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Regulation on maturation and function of dendritic cells by *Ganoderma lucidum* polysaccharides

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Abstract

Ganoderma lucidum polysaccharides (*Gl*-PS) exhibits a variety of immunomodulatory activities, and dendritic cells (DC) are professional antigen presenting cells, which are pivotal for initiation of primary immune response. In this study, the regulatory effects of *Gl*-PS on maturation and function of cultured murine bone marrow derived DC were investigated in vitro. *Gl*-PS (0.8, 3.2, or 12.8 μg/ml) could increase the co-expression of CD11c and I-A/I-E molecules on DC surface, promote mRNA expression of cytokine IL-12 p40 in DC and augment protein production of IL-12 P40 in culture supernatants. The lymphocyte proliferation of mixed lymphocyte culture (MLC) induced by mature DC was enhanced by *Gl*-PS, either. *Gl*-PS was shown to promote not only the maturation of cultured murine bone marrow derived DC in vitro, but also the immune response initiation induced by DC.

Keywords: *Ganoderma lucidum* polysaccharides (*Gl*-PS); Dendritic cells (DC); Mixed lymphocyte culture (MLC)

1. Introduction

Ganoderma lucidum polysaccharides (*Gl*-PS), one of the main efficacious ingredient of *G. lucidum* (Leyss, Ex Fr.) Karst (*Gl*), has been under modern pharmacological research in recent 30 years, and been reported to be effective in modulating immune functions, inhibiting tumor growth [1–4]. The immuno-modulation effects of *Gl*-PS were extensive, including promoting the function of mononuclear phygocyte system, humoral immunity and cellular immunity, and the site of *Gl*-PS were speculated to be located in the course of immune precursor cells proliferation and differentiating to effector cells. Recently, the antitumor effects of *Gl*-PS have been deeply investigated [2,5–8] and were believed to be going through immune mechanisms.

Dendritic cells (DC), a kind of important professional antigen-presenting cells (APC), are crucial for the initiation of primary immune response of both helper and cytotoxic T lymphocytes [9–12]. The property distinguishes them from all other APC is that it bearing sole responsibility for the stimulation of virgin T lymphocytes. Only small number of DC could be isolated from peripheral blood and secondary lymphoid organs, including spleen and lymph nodes [13]. Fortunately, large amount of DC now could be generated in vitro from bone marrow derived progenitor cells in the presence of GM-CSF and IL-4 [14], and they are therefore used to study the induction of T lymphocytes and anti-tumor immunity [15]. In the present study, we established the culture of murine bone marrow derived DC in vitro, and further explored whether *Gl*-PS has regulatory effects on maturation and function of DC.

2. Materials and methods

2.1. Mice

Male or female C57BL/6j (H-2^b) (Grade II, Certificate Number SCXK11-00-0004) and BALB/c (H-2^d) mice (Grade II, Certificate Number SCXK11-00-0004) were purchased from the Department of Experimental Animal, Health Science Center, Peking University, Beijing, China. Mice were used at 6–8 week of age.

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2.2. Drugs

Gl-PS were isolated from boiling water extract of *Gl*, followed by ethanol precipitation, dialysis and protein depletion using Sevag method. It was a kind of polysaccharides peptide with molecular weight of 584 900, and the ratio of polysaccharides to peptides is 93.51:6.49%. The polysaccharides were consisted of D-rhamnose, D-xylose, D-fructose, D-galactose, D-mannose and D-glucose with molar ratio of 0.793:0.964:2.944:0.167:0.389:7.94 and linked together by β-glycosidic linkages. The peptides contained 16 kinds of amino acid. *Gl*-PS was a kind of hazel powder and dissolved in serum-free RPMI media 1640 (Gibco BRL), then filtered through a 0.22 μm filter and stored at 4 °C. They were further diluted to indicated concentrations (0.8, 3.2, or 12.8 μg/ml) prior to each assay.

