbarum* polysaccharide for Lewis lung cancer. ***Chin J Integrated Trad West Med.*** 11: 582, 611-2.

Lu H, Zhang H, Zhang D. 2002. Effects of *Lycium barbarum* polysaccharides on PG cell proliferation and apoptosis. ***J Ningxia Med Coll.*** 24:10-11,14.

Luo Q, Cai Y, Yan J, Sun M, Corke H. 2004. Hypoglycemic and hypolipidemic effects and antioxidant activity of fruit extracts from *Lycium barbarum*. ***Life Sci.*** 76 137-149

Luo Q, Li J, Zhang S. 1997. Effect of *Lycium barbarum* polysaccharides-X on reducing blood glucose in diabetic rabbits. ***Acta Nutri Sinica.*** 19:173-177.

Luo Q, Li Z, Huang X, Yan J, Zhang S, Cai YZ. 2006. *Lycium barbarum* polysaccharides: Protective effects against heat-induced damage of rat testes and H_2O_2-induced DNA damage in mouse testicular cells and beneficial effect on sexual behavior and reproductive function of hemicastrated rats. ***Life Sci.*** 79:613-21.

Luo Q, Yan J, Li J, Zhang S. 1999. The comparative study on the antifatigue effects of crude and pure *Lycium barbarum* polysaccharides. ***Acta Nutr Sin.*** 21:310-7.

Luttrell LM, van Biesen T, Hawes BE, Koch WJ, Krueger KM, Touhara K et al. 1997. G-protein-coupled receptors and their regulation: activation of the MAP kinase signaling pathway by G-protein-coupled receptors. ***Adv Second Messenger Phosphoprotein Res.*** 31:263-77.

Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N et al. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. ***J Immunol Methods.*** 223:77-92.

Ma J, Chen T, Mandelin J, Ceponis A, Miller NE, Hukkanen M et al. 2003. Regulation of macrophage activation. ***Cell Mol Life Sci.*** 60:2334-46.

Ma X, Zhao S. 2003. Comparison of the immunomodulatory effects of six polysaccharides on mice. ***Pharmacol Clin Chin Materia Medica.*** 19(4):14-5.

Ma X, Zhou Y, Yan X. 1996. Immuno-enhancing effects of polysaccharides from Chinese medicinal herbs. ***Chin J Exp Clin Immunol.*** 8(2):40.

Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA et al. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. ***J Immunol.*** 154:5071-9.

Macian F. 2005. NFAT proteins: key regulators of T-cell development and function. ***Nat Rev Immunol.*** 5:472-84.

Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. 2002. Transcriptional mechanisms underlying lymphocyte tolerance. ***Cell.*** 109:719-731.

Macian F, Lopez-Rodriguez C, Rao A. 2001. Partners in transcription: NFAT and AP-1. ***Oncogene.*** 20:2476-89.

Maldonado-López R, De Smedt T, Michel P, Godfroid J, Pajak B, Heirman C et al. 1999. $CD8alpha^+$ and $CD8alpha^-$ subclasses of dendritic cells direct the development of distinct T helper cells *in vivo*. ***J Exp Med.*** 189:587-92.

Manicassamy S, Gupta S, Sun Z. 2006. Selective function of PKC-theta in T cells. ***Cell Mol Immunol.*** 3:263-70.

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. ***Trends Immunol.*** 23:549-55.

Martinez FO, Gordon S, Locati M, Mantovani A. 2006. Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression. ***J Immunol.*** 177:7303-11.

Martinez FO, Sica A, Mantovani A, Locati M. 2008. Macrophage activation and polarization. ***Front Biosci.*** 13:453-61.

McCoy CE, O'Neill LA. 2008. The role of toll-like receptors in macrophages. ***Front Biosci.*** 13:62-70.

Meng XZ, Xi JP, Li LP, Lei JY, Meng SJ. 1999. Current studies on *Lycium barbarum* polysaccharide in chemistry. ***J Ningxia Agri Forensic Sci Tec.*** 4: 22-25.

Miggin SM, O'Neill LA. 2006. New insights into the regulation of TLR signaling. ***J Leukoc Biol.*** 80:220-6.

Miosge L, Zamoyska R. 2007. Signalling in T-cell development: is it all location, location, location? ***Curr Opin Immunol.*** 19:194-9.

