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Research Article

Acetylation Modification Improves Immunoregulatory Effect of Polysaccharide from Seeds of *Plantago asiatica* L.

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The current study was conducted to investigate effects of acetylated *Plantago asiatica* L. polysaccharides (Ac-PLCPs) on their immunoregulatory activities in bone-marrow-derived dendritic cells (DCs) model. Influences of natural *Plantago asiatica* L. crude polysaccharide (PLCP) and Ac-PLCPs on inducing phenotypic and functional maturation on DCs were determined. The results showed that Ac-PLCPs with degree of substitution (DS) from 0.06 to 0.1 could not only stimulate the expression of surface molecules major histocompatibility complex class II (MHC II), cluster of differentiation 86 (CD86), and CD80 on DCs ($P < 0.01$) but also increase the secretion of cytokine IL-12p70 ($P < 0.01$). The endocytosis activity of DCs was attenuated by Ac-PLCPs treatment ($P < 0.01$), while the mRNA expressions of chemokine receptors CCR7 and CXCR4 in DCs were significantly increased ($P < 0.01$). Besides, DCs treated with the Ac-PLCPs showed extremely strong T cell proliferation stimulating activity ($P < 0.01$). These data showed that Ac-PLCPs had higher maturation-stimulating activity on DCs than PLCP, which indicated that acetylation modification improved the immunoregulatory effect of PLCP.

1. Introduction

Bioactive polysaccharides have attracted extensive attention worldwide, and they are now considered as one functional component of traditional medicine. A great deal of researches indicate that bioactivity of polysaccharide is closely correlated with various structural parameters, such as molecular weight, monosaccharide composition, branches, conformation, and substituent group. Recently, a growing body of research implicates that appropriate structure modification can improve bioactivity of polysaccharide [1]. So far, lots of chemical modifications have been applied to modulate physicochemical or biological properties of polysaccharides, such as carboxymethylation [2, 3], sulfation [4], acetylation [5], phosphorylation [6], and oxidation [7] modification.

Acetyl content in some natural polysaccharides has been demonstrated to play an important role in their bioactivities. For example, the lymphocyte proliferation stimulating activity of polysaccharide from *Dendrobium nobile* mainly depended on the acetyl groups [8, 9]. Acetylated *Ulva pertusa* [10], *Laminaria japonica* polysaccharide [11], and *Enteromorpha linza* polysaccharide [12] have higher antioxidant activity. Acetylated *Grifola frondosa* polysaccharide peptides can inhibit glioma C6 cell line *in vitro*, and the combination treatment of cyclophosphamide and the acetylated polysaccharide peptides can significantly decrease total sialic acid levels in tumor-bear mice [13]. Acetyl fucoidan from *Cladosiphon okamuranus* could activate murine macrophage cell line, RAW 264.7 cells, through membrane receptor, toll-like receptor 4 (TLR4), cluster of differentiation 14 (CD14), and scavenger receptor class A [14].

Psyllium is a common name used for the plant genus *Plantago*. In western countries, dietary fiber from psyllium has been used extensively as both pharmacological supplements and food ingredients. Ripe seeds of *Plantago asiatica* L. are a traditional medicine used as antipyretic, diuretic, and expectorant treatment in China. Our previous study showed that polysaccharide from seeds of *Plantago asiatica* L. was a high branched heteroxylan consisting of β-1,4-linked Xyl*p* backbone with side chains attached to O-2 or O-3 position. And its side chains consist of β-T-linked Xyl*p*, α-T-linked Ara*f*, α-T-linked GlcA*p*, β-Xyl*p*-(1→3)-α-Ara*f*, and α-Ara*f*-(1→3)-β-Xyl*p* [15]. In particular, the polysaccharide was a potential immune adjuvant since it could promote

maturation of mouse bone-marrow-derived dendritic cells *in vitro* [16].

In the present study, *Plantago asiatica* L. crude polysaccharide (PLCP) were acetylated using acetic anhydride as acetylation reagent, and bioactivities of the acetylated polysaccharides (Ac-PLCP) were investigated on dendritic cells model.

2. Materials and Methods

2.1. Preparation of Acetylated Plantago asiatica L. Polysaccharides. PLCP was prepared as previously described [17]. Briefly, water-soluble polysaccharide was extracted from the seeds of *Plantago asiatica* L. by boiling water for 2 h and precipitated with 80% (v/v) ethanol. Then the polysaccharide was deproteinised with sevage reagent, a mixture of chloroform and n-butyl alcohol at a ratio of 4 : 1 (v/v), dialysed against double distilled water for 48 h (Mw cut-off, 8000–14,000 kDa), and obtained by freeze-drying.

Acetylation modification of PLCP was carried out using acetic anhydride as acetylation reagent [18]. One hundred milligram PLCP was firstly dissolved in 10 mL distilled water (pH 9.0). The solution was maintained at 30°C with continuous stirring. Acetic anhydride (400–2000 μL) was added to the solution dropwise within 2 h (pH 8.5–9.0). After the modification reaction, the mixture was neutralized with 1 M HCl and dialysed against double distilled water for 48 h (Mw cut-off: 8000–14,000 kDa). The acetylated PLCP (AC-PLCP) was obtained by freeze-drying.

The degrees of substitution (DS) of the acetyl group in the Ac-PLCPs were determined as described [19]. Briefly, 40 mg Ac-PLCP was dissolved in 20 mL 0.01 M NaOH. The solution was maintained at 50°C for 2 h, then excess NaOH was back-titrated with 0.01 M HCl using phenolphthalein indicator. Four Ac-PLPCs with gradient degrees of substitution, that is, DS of 0.027 (Ac-PLCP I), 0.059 (Ac-PLCP II), 0.082 (Ac-PLCP III), and 0.11 (Ac-PLCP IV), were selected to investigate the immune regulatory activity in dendritic cell model.