2.3. Preparation and culture of bone marrow derived DC

DC were prepared as described previously [14], with minor modifications. Briefly, bone marrow was flushed from the femura and tibiae of C57BL/6j mice, and DC were grown from precursors at a starting concentration of 2×10^6 cells per ml in complete RPMI media 1640 (RPMI media 1640 supplemented with 10% inactivated fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin) and cultured in six-well flat bottom plates (Falcon) at 37 °C, 5% CO_2 for 3 h and then non-adherent cells were washed out. 20 ng/ml rmGM-CSF (R&D Systems) and 20 ng/ml rmIL-4 (R&D Systems) were given to the culture. On day 3 and 5, half of the medium was replaced with fresh medium. On day 5, 1 μg/ml lipopolysaccharide (LPS, Sigma) was added into the culture. On day 7, non-adherent cells and culture supernatants were collected for further experiments and analysis. Bone marrow cells without cytokines stimulation were used as control.

2.4. Stimulation of DC by Gl-PS

DC were cultured as described above, except that on day 5, non-adherent cells were incubated at a concentration of 3×10^3/ml with indicated concentration of *Gl*-PS. Serum-free RPMI media 1640 were used as control.

2.5. Immunofluorescence staining and flow cytometry

Cell surface expression of I-A/I-E or CD11c was determined by immunofluorescence staining. Cells were washed twice with ice cold FACScan buffer (PBS containing 2% FCS and 0.1% sodium azide). The same buffer was used throughout for the incubation with antibodies as well as for all washes. Twenty percent of mixed serum of mice and rat were used to prevent non-specific antibody binding. R-PE conjugated anti-mouse I-A/I-E (06355A, Clone: M5/114.15.2, BD PharMingen) or FITC conjugated anti-mouse CD11c (09704A, Clone: HL3, BD PharMingen) were added, respectively, to the cells and the samples were left on ice for 45 min in the dark. The cells were then washed twice and fixed with 1% paraformaldehyde in FACScan buffer. The fluorescence on cell surface was detected under fluorescence microscope (Olympus). Cell surface co-expression of I-A/I-E and CD11c were determined by flow cytometric analysis. Immunofluorescence staining were carried out as above, except that R-PE conjugated anti-mouse I-A/I-E and FITC conjugated anti-mouse CD11c were added at the same time. Fluorescence profiles were generated on a FACScan flow cytometer (Becton Dickinson). Histogram and density plots were produced by the CellQuest software package. Dead cells and debris were gated out.

2.6. RNA extraction and semi quantitative RT-PCR

Cells were collected and washed once with diethyl pyrocarbonate (DEPC)-treated PBS, and the total RNA were extracted using TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol. RNA precipitates were dissolved in DEPC-treated water containing RNasin(R) Ribonuclease Inhibitor (Promega). The concentration of RNA was determined spectrophotometrically. The mRNA expression of IL-12 p40 in DC was performed by reverse transcription polymerase chain reaction (RT-PCR) using Access RT-PCR system (Promega) according to the manufacturer's protocol. To ensure that equal amounts of starting material were used in each RT-PCR reaction, the RNA was reverse transcripted and amplified with β-actin-specific primers. The cycles number of PCR amplification was 28, which was chosen to ensure that amplification of all specific cDNA products was exponential (data not shown). Specific primers sequences of IL-12 p40 unit [16] and β-actin [17] were as follow and the size of production were 312 and 478 bp, respectively:

- IL-12 p40 sense primer: 5′-cgtgctcatggctggtgcaaag-3′.
- IL-12 p40 antisense primer: 5′-cttcatctgcaagttcttgggc-3′.
- β-actin sense primer: 5′-agggaaatcgtgggtgacatcaaa-3′.
- β-actin antisense primer: 5′-actcatcgtactcctgcttgctga-3′.

All of the products were electrophoresed on 2% agarose gel and stained with ethidium bromide. By using Gel Doc 2000 system and Quantity One software (BIO-RAD), the two coamplified bands were quantified and expressed as a ratio of intensity (IL-12 p40 to β-actin).

2.7. Cytokine assay

The level of mouse IL-12 P40 unit in culture supernatants was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Jingmei Biotech) according to the manufacturer's protocol. Microtiter plates were coated with specific antibodies to capture IL-12 P40 in DC culture supernatants. A second layer antibody was then added. Cytokine concentrations were determined with a standard curve derived from known amounts of the relevant cytokine using absorbance readings at 450 nm on a spectrophotometer (BIO-RAD). The minimum detection level of IL-12 P40 was 24 pg/ml.