Miyazaki T, Liu ZJ, Kawahara A, Minami Y, Yamada K, Tsujimoto Y et al. 1995. Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. ***Cell.*** 81:223-31.

Mor A, Philips MR. 2006. Compartmentalized Ras/MAPK signaling. ***Annu Rev Immunol.*** 24:771-800.

Morelli AE, Thomson AW. 2007.Tolerogenic dendritic cells and the quest for transplant tolerance. ***Nat Rev Immunol.*** 7:610-21.

Mosser DM. 2003. The many faces of macrophage activation. ***J Leukoc Biol.*** 73:209-12.

Murphy KM, Reiner ST. 2002. The lineage decisions of helper T cells. ***Nat Rev Immunol.*** 2:933-944.

Niimi M, Shirasugi N, Ikeda Y, Kan S, Takami H, Hamano K. 2001. Operational tolerance induced by pretreatment with donor dendritic cells under blockade of CD40 pathway. ***Transplantation.*** 72:1556-62.

O'Neill LA, Bowie AG. 2007. The family of five TIR-domain-containing adaptors in Toll-like receptor signaling. ***Nat Rev Immunol.*** 7:353-64.

O'Neill LA, Dinarello CA. 2000. The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. ***Immunol Today.*** 21:206-9.

Ooi VEC, Liu F. 2000. Immunomodulation and anticancer activity of polysaccharide protein complex. ***Curr Med Chem.*** 7:715-729.

Osada T, Clay TM, Woo CY, Morse MA, Lyerly HK. 2006. Dendritic cell-based immunotherapy. ***Int Rev Immunol.*** 25:377-413.

Palacios EH, Weiss A. 2004. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. ***Oncogene.*** 23:7990-8000.

Pan T, Mao Z, Zhang J, Qiu H, Liu D. 2002. Research and development of *Lycium barbarum* polysccharides. ***Chin Food Additives.*** 4:21-4.

Pawson T. 1994. Tyrosine kinase signalling pathways. ***Princess Takamatsu Symp.*** 24:303-22.

Peng SL, Gerth AJ, Ranger AM, Glimcher LH. 2001. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. ***Immunity.*** 14, 13-20.

Peng X, Tian G. 2001. Structural characterization of the glycan part of glycoconjugate LbGp2 from *Lycium barbarum* L. ***Carbohydr Res.*** 331:95-9.

Peng X, Xiu D, Peng J, Liu C. 2002. Effects of *Lycium barbarum* polysaccharide on hippocampal activity in animals. ***J Ningxia Med Coll.*** 24:79-81.

Pfeifhofer C, Kofler K, Gruber T, Tabrizi NG, Lutz C, Maly K et al. 2003. Protein kinase C-θ ffects Ca^{2+} mobilization and NFAT cell activation in primary mouse T cells. ***J Exp Med.*** 197:1525-35.

Plüddemann A, Neyen C, Gordon S. 2007. Macrophage scavenger receptors and host-derived ligands. ***Methods.*** 43:207-17.

Pylkkänen L, Gullstén H, Majuri ML, Andersson U, Vanhala E, Määttä J et al. 2004. Exposure to *Aspergillus fumigatus* spores induces chemokine expression in mouse macrophages. ***Toxicol.*** 200:255-63.

Qi C, Zhang Y, Chen B, Gu G. 1999. Effects of *Lycium barbarum* polysaccharides on the immune function of normal and aged mice and it's possible mechanism *in vitro*. ***Chin J Immunol.*** 15:419-24.

Qi C, Zhang Y, Wei C, Ru X, Zhao X, Huang L et al. 2001. Immunoactivity of the crude polysaccharides from the fruit of *Lycium barbarum* L.. ***Chin J Pharmacol Toxicol.*** 15:180-4.

Qian Y, Bai H, Yin J. 1989. Immunomodulatory activities of anti-aging compound *Lycium barbarum* L. ***J Beijing Med Univ.*** 21:31.

Qian YK, Cheng HT, Richardson A. 1988. Regulation of immune cells and cytokines by Chinese medicinal herbs (Kusen and *Lycium barbarum*). ***Chin J Microbiol Immunol.*** 8:312-316.

Qin X, Yamauchi R, Aizawa K, Inakuma T, Kato K. 2001. Structural features of arabinogalactan-proteins from the fruit of *Lycium chinense* Mill. ***Carbohydr Res.*** 333:79-85.