In addition, the bacterial endotoxins in PLCP and Ac-PLCPs were determined using tachypleus amebocyte lysate test kit (Chinese Horseshoe Crab Reagent Manufactory, Co., Fujian Province, China). The quantity of endotoxin was estimated to be ≤0.015 endotoxin unit (EU) per mg in all polysaccharide samples.

2.2. Animals. 4- to 6-week-old BALB/c (H-2K^d and I-A^d) and C57BL/6 (H-2K^d and I-A^d) mice were purchased from Hunan SJA Laboratory Animal, Co. (Hunan Province, China). All animals were kept according to the Care and Use of Laboratory Animals Guidelines published by the United States National Institute of Health (NIH Publication 85-23, 1996). All experimental procedures were approved by the Animal Ethnics Committee, Nanchang University.

2.3. Generation of Bone-Marrow-Derived Dendritic Cells (DCs). Bone marrow of BALB/c mice was sacrificed to obtain dendritic cells. According to our previous report [16], bone-marrow cells were flushed out with RPMI 1640 medium, then recombinant murine granulocyte macrophage-colony stimulating factor (rmGM-CSF, 10 ng/mL) combined with recombinant murine interleukin-4 (rmIL-4, 10 ng/mL, both from R&D system) was used to generate immature dendritic cells.

2.4. Determination of Phenotypic and Functional Maturation of DCs. Immature DCs were stimulated with the PLCP or Ac-PLCPs (100 μg/mL) for 48 h, and then the phenotypic and functional maturation of DCs were determined according to the methods reported previously [20].

Briefly, the cells were collected and incubated with FITC-conjugated monoclonal antibody [anti-major histocompatibility complex (MHC) class II, anti-CD80 (B7-1), and anti-CD86 (B7-2), eBioscience] for 1 h, and then the mean fluorescence intensity was determined by using FACSCalibur flow cytometer (BD Biosciences, USA). The endocytosis activity of DCs was also determined by flow cytometry using FITC-dextran (40,000 Da, Sigma). The quantity of the cytokine IL-12p70 in the culture supernatant was measured using Mouse IL-12p70 Enzyme Immunoassay kit (Wuhan Boster Biological Technology, China).

The migration activity of DCs was investigated by determining the mRNA expression of chemokine mRNA of CCR7 and CXCR4. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Then cDNA was synthesized using RevertAid ™ First Strand cDNA Synthesis Kit (Thermo Scientific, Maryland, USA). PCR products were analyzed by GoldView-agarose gel electrophoresis on 2% (w/v) agarose gel and visualized under UV light. The signals of target genes were measured by scanning densitometry and normalized to β-actin using Quantity One software. The forward and reverse PCR primers used for CCR7 were 5′-GCCTTCCTGTGTGATTTCTACAG-3′ and 5-TCACCTTCTCTCCTTTCTGTCAC-3′; for CXCR4 were 5′-TGTTGCCATGGAACCGATCA-3′ and 5′-GGATCC-AGACGCCCACATAG-3′; for β-actin were 5′-TGGCAC-CACACCTTCTACAATG-3′ and 5′-CCTGCTTGCTGA-TCCACATCTG-3′, respectively. Condition of reverse transcription system was 91°C for 5 min, 4°C for 5 min, 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 4°C for 5 min. Condition of PCR system was initial denaturation at 94°C × 3 min, followed by 25 cycles of denaturation at 94°C × 30 s, annealing at 60°C × 30 s, extension at 72°C × 2 min, and final extension at 72°C for 10 min.

Besides, splenic T lymphocytes were purified from C57BL/6 (H-2K^d and I-A^d) mice using Pan T Cell Isolation kit II (Miltenyi Biotec, Inc., Auburn, USA) and then cocultured with DCs for another 48 h. The proliferation of T cells was determined using MTT Cell Proliferation and Cytotoxicity Assay kit (Beyotime, Shanghai, China).

2.5. Statistical Analysis. Results data were analyzed using SPSS statistical software (version 17.0) and expressed as mean ± standard deviation (SD) of the indicated number of experiments. The statistical significance was estimated using a Student's t-test. $P < 0.05$ and $P < 0.01$ were considered as statistically significant and highly significant, respectively.