2.8. Mixed lymphocyte culture (MLC) induced by DC

Mature DC was pretreated with 25 μg/ml mitomycin and mononuclear lymphocytes from splenocytes were isolated by Ficoll-Urografin density gradient. H-2^d BALB/c responder spleen lymphocytes (1×10^6 cells per well) were cultured with H-2^b C57BL/6j inducer DC (1×10^3 cells per well). The cells were plated into 96-well flat bottom tissue culture plates (Falcon) and stimulated with *Gl*-PS at indicated concentrations for 5 days. Serum-free RPMI media 1640 were used as control. Cell proliferation was estimated based on the cellular reduction of tetrazolium salt MTT by the mitochondrial dehydrogenase of viable cells into a blue formazan product that can be measured spectrophotometrically [18].

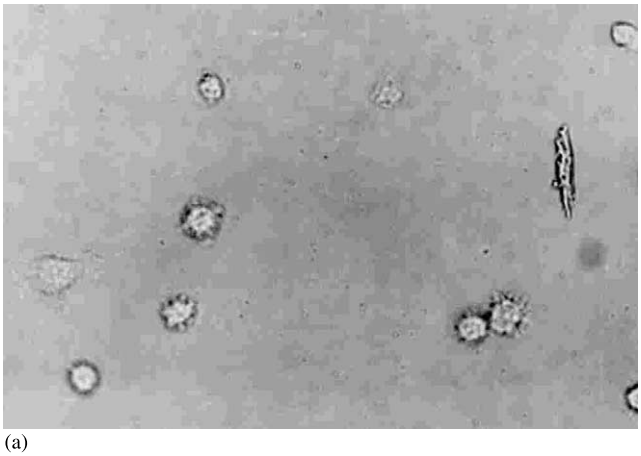
2.9. Statistical analysis

Statistical significance was determined by one-way ANOVA followed by least-significant difference (LSD). *P* values below 0.05 were considered significant.

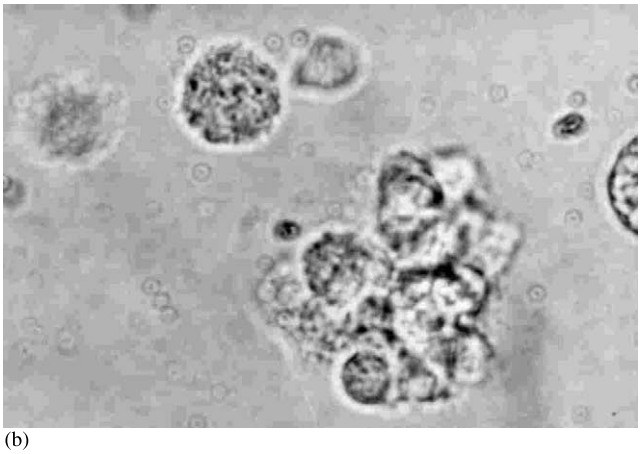
3. Results

3.1. Maturation of bone marrow derived DC

When bone-marrow derived DC were cultured for 7 days, it was enlarged and displayed typical stellate morphology under microscopy (Fig. 1); CD11c and I-A/I-E were expressed under immunofluorescence microscopy (Fig. 2); Flow cytometric analysis showed that CD11c and I-A/I-E molecules were co-expressed on cell surface, while none was observed on bone-marrow cells without cytokines stimulation (Fig. 3); RT-PCR displayed that mRNA of IL-12 p40 was highly expressed (Fig. 4), and the protein level of IL-12 P40 in culture supernatants were 704.92 pg/ml with ELISA assay, which was undetectable in bone-marrow cells without cytokines stimulation.