Qiu S, Li H, Pang Q. 2001. Effect of LBP on IL-2 production by mouse lymphocytes. ***Chin J Trad Med Sci Tec.*** 8:166.

Quintana A, Griesemer D, Schwarz EC, Hoth M. 2005. Calcium-dependent activation of T-lymphocytes. ***Pflugers Arch.*** 450:1-12.

Reis e Sousa C. 2006. Dendritic cells in a mature age. ***Nat Rev Immunol.*** 6:476-83.

Richie LI, Ebert PJR, Wu LC, Krummel MF, Owen JJT, Davis MM. 2002. Imaging synapse formation during thymocyte selection: inability of CD3z to form a stable central accumulation during negative selection. ***Immunity.*** 16:1-20.

Riley JL, June CH. 2005. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. ***Blood.*** 105:13-21.

Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD et al. 2005. The evolution of vertebrate Toll-like receptors. ***Proc Natl Acad Sci U S A.*** 102:9577-82.

Roake JA, Rao AS, Morris PJ, Larsen CP, Hankins DF, Austyn JM. 1995. Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. ***J Exp Med.*** 181:2237-47.

Robertson MJ, Ritz J. 1996. Interleukin 12: Basic Biology and Potential Applications in Cancer Treatment. ***Oncologist.*** 1(1 & 2):88-97.

Rudolph MG, Stanfield RL, Wilson IA. 2006. How TCRs bind MHCs, peptides, and coreceptors. ***Annu Rev Immunol.*** 24:419-66.

Rutella S, Danese S, Leone G. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. ***Blood.*** 108:1435-40.

Sallusto F, Lanzavecchia A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. ***J Exp Med.*** 179:1109-18.

Sanzen I, Imanishi N, Takamatsu N, Konosu S, Mantani N, Terasawa K et al. 2001. Nitric oxide-mediated antitumor activity induced by the extract from *Grifola frondosa* (Maitake mushroom) in a macrophage cell line, RAW264.7. ***J Exp Clin Cancer Res*** 20:591-7.

Savignac M, Mellström B, Naranjo JR. 2007. Calcium-dependent transcription of cytokine genes in T lymphocytes. ***Pflugers Arch.*** 454:523-33.

Schepetkin IA, Quinn MT. 2006. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. ***Int Immunopharmacol.*** 6:317-33.

Schmitz ML, Bacher S, Dienz O. 2003. NF-kappaB activation pathways induced by T cell costimulation. ***FASEB J.*** 17:2187-93.

Schnare M, Holt AC, Takeda K, Akira S, Medzhitov R. 2000. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. ***Curr Biol.*** 10:1139-42.

Schroder K, Hertzog PJ, Ravasi T, Hume DA. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. ***J Leukoc Biol.*** 75:163-89.

Schuler G, Steinman RM. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. ***J Exp Med.*** 161:526-46.

Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P et al. 2002. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. ***J Exp Med.*** 195:1279-88.

Segura M, Su Z, Piccirillo C, Stevenson MM. 2007. Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. ***Eur J Immunol.*** 37:1887-904.

Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. 1988. Identification of a putative regulator of early T cell activation genes. ***Science.*** 241:202-5.

Shortman K, Naik SH. 2007. Steady-state and inflammatory dendritic-cell development. ***Nat Rev Immunol.*** 7:19-30.

Siebenlist U, Brown K, Claudio E. 2005. Control of lymphocyte development by nuclear factor-kappaB. ***Nat Rev Immunol.*** 5:435-45.

Snyder JT, Alexander-Miller MA, Berzofskyl JA, Belyakov IM. 2003. Molecular mechanisms and biological significance of CTL avidity. ***Curr HIV Res.*** 1:287-94.

Song Y, Zeng M, Lu L, Fan Z, Mao Y, Yang W et al. 2002. Morphological observation of protective effects of *Lycium barbarum* polysaccharide on experimental alcoholic liver disease in rats. ***J Med Postgraduates.*** 15:13-6.

Spörri R, Reis e Sousa C. 2005. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. ***Nat Immunol.*** 6:163-70.

Staska LM, Davies CJ, Brown WC, McGuire TC, Suarez CE, Park JY et al. 2005. Identification of vaccine candidate peptides in the NcSRS2 surface protein of *Neospora caninum* by using $CD4^+$ cytotoxic T lymphocytes and gamma interferon-secreting T lymphocytes of infected Holstein cattle. ***Infect Immun.*** 73:1321-9.