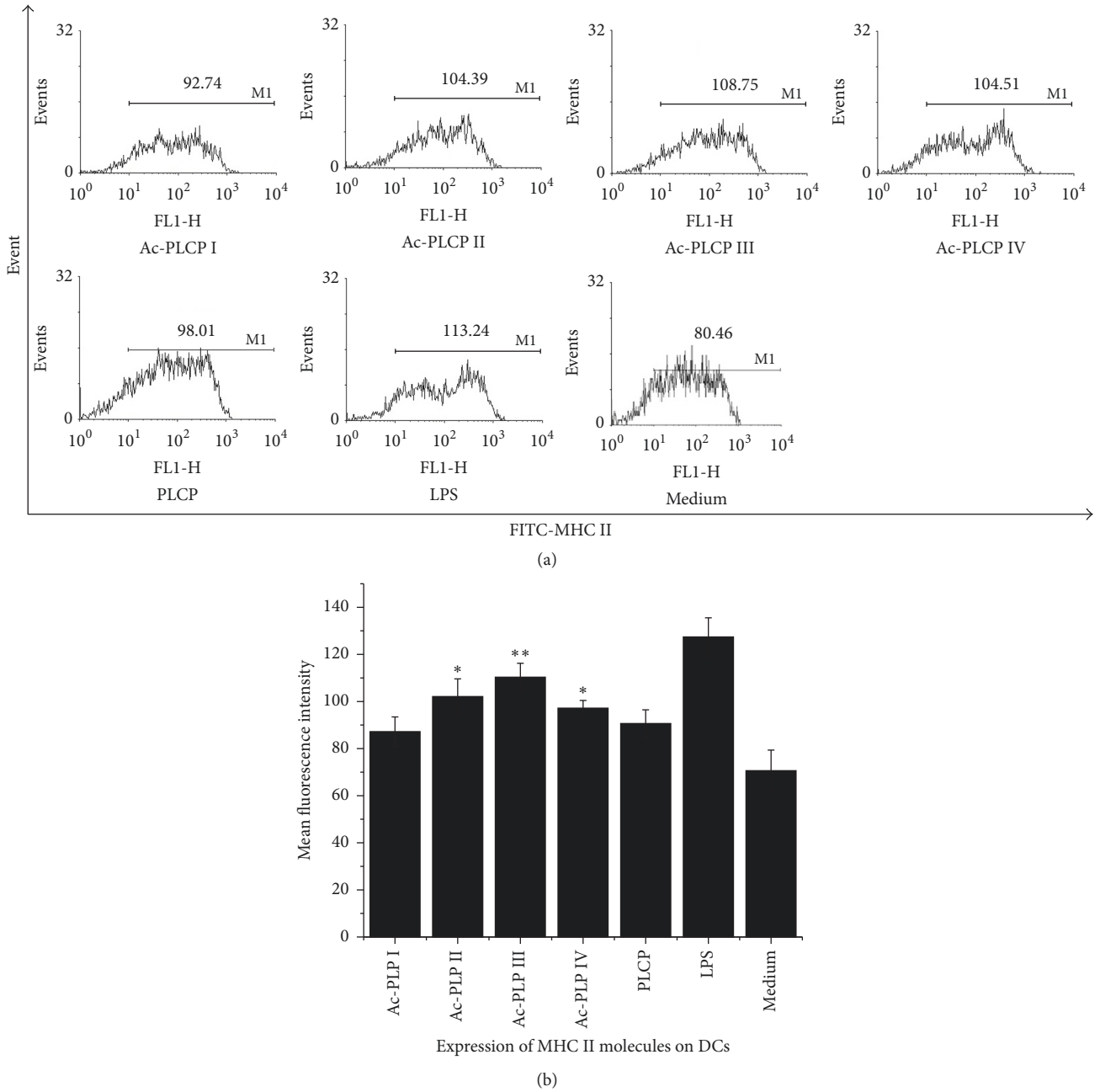
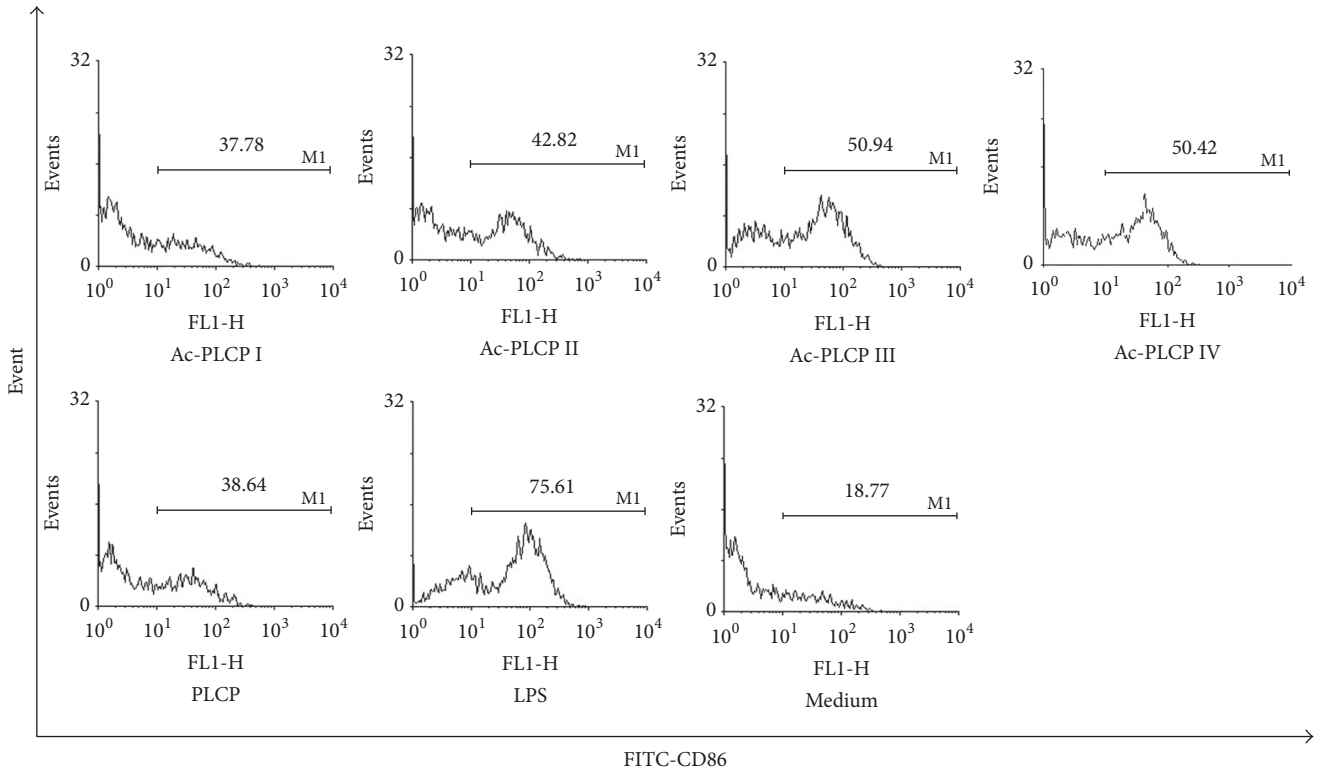