(a)



(b)

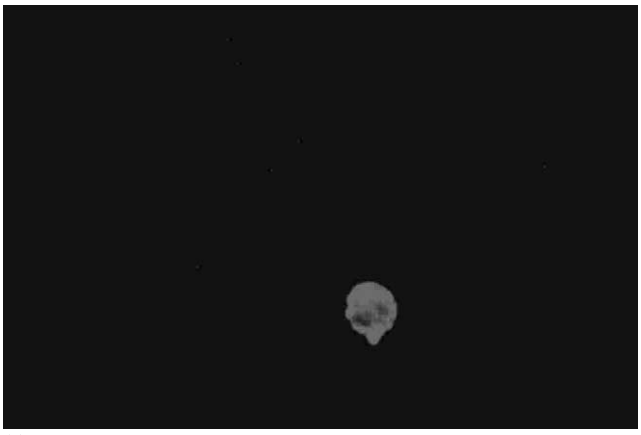
Fig. 1. Morphology of mature DC on day 7. DC were grown from precursors of bone marrow of C57BL/6j mice in complete RPMI media 1640 containing 20 ng/ml rmGM-CSF and 20 ng/ml rmIL-4. One μg/ml LPS was added into the culture on day 5. Non-adherent cells were photographed under a microscope on day 7. (a) 10 × 20 and (b) 10 × 40.

3.2. Regulation of Gl-PS on DC surface phenotype

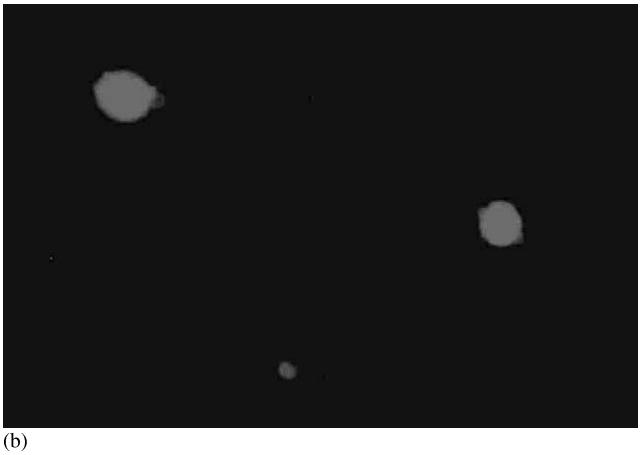
Gl-PS (0.8, 3.2, or 12.8 μg/ml) treated DC showed an increased co-expression of CD11c and I-A/I-E molecules on cell surface, and the cell ratios were 46.1, 51.0 or 51.4%, respectively, compared with 44.9% of RPMI medial 1640 (Fig. 5).

3.3. Regulation of Gl-PS on IL-12 p40 mRNA expression of DC

The mRNA of IL-12 p40 were highly expressed in DC treated with or without *Gl*-PS. *Gl*-PS (0.8, 3.2, or 12.8 μg/ml) could increase the mRNA expression of IL-12 p40 of DC. The intensity ratio of IL-12 p40 to β-actin in RPMI media 1640 treated DC was 65.4 ± 5.2%, while for DC treated with three concentrations of *Gl*-PS, they were 65.5 ± 6.0, 73.9 + 7.5 or 76.9 ± 10.8%, respectively (Fig. 6).



(a)



(b)

Fig. 2. Immunofluorescence staining on DC surface. DC were grown from precursors of bone marrow of C57BL/6j mice in complete RPMI media 1640 containing 20 ng/ml rmGM-CSF and 20 ng/ml rmIL-4. One μg/ml LPS was added into the culture on day 5. On day 7, non-adherent cells were stained with (a) FITC conjugated anti-mouse CD11c or (b) R-PE conjugated anti-mouse I-A/I-E on ice for 45 min in the dark. The fluorescence on cell surface was detected under fluorescence microscope (Olympus) (10 × 20).

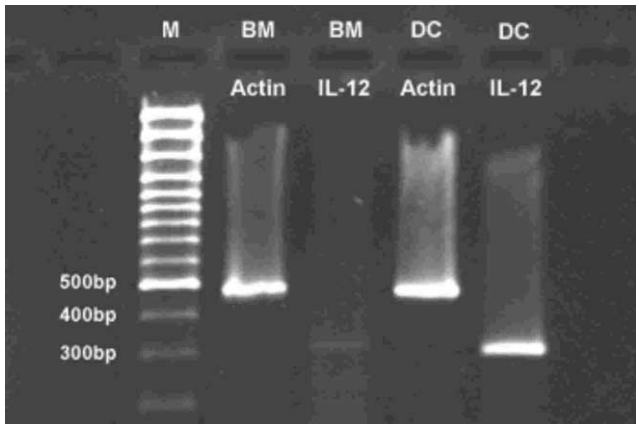


Fig. 4. mRNA expression of IL-12 p40 in bone marrow cells (BM) without stimulation and DC on day 7. DC were grown from precursors of bone-marrow of C57BL/6j mice in complete RPMI media 1640 containing 20 ng/ml rmGM-CSF and 20 ng/ml rmIL-4. One μg/ml LPS was added into the culture on day 5. On day 7, non-adherent cells were collected, and total RNA were extracted using TRIzol Reagent. The mRNA expression of IL-12 p40 was performed by RT-PCR using Access RT-PCR system, and β-actin was used as internal control. All of the products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