Steinman RM, Hawiger D, Nussenzweig MC. 2003. Tolerogenic dendritic cells. ***Annu Rev Immunol.*** 21:685-711.

Steinman RM, Witmer MD. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. ***Proc Natl Acad Sci U S A.*** 75:5132-6.

Sullivan PG, Brown MR. 2005. Mitochondrial aging and dysfunction in Alzheimer's disease. ***Prog Neuro-Psychopharmacol Biol Psychiat.*** 29:407-10.

Sun Z, Arendt CW, Ellmeier W Schaeffer EM, Sunshine MJ, Gandhi L et al. 2000. PKC-theta is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. ***Nature.*** 404:402-7.

Tak PP, Firestein GS. 2001. NF-κB: a key role in inflammatory diseases. ***J Clin Invest.*** 107:7-11

Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. 2005. Macrophage receptors and immune recognition. ***Annu Rev Immunol.*** 23:901-44.

Tian G, Wang C, Feng Y. 1995. Isolation, purification and properties of LbGp and characterization of its glycan-peptide bond. ***Acta Biochim Biophys Sin.*** 27:201-6.

Tian L, Wang M, Chen W. 2005. Inhibition effects of *Lycium barbarum* polysaccharides on α-glucosidase. ***Pharmacol Clin Chin Materia Medica***. 21(3):23-5.

Tian L, Wang M, Chen W. 2006. Inhibition effects of *Lycium barbarum* polysaccharides on α-glucosidase. ***West Chin J Pharma Sci.*** 21:131-3.

Tian M, Wang M. 2006. Studies on extraction, isolation and composition of *Lycium barbarum* polysaccharides. ***J Chin Meteria Madica.*** 31:1603-7.

Trinchieri G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. ***Nat Rev Immunol.*** 3:133-46.

Tschopp J, Martinon F, Hofmann K. 1999. Apoptosis: silencing the death receptors. ***Curr Biol.*** 9: R381-384.

Tsirogianni AK, Moutsopoulos NM, Moutsopoulos HM. 2006. Wound healing: immunological aspects. ***Injury.*** 37 Suppl 1:S5-12.

Tu L, Fang TC, Artis D, Shestova O, Pross SE, Maillard I et al. 2005. Notch signaling is an important regulator of type 2 immunity. ***J Exp Med.*** 202:1037-42.

Uematsu S, Akira S. 2008. Toll-like receptors (TLRs) and their ligands. ***Handb Exp Pharmacol.*** 183:1-20.

Underhill DM, Ozinsky A. 2002. Phagocytosis of microbes: complexity in action. ***Annu Rev Immunol.*** 20:825-52.

van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G, Meerschaut S, Beschin A, Raes G et al. 2006. Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. ***Immunobiol.*** 211:487-501.

van Vliet SJ, den Dunnen J, Gringhuis SI, Geijtenbeek TB, van Kooyk Y. 2007. Innate signaling and regulation of dendritic cell immunity. ***Curr Opin Immunol.*** 19:435-40.

Vetter IR, Wittinghofer A. 2001. The guanine nucleotide-binding switch in three dimensions. ***Science.*** 294:1299-304.

Villadangos JA, Schnorrer P. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets *in vivo*. ***Nat Rev Immunol.*** 7:543-55.

Vremec D, Pooley J, Hochrein H, Wu L, Shortman K. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. ***J Immunol.*** 164:2978-86.

Wagner EF, Eferl R. 2005. Fos/AP-1 proteins in bone and the immune system. ***Immunol Rev.*** 208:126-40.

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. ***Annu Rev Immunol.*** 17:331-67.

Wang B, Xing S, Zhou J. 1990. Effect of *Lycium barbarum* polysaccharides on the immune responses of T, CTL and NK cells in normal and cyclophosphamide-treated mice. ***Chin J Pharmacol Toxicol.*** 4:39.

Wang J, Roderiquez G, Oravecz T, Norcross MA. 1998. Cytokine regulation of human immunodeficiency virus type 1 entry and replication in human monocytes/macrophages through modulation of CCR5 expression. ***J Virol.*** 72:7642-7.

Wang J, Wang H, Zhang M, Zhang S. 2002a. Anti-peroxidation effects of polysaccharide-3 from *Fructus lycii* on mice. ***Chin J Vet Sci.*** 22:267-8.