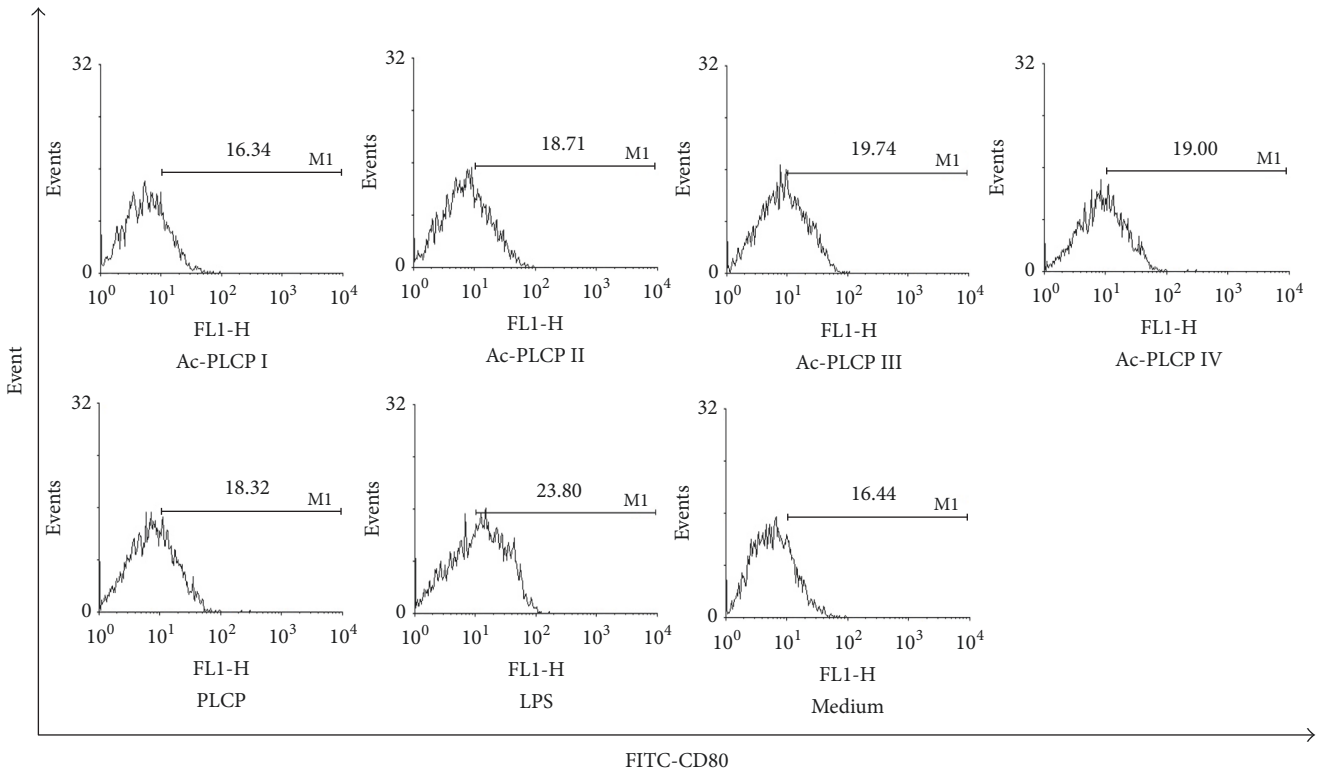
Figure 1: Ac-PLCP increased the expression of MHC II molecules on DCs. Immature DCs were treated with 100 μg/mL PLCP or Ac-PLCPs for 48 h. Then, the expression levels of MHC II molecules were determined by flow cytometry. The data were shown as mean fluorescence intensity (MFI). Compared with medium group, DCs treated with PLCP expressed higher level of MHC II molecules. While compared with the PLCP group, significant increases in MFI were observed in Ac-PLCP II, III, and IV groups. The results shown were from one representative experiment of three independent experiments performed. $^{*}P < 0.05$ compared to the PLCP group; $^{**}P < 0.01$ compared to the PLCP group.

3. Results

3.1. Ac-PLCP Enhanced Expression of MHC II, CD80, and CD86 Molecules in DCs. Immune responses were initiated through the specific recognition of antigens by lymphocytes, and this progress required stable adhesion of the T cells to the antigen-presenting cells (APCs), efficient antigen presentation, and transduction of signals from the cell surface to the nucleus of T cell. In particular, T cells could only recognize and respond to peptide antigens bound to and displayed by MHC molecules. Flow cytometry was applied to determine the expression of MHC II on DCs, and the data were shown as mean fluorescence intensity (MFI) in Figure 1. After incubation with PLCP, the expression of MHC II on DCs was increased from 80.46 to 98.01. Meanwhile, the expressions of MHC II in Ac-PLCP II, III, and IV groups were 104.39 ($P < 0.05$), 108.75 ($P < 0.01$), and 104.51 ($P < 0.05$), respectively, which were significantly higher than that of PLCP