3.4. Regulation of Gl-PS on IL-12 P40 production of DC

The production of IL-12 P40 in culture supernatants of DC were augmented by *Gl*-PS (0.8, 3.2, or 12.8 μg/ml), which were 123.8 ± 12.9, 143.0 ± 14.6, or 158.5 ± 14.7 pg/ml, respectively, while it was 120.8 ± 7.1 pg/ml in DC treated with RPMI media 1640, and significant difference was observed between RPMI media 1640 control and 12.8 μg/ml *Gl*-PS group (Fig. 7).

3.5. Regulation of Gl-PS on MLC induced by DC

The effects of *Gl*-PS on MLC reactions induced by DC were illustrated in Fig. 8. With their concentrations

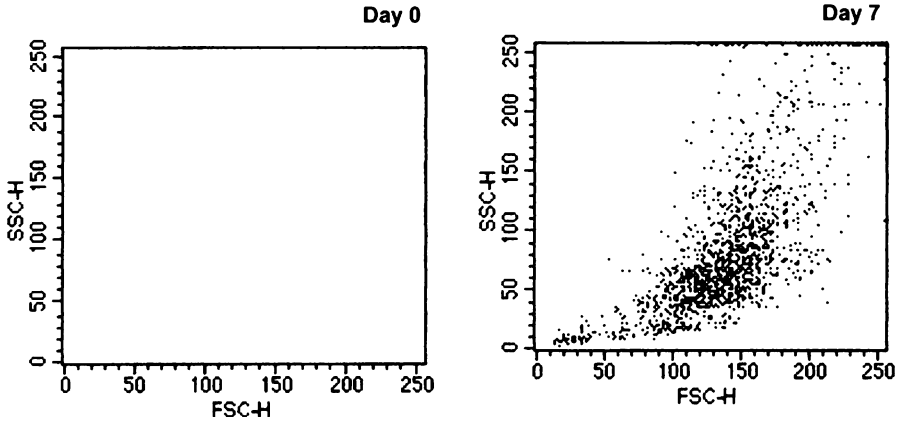


Fig. 3. Flow cytometric analysis of bone marrow cells without stimulation (Day 0) and DC on day 7 (Day 7). DC were grown from precursors of bone marrow of C57BL/6j mice in complete RPMI media 1640 containing 20 ng/ml rmGM-CSF and 20 ng/ml rmIL-4. One μg/ml LPS was added into the culture on day 5. On day 7, non-adherent cells were stained with R-PE conjugated anti-mouse I-A/I-E and FITC conjugated anti-mouse CDllc on ice for 45 min in the dark. Fluorescence profiles were generated on a FACScan flow cytometer (Becton Dickinson). Histogram and density plots were produced by the CellQuest software package. FSC-H: FITC conjugated anti-mouse CDllc; SSC-H: R-PE conjugated anti-mouse I-A/I-E.