Wang J, Wang J, Li L, Zhang M, Zhang S. 2002b. Isolation, purification, and structure of *Lycium barbarum* polysaccharide LBP2a. ***Food Sci.*** 23(6): 44-48.

Wang L, Li J, Qian Y. 1995. Effect of LBP2 on the recovery of immunological function of the irradiation damaged mice. ***J Shanghai Immunol.*** 15:209-12.

Wang L, Wang G, Zhao Y, Qian Y. 1998. Effect of *Lycium barbarum* polysaccharides on rat peritoneal macrophage function *in vitro*. ***J Shanghai Immunol.*** 18-219.

Wang Q, Chen T. 1991. Polysaccharide content in *Lycium barbarum* fruit. ***Chin Trad Herb Drugs.*** 22:67-8.

Wang S, Chen L. 2004. T lymphocyte co-signaling pathways of the B7-CD28 family. ***Cell Mol Immunol.*** 1:37-42.

Wang X, Hu Q, Guan Y, Zhao C, Zhao W, W R. 1997. Induction of cytotoxic factors from mouse peritoneal macrophages by *Lycium barbarum* polysaccharides. ***Acta Chin Med Pharmacol.*** 37-8.

Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. 2003. The biology of IL-12: coordinating innate and adaptive immune responses. ***Cytokine Growth Factor Rev.*** 14:361-8.

Weber ML, Krammer P. 2003. Regulation of IL-4 gene expression by T cells and the therapeutic perspectives. ***Nat Rev Immunol.*** 3:534-543.

Weil R, Israel A. 2004. T-cell-receptor- and B-cell-receptor-mediated activation of NF-κB in lymphocytes. ***Curr Opin Immunol.*** 16:374-81.

Weston CR, Davis RJ. 2002. The JNK signal transduction pathway. ***Curr Opin Genet Dev.*** 12:14-21.

Weston CR, Davis RJ. 2007. The JNK signal transduction pathway. ***Curr Opin Cell Biol.*** 19:142-9.

Wilson NS, Villadangos JA. 2005. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. ***Adv Immunol***. 86:241-305.

Winter H, Fox BA. 1999. Adoptive cellular immunotherapy of cancer. ***Curr Opin Mol Ther.*** 1:89-97.

Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L et al. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. ***J Exp Med.*** 185:317-28.

Wu H, Guo H, Zhao R. 2006. Effect of *Lycium barbarum* polysaccharide on the improvement of antioxidant ability and DNA damage in NIDDM rats. ***Yakugaku Zasshi.*** 126:365-71.

Wu L. 2006. T lineage progenitors: the earliest steps en route to T lymphocytes. ***Curr Opin Immunol.*** 18:121-6.

Wu SJ, Ng LT, Lin CC. 2004. Antioxidant activities of some common ingredients of traditional Chinese medicine, *Angelica sinensis*, *Lycium barbarum* and *Poria cocos*. ***Phytother Res.*** 18:1008-12.

Xiao L, Bai X, Yang X. 2006. Effect of *Lycium barbarum* polysaccharide on apotosis of human lung cancer cells. ***J Math Med.*** 19:130-1.

Xin YF, Zhou GL, Deng ZY, Chen YX, Wu YG, Xu PS, Xuan YX. 2007. Protective effect of Lycium barbarum on doxorubicin-induced cardiotoxicity. ***Phytother Res.*** 21:1020-4.

Xing J. 1989. Effects of Ningxia wolfberry on the immune function and complement activity in Wistar rats. ***Bulletin Med Res.*** 18:4.

Yan ZQ, Hansson GK. 2007. Innate immunity, macrophage activation, and atherosclerosis. ***Immunol Rev.*** 219:187-203.

Yankner BA, Lu T, Loerch P. 2008. The aging brain. ***Annu Rev Pathol.*** 3:41-66.

Ye M, Graf T. 2007. Early decisions in lymphoid development. ***Curr Opin Immunol.*** 19:123-8.

Young G, Lawrence R, Schreuder M. 2005. Discovery of the Ultimate Superfood. ***Essential Science Publishing.***

Young HA. 2006. Unraveling the pros and cons of interferon-gamma gene regulation. ***Immunity.*** 24:506-7.

Yu MS, Ho YS, So KF, Yuen WH, Chang RC. 2006. Cytoprotective effects of *Lycium barbarum* against reducing stress on endoplasmic reticulum. ***Int J Mol Med.*** 17:1157-61.