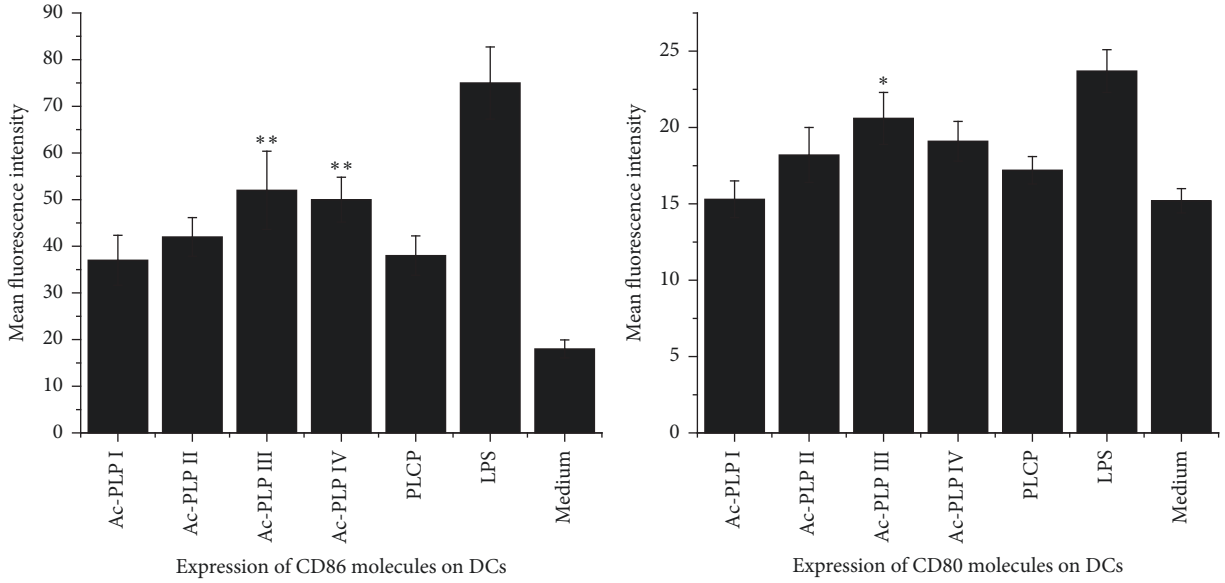


(a)



(b)

Figure 2: Continued.



(c)

Figure 2: Ac-PLCP increased the expression of costimulating molecules on DC. DCs treated with Ac-PLCPs III and IV expressed a higher level of CD86 (a) and CD80 (b) molecules than PLCP group. The results shown were from one representative experiment of three independent experiments performed. $^{*}P < 0.05$ compared to the PLCP group; $^{**}P < 0.01$ compared to the PLCP group.

group. Costimulating molecules are necessary to ensure an effective immune response. The expressions of CD86 and CD80 costimulating molecules were also measured using flow cytometry. As shown in Figure 2(a), the expression levels of CD86 in DCs in Ac-PLCP I, II, III, and IV groups were 37.78, 42.82, 50.94, and 50.42, respectively, which were much higher than that in the control group (18.77) ($P < 0.01$). In particular, the expression levels of CD86 in Ac-PLCPs III and IV were significantly higher than PLCP group ($P < 0.01$). As shown in Figure 2(b), the expression of CD80 molecules was notably stimulated by Ac-PLCP III (19.74) compared with PLCP group (16.44) ($P < 0.05$).

3.2. Ac-PLCP Stimulated Secretion of IL-12p70 in DCs. Beyond interaction with T cells through surface molecules, DCs also regulate the immune response by secreting cytokines. As shown in Figure 3, PLCP increased the secretion of IL-12p70 in DCs from 20.38 pg/mL (control group) to 49.97 pg/mL (PLCP group), while the cytokine levels of IL-12p70 in the acetylated polysaccharides-treated groups were 33.11 pg/mL (Ac-PLCP I), 60.51 pg/mL (Ac-PLCP II), 96.43 pg/mL (Ac-PLCP III), and 81.06 pg/mL (Ac-PLCP IV), respectively. Compared with PLCP group, Ac-PLCP III and Ac-PLCP IV groups were significantly enhanced ($P < 0.01$).

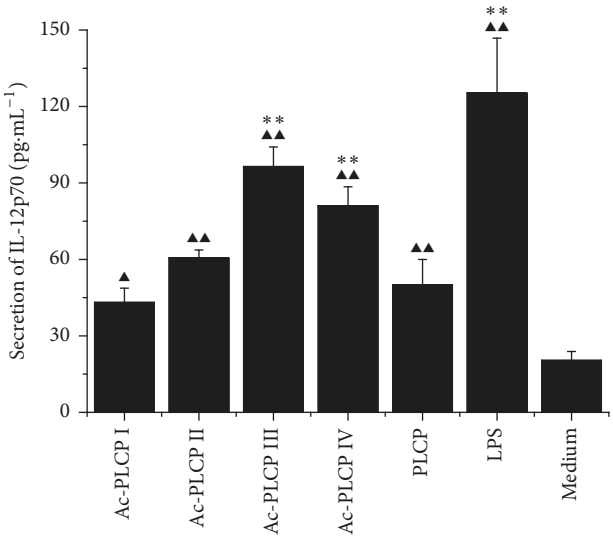


Figure 3: Ac-PLCP enhanced the secretion of IL-12p70 cytokine on DCs. Quantity of IL-12p70 in the culture supernatant was determined by ELISA method. Compared with PLCP group, the secretion of DCs in the Ac-PLCPs III and IV was significantly higher than that in PLCP group. $^{**}P < 0.01$ compared to the PLCP group; $^{▲}P < 0.05$ compared to the medium group; $^{▲▲}P < 0.01$ compared to the medium group.