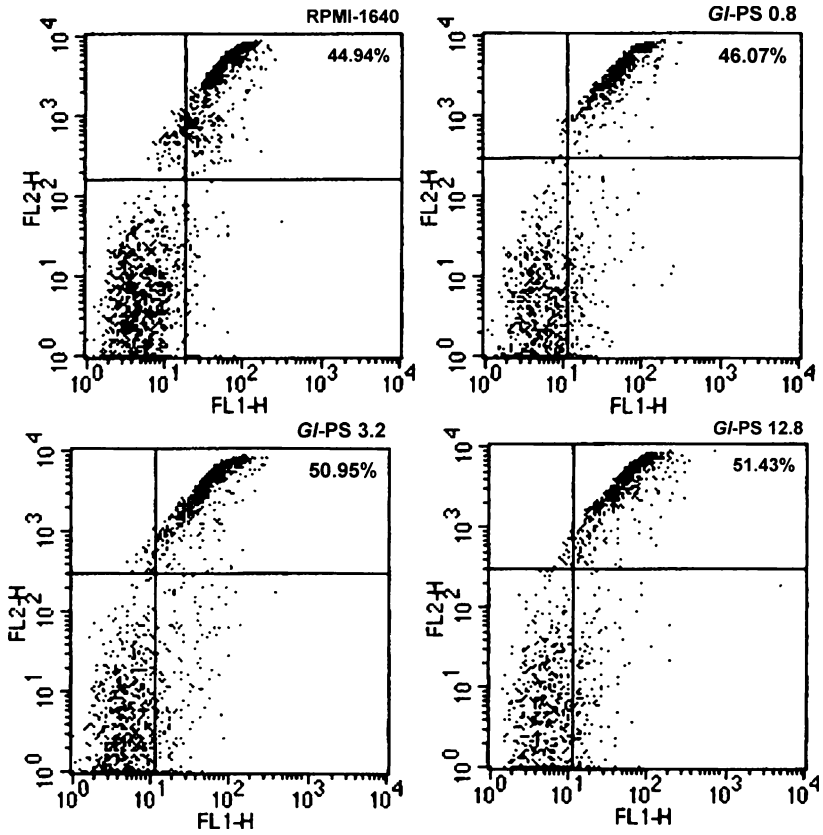


Fig. 5. Flow cytometric analysis of DC treated with RPMI media 1640 or *Gl*-PS. On day 5 of DC culture, non-adherent cells were incubated at a concentration of 3×10^3/ml with 1 μg/ml LPS and indicated concentration of *Gl*-PS (0.8, 3.2, or 12.8 μg/ml). Serum-free RPMI media 1640 were used as control. On day 7, non-adherent cells were stained with R-PE conjugated anti-mouse I-A/I-E and FITC conjugated anti-mouse CDllc on ice for 45 min in the dark. Fluorescence profiles were generated on a FACScan flow cytometer (Becton Dickinson). Histogram and density plots were produced by the CellQuest software package. FL1-H: FITC conjugated anti-mouse CDllc; FL2-H: R-PE conjugated anti-mouse I-A/I-E.

ranging within 0.8–12.8 μg/ml, MLC reactions were significantly improved. Compared with RPMI media 1640 control, the lymphocytes proliferation was increased by 11.6, 20.9 or 20.4%, respectively (RPMI media 1640 control served as 100%).

4. Discussion

DC can differentiate from immature to mature stages. Immature DC has a high capability for antigen capture and processing. The immature cells differentiate to mature cells in response to cytokines such as TNFα or pathogen products such as LPS [9]. GM-CSF was used to stimulate the precursors differentiating to immature DC, IL-4 acted as a prevention of immature DC differentiating to macrophages, while LPS promoted the maturation of DC. Mature DC demonstrate characteristic morphology, with enlarged size and numerous cytoplasmic processes giving rise to a stellate appearance, and maturation of DC is characterized by a decreased antigen processing capacity, an increased cell surface expression of MHCII and co-stimulatory molecules, and secretion of IL-12, priming strong stimulation of $CD4^+$ and $CD8^+$ T lymphocytes growth and differentiation [9–12]. Typical stellate morphology, co-expression of cell surface phenotype of I-A/I-E (MHC II), a marker of maturation, and CD11c, a kind of DC marker, mRNA expression and protein secretion of IL-12 p40 unit indicated the maturation of cultured DC. Mature DC could secret IL-12 P70, the active protein, and then priming Thl type lymphocytes transformation. IL-12 P40 is a subunit of IL-12 P70, whose expression is inducible and correlated with the production of bioactive P70 by DC; while another covalently linked subunit, IL-12 P35, is encoded on separate genes and constitutively expressed by a variety of cell types [19,20]. Several researchers have demonstrated the immunomodulatory effects of *Gl*-PS on T lymphocytes [21–25]. Autologous mixed lymphocyte reaction (AMLR) is a kind of specific immune reaction model stimulated by autologous antigen and depletion of adherent cells of splenocytes decreased the effect of *GI*-B on automatic proliferation and IL-2 production [25]. Since adherent cells of splenocytes include DC, we presumed that immunomodulatory effects of *Gl*-PS maybe have relation to DC.