Yu MS, Lai CS, Ho YS, Zee SY, So KF, Yuen WH et al. 2007. Characterization of the effects of anti-aging medicine Fructus lycii on beta-amyloid peptide neurotoxicity. ***Int J Mol Med.*** 20:261-8.

Yu MS, Leung SK, Lai SW, Che CM, Zee SY, So KF et al. 2005. Neuroprotective effects of anti-aging oriental medicine *Lycium barbarum* against beta-amyloid peptide neurotoxicity. ***Exp Gerontol.*** 40:716-27.

Zebisch A, Czernilofsky AP, Keri G, Smigelskaite J, Sill H, Troppmair J. 2007. Signaling through RAS-RAF-MEK-ERK: from basics to bedside. ***Curr Med Chem.*** 14:601-23.

Zenner G, Dirk zur Hausen J, Burn P, Mustelin T. 1995. Towards unraveling the complexity of T cell signal transduction. ***Bioessays.*** 17:967-75.

Zhang H, Wang Y. 2005. The protective effects of *Lycium barbarum* polysaccharides on alloxan-induced rat pancreatic islets damage. ***Chin J Cell Biol.*** 27:173-7.

Zhang M, Chen H, Huang J, Li Z, Zhu C, Zhang S. 2005. Effect of *Lycium barbarum* polysaccharide on human hepatoma QGY7703 cells: inhibition of proliferation and induction of apoptosis. ***Life Sci.*** 76:2115-24.

Zhang M, Hu Q, Jia B. 1994. Effects of *Lycium barbarum* polysaccharides on the peritoneal macrophages with quantitative cytochemical studies in mice. ***J Ningxia Med Coll.*** 16:307-10.

Zhang M, Wang J, Gan L, Zhang S. 2003a. Constituents and physical activities of *Lycium barbarum* polysaccharide-4. ***Food Ferment indust.*** 29(2):22-5.

Zhang M, Zhu C, Shi C, Xiao J, Zhang S. 2003b. Isolation, purification of *Lycium barbarum* polysaccharide-4 and its defatting effects on monosodium glutamate induced obesity on female mice. ***Nutr Hyg.*** 24:114-7.

Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. ***Cell.*** 92:83-92.

Zhang X, Li J. 1997a. Effects of *Lycium barbarum* polysaccharides on cell membrane mobility and the activity of protein kinase C *in vitro*. ***J Beijing Med Univ.*** 29:118-20.

Zhang X, Mosser D. 2008. Macrophage activation by endogenous danger signals. ***J Pathol.*** 214:161-78.

Zhang X, Xiang S, Cui X, Qian Y. 1997b. Effect of *Lycium barbarum* polysaccharides on the lymphocyte signal transduction system in mice. ***Chin J Immunol.*** 13:289-92.

Zhang Y，Xiang S，Zhou J. 1989. Effects of *Lycium barbarum* polysaccharides and their combination with *Corynebacterium parvum* on the peritoneal macrophages in mice. ***Chin J Pharmacol Toxicol.*** 3:169-74.

Zhao C, He Y, Li R, Cui G. 1996. Chemistry and pharmacological activity of peptidoglycan from *Lycium barbarum* L.. ***Chin Chem Lett.*** 7:1009-10.

Zhao C, Li R, He Y, Chui G. 1997. Studies on the chemistry of *Lycium barbarum* polysaccharides. ***J Beijing Med Univ.*** 29:231-2, 240.

Zhao H, Alexeev A, Chang E, Greenburg G, Bojanowski K. 2005. *Lycium barbarum* glycoconjugates: effect on human skin and cultured dermal fibroblasts. ***Phytomedicine.*** 12:131-7.

Zhao R, Li Q, Gao D, Xiao B, Liu L. 2007. Efect of *Lycium barbarum* polysaccharide on morphogeny and function of pancreatic islet B cells in N I DDM mice. ***J Yanshan Univ.*** 31:344-7.

Zhao R, Li Q, Xiao B. 2005. Effect of *Lycium barbarum* polysaccharide on the improvement of insulin resistance in NIDDM rats. ***Yakugaku Zasshi.*** 125:981-8.

Zhou Y, Tong S, Wang N, Zhao J, Li X. 2000. Effect of *Lycium barbarum* polysaccharides on the activities of intracellular enzymes and amount of NO produced by mouse macrophages. ***J Shandong Trad Chin Med.*** 19:361-2.