3.3. Ac-PLCP Attenuated the Endocytosis of DCs. Although DCs are found in almost all tissues in the body, most of the cells exist in immature states. The immature DCs are not professional in antigen peptide presenting but antigen uptaking. However, once the immature DCs receive mature signal, they would develop maturation and transform from antigen-uptake cells into antigen-presenting cells. As shown in Figure 4, DCs in the control group (medium, 37°C) showed a dramatically high uptake of FITC-dextran. Compared with the control group, DCs in the PLCP and Ac-PLCP groups exhibited an attenuated endocytosis. Particularly, the MFI determined in the Ac-PLCP III (55.17) and IV groups (56.28) was significantly lower than PLCP group (67.60) ($P < 0.01$).

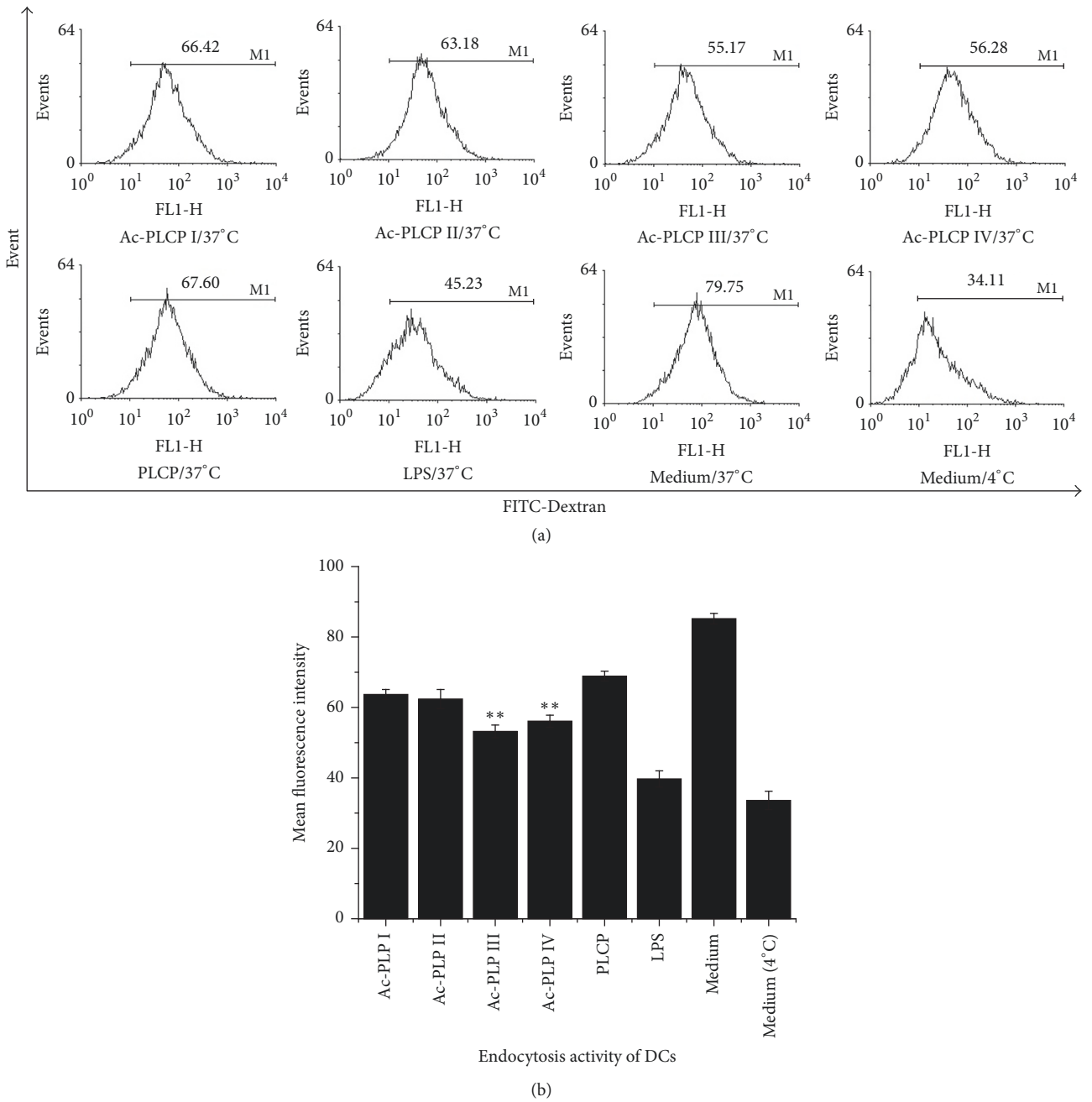


Figure 4: Ac-PLCP attenuated the endocytosis activity of DCs. The endocytosis activity was also determined by flow cytometry. The uptake of FITC-dextran of DCs in medium group was extremely high. However, endocytosis activity was significantly attenuated by PLCP treatment. In particular, DCs in Ac-PLCP III and IV groups exhibited much weaker uptake of FITC-dextran. The results shown were from one representative experiment of three independent experiments performed. $^{**}P < 0.01$ compared to the PLCP group.