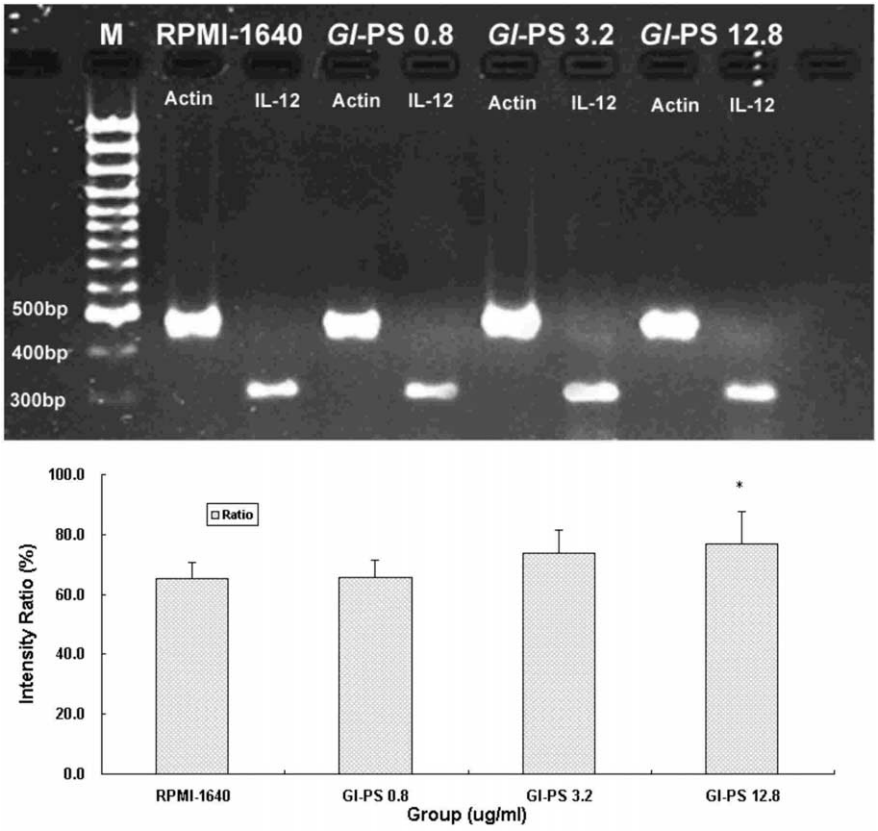


Fig. 6. mRNA expression of IL-12 p40 in DC treated with RPMI media 1640 or *Gl*-PS. On day 5 of DC culture, non-adherent cells were incubated at a concentration of 3×10^3/ml with 1 μg/ml LPS and indicated concentration of *Gl*-PS (0.8, 3.2, or 12.8 μg/ml). Serum-free RPMI media 1640 was used as control. On day 7, non-adherent cells were collected, and total RNA were extracted using TRIzol Reagent. The expression of IL-12 p40 mRNA was performed by RT-PCR using Access RT-PCR system, and β-actin was used as internal control. All of the products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The two coamplified bands were quantified by using Gel Doc 2000 system and Quantity One software (BIO-RAD), and expressed as a ratio of intensity (IL-12 p40 to β-actin). $^*P < 0.05$ vs. RPMI media 1640.

Our research showed that *Gl*-PS up-regulated the co-expression of I-A/I-E and CDllc on DC surface, mRNA expression and protein secretion of IL-12 p40 unit, which indicated that *Gl*-PS could promote the maturation of DC in the presence of LPS. On the other hand, the up-regulation of co-expression of I-A/I-E and CDllc on DC surface also indicated the mechanism of *Gl*-PS promoting the maturation of DC maybe have relation to its' effect on I-A/I-E expression, Thurnher et al. [26] showed that DC differentiation into $CD83^+$ dendritic