Zhou Z. 1991. Effect of *Lycium barbarum* polysaccharide on erythropoiesis and colony stimulating factors in normal mice. ***Chin J Hematol.*** 17:204-7.

Zhou Z, Zhou J, Xing S. 1991. Effects of *Lycium barbarum* polysaccharides on proliferation and differentiation of hemopoietic stem cells and granulocyte-monocyte progenitors in normal mice. ***Chin J Hematol.*** 5:44-6.

Zhu C, Zhang S. 2006a. Antitumor activity of *Lycium barbarum* polysaccharides in hepatoma H22-bearing mice. ***Chin J Pub Health.*** 22:717-8.

Zhu C, Zhang S. 2006b. The antitumor and immunoenhancement activity of *Lycium barbarum* polysaccharides in hepatoma H22-bearing mice. ***Acta Nutri Sinica.*** 28:182-3.

APPENDICES

Appendix I: Buffers and Solutions

The general format of this section is as such:

Chemicals required; Working concentration; Amount used.

Autoclaving for buffers and solutions was done at 121ºC for 15 min.

Buffers and Solutions for LBP Isolation and Characterization

Sevag Reagent

$CHCl_3$	160 ml
n-BuOH	40 ml

1 M NaCl

NaCl	58.5 g
Milli-Q water	Make up to 1000 ml

1 M KCl

KCl	74.55 g
Milli-Q water	Make up to 1000 ml

5% Phenol

Phenol	5.0 g
Milli-Q water	Make up to 100 ml

Protease Digestion Buffer

SDS	0.2 %
EDTA	10 mM

5× Sample Loading Buffer

SDS	10%
β-mercaptoethanol	10 mM
Glycerol	20% (v/v)
Tris-HCl	0.2 M, pH6.8
Bromophenol Blue	0.05%

10% Acrylamide Solution

Acrylamide	10 g
Bisacrylamide	2.5 g
Milli-Q water	Make up to 100 ml

8× Non-denaturing Stacking Gel Buffer (pH 6.8)

Tris Base	0.47 M (5.7 g)
TEMED	0.46 ml

The pH was adjusted to 6.8 with 1M H_3PO_4 and made up to 100 ml.

4× Non-denaturing Resolving Gel Buffer (pH 8.9)

Tris Base	1.5 M (18.2 g)
TEMED	0.23 ml

The pH was adjusted to 8.9 with 1M HCl and made up to 100 ml.

10% APS

Ammonium Persulfate	0.1 g
Milli-Q water	Make up to 1 ml

The solution was filtered and stored at 4ºC no longer than 2 weeks

Coomassie Blue Staining Solution

Coomassie Blue R250	0.6 g
Methanol	250 ml
Glacial Acetic Acid	50 ml
RO H_2O	200 ml

Destaining Solution

Methanol	400 ml
Glacial Acetic Acid	80 ml
RO H_2O	200 ml

10× Tris-Glycine SDS-PAGE Runing Buffer

Tris Base	250 mM	30.3 g
Glycine	1.92 M	144 g
SDS	10%	10 g
RO H_2O	Make up to 1000 ml	

10× Tris-Glycine Buffer

Tris Base	250 mM	30.3 g
Glycine	1.92 M	144 g
RO H_2O	Make up to 1000 ml, pH 8.3	

10× Non-Denaturing Gel Running Buffer (pH 8.3)

Tris base	50 mM	6.06 g
Glycine	384 mM	28.8 g
RO H_2O	Make up to 1000 ml, pH 8.3	

Buffers and Solutions for Cell Culture

RPMI-1640 Medium

RPMI-1640 medium powder	1 packet
HEPES	37.5 g
L-glutamine	3 g
Pyruvic acid sodium salt	1.1 g
Glucose	10 g
$NaHCO_3$	20 g

One packet of RPMI-1640 powder was dissolved in 5 L of ddH_2O followed by the addition of HEPES, L-glutamine, pyruvic acid sodium salt, and glucose. $NaHCO_3$ was added and the pH was adjusted to 7.20. The final volume was brought to 10 L. The medium was filtered through a 0.22-μm filter, aliquoted, and stored at 4ºC.