3.4. Ac-PLCP Increased Expressions of CCR7 and CXCR4 Chemokine mRNA in DCs. The mRNA expression levels of chemokine receptors CCR7 and CXCR4 were analyzed using reverse transcription PCR to investigate the migration capability of DCs. As shown in Figure 5, notable increase was observed in the expressions of both CCR7 and CXCR4 mRNA in Ac-PLCP III and Ac-PLCP IV groups. In particular, the chemokine mRNA expression level in Ac-PLCP III or IV group was significantly higher than PLCP group ($P < 0.01$).

3.5. Ac-PLCP Treated DCs Stimulated T Lymphocyte Proliferation. Splenic T lymphocyte was purified and cocultured with DCs at a ratio of 10 : 1 for 48 h, and then the proliferation of T cells was determined by MTT assay. The proliferation index was calculated as absorbance ratio of polysaccharide group to control group. As shown in Figure 6, compared with control group, the proliferation index was significantly increased in all the polysaccharide groups ($P < 0.01$). The proliferation indexes in Ac-PLCP I and Ac-PLCP II groups were 124.21%

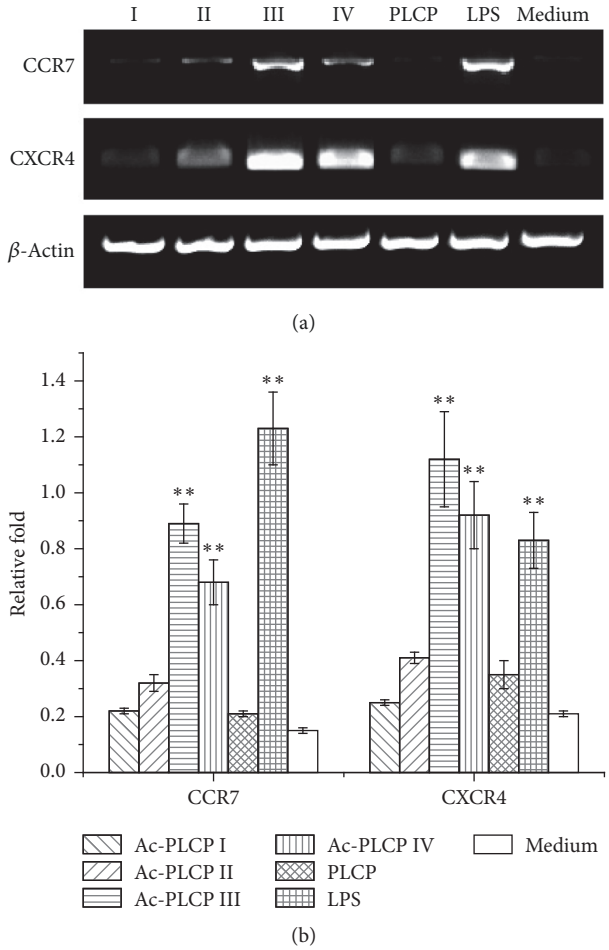


Figure 5: mRNA synthesis levels of CCR7 and CXCR4 were determined through reverse transcriptase PCR. The PCR product was analyzed by 1.5% agarose gel electrophoresis. The mRNA expression levels of both CCR7 and CXCR4 were significantly increased in Ac-PLCP III and IV groups. Besides, the expression of β-actin in each group was determined as internal control, and there was no significant difference among all the groups. $^{**}P < 0.01$ compared with PLCP group.

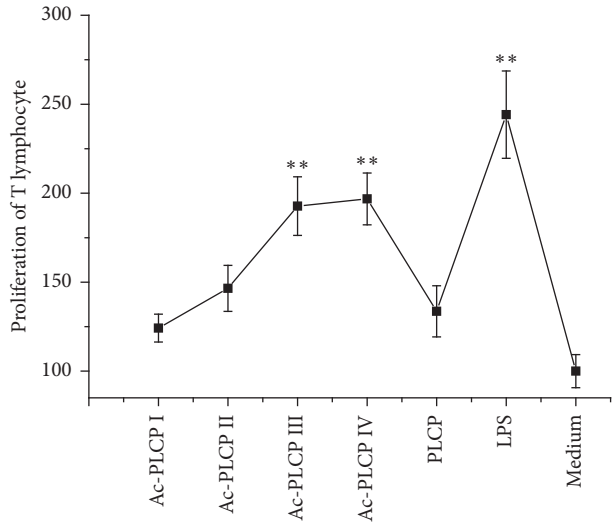


Figure 6: DCs treated with Ac-PLCPs showed stronger activity on stimulating T lymphocyte proliferation. T lymphocytes were obtained from C57BL/6 (H-2K^d and I-A^d) mice and cocultured with DCs in 96-well plate at the ratio of 10 : 1 for 48 h. The absorption value of the medium group was set at control values in the calculation of % proliferation. All the polysaccharide-treated DCs showed extremely strong T cell proliferation stimulating activity. Compared with PLCP group, DCs in Ac-PLCPs III and IV exhibited significantly enhanced T cell proliferation stimulating activity. $^{**}P < 0.01$ compared to the PLCP group.

and 146.52%. And the proliferation indexes in Ac-PLCP III and IV groups were 192.75% and 196.81%, respectively, which were dramatically higher than that in PLCP group (133.62%) ($P < 0.01$).