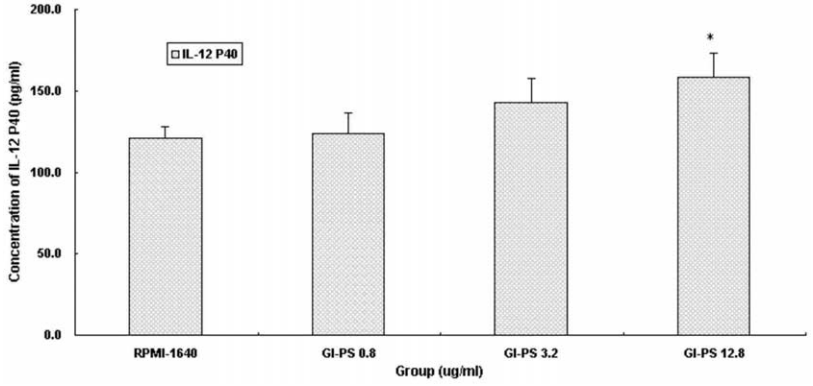


Fig. 7. Production of IL-12 P40 in culture supernatants of DC treated with RPMI media 1640 or G*l*-PS. On day 5 of DC culture, non-adherent cells were incubated at a concentration of 3×10^3/ml with 1 μg/ml LPS and indicated concentration of *Gl*-PS (0.8, 3.2, or 12.8 μg/ml). Serum-free RPMI media 1640 was used as control. On day 7, culture supernatants were collected for detecting the level of mouse IL-12 P40 (ELISA). Cytokine concentrations were determined with a standard curve derived from known amounts of the IL-12 P40. The minimum detection level of IL-12 P40 was 24 pg/ml. $^*P < 0.05$ vs. RPMI media 1640.

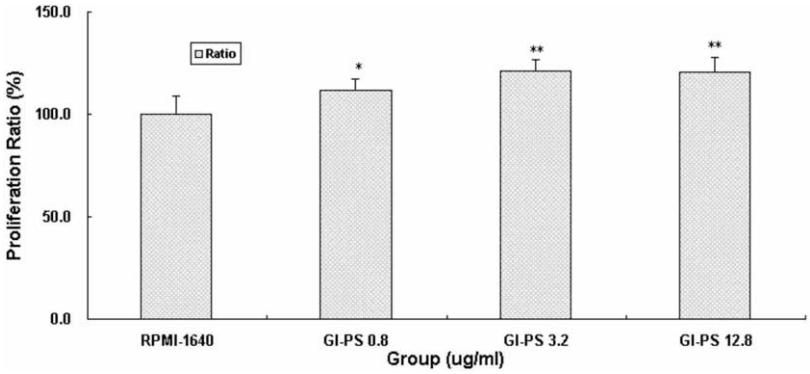


Fig. 8. Lymphocytes proliferation of MLC induced by DC. Mature DC was pretreated with 25 μg/ml mitomycin and mononuclear lymphocytes from splenocytes were isolated by FicolI–Urografin density gradient. H-2^d BALB/c responder spleen lymphocytes (1×10^6 cells per well) were cultured with H-2^b C57BL/6j inducer mature DC (1×10^3 cells per well). The cells were plated into 96-well flat bottom tissue culture plates (Falcon) and stimulated with 67-PS at indicated concentrations for 5 days. Serum-free RPMI media 1640 were used as control. Cell proliferation was estimated by MTT method. *$P < 0.05$ vs. RPMI-1640; **$P < 0.01$ vs. RPMI-1640.

cells required a low cell concentration (5×10^5/ml) when treated with TNFα, and the expression of CD83 was suppressed (1×10^6/ml) or almost prevented (2×10^6/ml) at higher cell density, inhibiting the maturation of DC. Our results were obtained when the concentration of DC was 3×10/ml, indicating that *Gl*-PS maybe more effective when the immune function was lower.

The primary function of DC includes activation and differentiation of naive T lymphocytes [9–12]. The one-way MLC we used depleted the stimulation of T lymphocytes on DC, and made it possible to focus on T lymphocyte proliferation induced by DC. Mature DC has potent activity of priming T lymphocytes, only a small number of DC was needed, which was much lower than routine MLC, approximately one in 1000 (1×10^4/ml:1×10^7/ml). Our results confirmed that Gl-PS could promote the proliferation of one-way MLC induced by DC, indicating the modulating effects of *Gl*-PS on innate immune response primed by mature DC.

Acknowledgements

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