DMEM Medium

DMEM medium powder	1 packet
HEPES	37.5 g
L-glutamine	3 g
Pyruvic acid sodium salt	1.1 g
Glucose	10 g
$NaHCO_3$	20 g

One packet of DMEM medium powder was dissolved in 5 L of ddH_2O followed by the addition of HEPES, L-glutamine, pyruvic acid sodium salt, and glucose. $NaHCO_3$ was

added and the pH was adjusted to 7.20. The final volume was brought to 10 L. The medium was filtered through a 0.22-μm filter, aliquoted, and stored at 4ºC.

FBS (Fetal Bovine Serum)

500 ml of fetal bovine serum (1 bottle) was thawed in a water bath at 37 ºC followed by heat inactivated at 56ºC for 30 min. The heat-inactivated FBS was then aliquoted, and stored at 4ºC.

Penicillin-Streptomycin Stock Solution (100×)

penicillin	10000 units/ml
streptomycin	10 mg/ml

Complete Growth Medium

Penicillin-Streptomycin stock	1 ml
FBS	10 ml
RPMI-1640 (or DMEM)	Make up to 100 ml

Freezing Medium

FBS	10 ml
DMSO	10 ml
RPMI-1640 (or DMEM)	80 ml

10× PBS

NaCl	800 g
KCl	20 g
Na_2HPO_4	115 g
KH_2PO_4	20 g
ddH_2O	Make up to 10 L

Buffers and Solutions for Flow Cytometery

FACS PBS (pH 7.4)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
1% FBS	10 ml
Sodium Azide	1 g
ddH_2O	900 ml

The pH was adjusted to 7.4 and the final volume was brought to 1000 ml with ddH_2O. The solution was filtered through 0.22-µm filter and stored at 4ºC.

2% Paraformaldehyde

2 g of paraformaldehyde was added into 80 ml FACS PBS (pH 7.4). The mixture was heated to 56ºC until dissolved. The final volume was brought to 100 ml. The solution was filtered through 0.22-µm filter and stored at 4ºC.

PI Staining Solution

Propidium Iodide	0.4 mg
RNase	1 mg
Triton X-100	1 ml
PBS	Make up to 10 ml

Buffers and Solutions for RT-PCR

10× dNTP Stock Solution

dATP (100 mM) stock	2 mM	30 µl
dTTP (100 mM) stock	2 mM	30 µl
dCTP (100 mM) stock	2 mM	30 µl
dGTP (100 mM) stock	2 mM	30 µl

The mixture was made up to 1.5 ml with 3 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA.

The stock was stored at -20ºC.

Buffers and Solutions for Luciferase Assay

Lysis Buffer

Tris-phosphate	25 mM
$MgCl_2$	8 mM
DTT	2 mM
Triton X-100	1 %
Glycerol	10 %
1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid	2 mM

Buffers and Solutions for ELISA

Coating Buffer (pH 6.5)

Na_2HPO_4	11.8 g
NaH_2PO_4	16.1 g
ddH_2O	Make up to 1000 ml

Assay Diluent

PBS	900 ml
FBS	100 ml

The pH was adjusted to 7.0 and the solution was stored at 4ºC.

Wash Buffer

PBS	999.5 ml
Tween-20	0.5 ml

Appendix II: Publications

International Journal Papers

1. **Chen Z**, Tan BK, Chan SH. Activation of T lymphocytes by polysaccharide-protein complex from *Lycium barbarum* L. ***Int Immunopharmacol***. 2008;8:1663-71.

2. **Chen Z**, Soo MY, Srinivasan N, Tan BK, Chan SH. *Lycium barbarum* polysaccharide-protein complex is a potent stimulus of macrophage activation. *Submitted.*

3. **Chen Z**, Lu J, Srinivasan N, Tan BK, Chan SH. Polysaccharide-protein complex from *Lycium barbarum* L. is a novel stimulus of dendritic cell immunogenicity. *In revision (**J Immunol**).*

Conference Papers

1. **Chen Z**, Tan BK, Tay SW, Chan SH. Activation of T lymphocytes by polysaccharide-protein complex from a Chinese medicinal nutrient, *Lycium barbarum* L. ***Exp Biol.*** *(abstract & oral presentation).* 2008. *San Diego, U.S.A.*

2. **Chen Z**, Soo MY, Srinivasan N, Tan BK, Chan SH. *Lycium barbarum* polysaccharide-protein complex enhances innate immunity by activating macrophages. ***1st Int Sin Symp Immunol.*** *(abstract & poster).* 2008. *Singapore.*