4. Discussion

DCs are professional antigen-presenting cells and essential mediators of innate and adaptive immune response, as well as tolerance [21]. Immature DCs are strategically located in dermis or mucosal system to capture antigen and then transport these antigens to regional lymph nodes, where they develop into mature and activate lymphocyte to initial immune response. However, immature DCs can weakly activate naïve T lymphocyte for lacking of antigen-presenting molecules. MHC molecules T cell receptor interaction and CD80(B7-1)/CD86(B7-2)-CD28 surface molecules interaction between APCs and T cells have been regarded as a key point to induce T cell activation [22]. Though immature dendritic cells synthesize large amounts of MHC II molecules, such MHC II molecules form $\alpha\beta$-dimers that exhibit intracellular distributions. The $\alpha\beta$-dimers are targeted to late endosomes and lysosomes where they reside unproductively with internalized antigens; however, after exposure to inflammatory mediators or microbial products, the newly formed immunogenic MHC II complexes are transported from lysosomes to plasma membrane [23]. In particular, the half-life of MHC II molecules increases from about 10 h to over 100 h during this process [24]. Ac-PLCPs III and IV were more effective in inducing DCs maturation than PLCP, since the expressions of MHC II molecules on DCs of the Ac-PLCP III and IV groups were significantly higher than that of PLCP group. Besides the primary signal delivered to T cells through MHC II molecules, the B7-CD28 interaction is one of the dominant costimulatory signals. Particularly, for naïve T cells that express only limited number of costimulatory receptors, CD28 is an indispensable receptor required for T cell priming [25]. The B7-CD28 costimulation signals could direct the development and function of T cells. Lenschow et al. found that effector and regulatory T cell responses were impaired in mice lacking CD28 or in mice lacking CD80 and CD86. [26]. Gimmi et al. reported that antigen presentation in absence of B7 costimulation results in human T cell clonal anergy [27]. We found that Ac-PLCP III and IV incubation significantly promoted the expression of CD86 molecules on DCs as well, which strongly suggests that the acetylated polysaccharides

could induce phenotypic maturation on DCs. Meanwhile, an obvious decline of endocytosis activity on DCs was also observed, which indicates that the immature dendritic cells transform from antigen-capturing cells into the mature antigen-presenting cells.

Besides the ligand-receptor interaction, DCs could regulate the polarization of T lymphocytes by secreting various cytokines. IL-12 is a proinflammatory molecule produced primarily by monocytes/macrophages and DCs. And IL-12 principally activates natural killer cells and induces the differentiation of native $CD4^+$ T cells to become interferon-γ-producing T helper 1 (Th1) effectors in cell-mediated immune responses [28]. IL-12 could also synergize with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells [29]. It was reported that $CD1c^+$ myeloid DC but not plasmacytoid DC (pDC) in humans can induce high level of cytotoxic molecules in naïve T cells by producing high amount of IL-12 [30]. The secretion of IL-12p70 of DCs was promoted by Ac-PLPC III and IV treatments, and the levels of IL-12p70 secretion in these two groups were significantly higher than PLCP group. Consistently, we also found that DCs incubated with Ac-PLPC III or IV could significantly stimulate proliferation of naïve T cell *in vitro*. Furthermore, DCs in Ac-PLCP III and IV groups showed an extremely strong effect on provoking sizeable mixed lymphocyte reaction when cocultured with splenic T cells, which indicates that the acetylated polysaccharides stimulated functional maturation on DCs. All these findings support that DCs treated with Ac-PLCPs III and IV may possess much more powerful immune regulatory activity than the natural polysaccharide.

Optimal encounter with naïve T cells for the presentation of antigens requires DCs to migrate to secondary lymphoid organs, which is governed by chemokine. The key chemokines directing DCs migration are chemokine (C-C motif) ligand 19 (CCL19), CCL21, and chemokine (C-X-C motif) ligand 12 (CXCL12) [31, 32]. As receptor for CCL19 and CCL21, CC chemokine receptor CCR7 has been found to mediate the migration of DCs from skin into lymphatic vessels [33]. However, the migration of skin DC was initiated by CXCL12-CXCR4 engagement [34]. For plasmacytoid DCs, the migration into splenic white pulp was regulated by CCR7 signal coordinate with CXCR4 signal [35]. And the chemokine signal also enhanced DCs maturation and survival [36]. The migration capability of DCs was enhanced by Ac-PLCP III and IV treatments evidenced by the increased mRNA expression levels of CCR7 and CXCR4.

Dendritic cells are equipped with a battery of pattern-recognition receptors (PRRs) that can detect molecular patterns of invading microorganisms or endogenous signals and alter the immune response. The most widely studied family of PRRs on DCs is Toll-like receptors. In particular, TLR-4 expressed on the surface of DCs was demonstrated to be one to recognize polysaccharides [37, 38]. DCs in intestinal mucosa tissue were found to be able to open the tight junctions between epithelial cells without breaking the integrity of the epithelial barrier [39], which provides a probable route for the polysaccharide to interact with DCs. We have also found that TLR-4 mediates the maturation of DCs induced by PLCP [16]. The acetylation modification may enhance the immunoregulatory activities of PLCP by improving the solubility of PLCP and decreasing the viscosity of the polysaccharide.

In conclusion, Ac-PLCPs exhibited higher maturation-stimulating activities on DCs compared with the natural polysaccharide. In particular, the Ac-PLCPs of DS ranging from 0.06 to 0.1 showed the best immunoregulatory activities. The current study also provided a new insight into the structure-activity relationship of PLCP and the utilization of the seeds of *Plantago asiatica* L.

Abbreviations

CD: Cluster of differentiation
Ac: Acetylated
DCs: Dendritic cells
DS: Degree of substitution
MHC: Major histocompatibility complex
PLCP: *Plantago asiatica* L. crude polysaccharide
IL-12: Interleukin-12.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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