International Immunopharmacology 22 (2014) 324–331

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Carboxymethylation enhances the maturation-inducing activity in dendritic cells of polysaccharide from the seeds of *Plantago asiatica* L.

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ARTICLE INFO

Article history:
Received 31 December 2013
Received in revised form 22 April 2014
Accepted 12 June 2014
Available online 26 June 2014

Keywords:
Carboxymethylation
Dendritic cells
Plantago asiatica L.
Polysaccharide

ABSTRACT

Carboxymethylation is a well-known modification process for polysaccharides. To evaluate the biological availability of carboxymethyl, polysaccharide from the seeds of *Plantago asiatica* L. (PLCP) was carboxymethylated (CM-PLCP) and the immunomodulatory activities of five CM-PLCPs of gradient degree of substitution (DS) from 0.40 to 0.62 were determined on dendritic cells (DCs) in vitro. Compared with DCs treated with PLCP, DCs treated with CM-PLCP of $DS_{0.50}$, $DS_{0.55}$, $DS_{0.62}$, as well as CD86 and CD80, expressed higher levels of MHCII, CD86 and CD80 surface molecules. In addition, the secretion of IL-12p70 and the mRNA of CCR7 and CXCR4 chemokines were increased, while the endocytosis activities were inhibited. Correspondingly, stronger mixed lymphocyte reactions were induced by the DCs treated with the CM-PLCPs. The results showed that carboxymethylation modification of relevant high DS can enhance the DC maturation-inducing function of PLCP, indicating the potential application of carboxymethylated polysaccharide as an immunotherapeutic adjuvant.

1. Introduction

In the past decades, numerous studies come to the viewpoint that natural polysaccharides have a broad spectrum of immunological function. Recently, semi-synthetic polysaccharides and derivatives from natural polysaccharides prepared by chemical or enzymatic modification have been reported to exhibit high biological properties [1]. For the advantage of low cost of the chemicals and the non-toxicity of the products, carboxymethylation reactions are applied to natural polymers, such as chitosan [2], starch [3] and kappa-carrageenan [4], to obtain biomaterials in a wide variety of fields.

Plantaginaceae is the largest genus of the cosmopolitan family and some *Plantago* species are confirmed to have beneficial bioactivities, such as anti-inflammatory, anti-cancer and anti-oxidant activities [5–8]. *Plantago asiatica* L., a traditional Chinese herbal medicine, is used in the treatment of many diseases. We have recently reported the structure and bioactivities of crude polysaccharide from the seeds of *P. asiatica* L. (PLCP). It contains three fractions, i.e., PLP-1, PLP-2 and PLP-3. The main fraction of PLP-2 is a highly branched heteroxylan which consisted of a β-1,4-linked Xylp backbone with side chains attached to *O*-2 or *O*-3 [9]. PLP-3 was found to be arabinoxylan which consisted of a β-1,4-linked Xylp backbone with short side chains attached to its *O*-2 (1,2,4-linked Xylp, 17.87%) or *O*-3 (1,3,4-linked Xylp, 24.24%) positions [10]. Interestingly, we found that PLCP could induce the maturation of dendritic cells (DCs) [11], which orchestrate the innate and adaptive immunity. So far, whether the carboxymethylation modification would influence the immunoregulation activity of PLCP is still unknown.

In the present study, the effect of five carboxymethylated PLCPs (CM-PLCPs) on DCs were determined. The main purpose was to determine whether carboxymethylation modification could raise the immune enhancement of PLCP.

2. Materials and methods

2.1. Preparation of PLCP and CM-PLCPs

The seeds of *P. asiatica* L. were purchased from Ji'an County, Jiangxi Province, China, and air dried before use. The species were identified by Dr. Cui-sheng Fan, Jiangxi University of Traditional Chinese Medicine, Nanchang, China.

Dried seeds of *P. asiatica* L. were extracted three times with tenfold volumes of distilled water at 90 °C for 2 h. The combined supernatant was concentrated at 55 °C. A fourfold volume of ethanol was added to the concentrated solution to precipitate polysaccharide. The precipitate was redissolved and deproteinized using the Sevag method. The solution was dialyzed (8000–14,000 kDa) against distilled water for 48 h and lyophilized to yield PLCP.

CM-PLCPs were prepared from PLCP in aqueous alkaline solution using monochloroacetic acid (MCA) as an etherifying agent [12]. Approximately 100 mg PLCP was dissolved in 10 mL distilled water, and then 10 mL of 10 M sodium hydroxide solution was added. The mixture

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http://dx.doi.org/10.1016/j.intimp.2014.06.027
1567-5769/

was blended for 10 min, and then MCA (1.89–2.84 g) was added. The mixture was heated to 50 °C, and maintained for 2 h. The mixture was neutralized with 3 M HCl and dialyzed (8000–14,000 kDa) against distilled water for 48 h. CM-PLCPs were obtained by freeze-drying. The degree of substitution (DS) of CM-PLCP was determined by the acid-washed method [13]. Five CM-PLCPs, CM-PLCPI (DS = 0.40), CM-PLCPII (DS = 0.44), CM-PLCPIII (DS = 0.50), CM-PLCPIV (DS = 0.55) and CM-PLCP V (DS = 0.62) were selected to determine the maturation inducing activity on DCs.

2.2. Detection of endotoxin

Gel-clot method of tachypleus amebocyte lysate test was applied to detect bacterial endotoxins in PLCP and CM-PLCPs. 100 μL of PLCP and CM-PLCPs solution (2 mg/mL) was mixed with 100 mL of tachypleus amebocyte lysate reagent (Chinese Horseshoe Crab Reagent Manufactory Co., Fujian Province, China) and incubated for 1 h at 37 °C. Each tube was then examined for gelation. The quantity of endotoxin was estimated to be ≤0.015 endotoxin unit (EU) per mg of PLCP and CM-PLCPs.

2.3. Animals

Female 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) and kept under specific pathogen-free conditions at 25 °C, 40–60% relative humidity and 12/12 h light/dark cycle.

All animals used in this study were cared for 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 involving the use of animals were approved by the Animal Care Review Committee, Nanchang University.

2.4. Preparation of DCs

DCs were generated from the bone marrow of BALB/c (H-2K^d and I-A^d) mice using granulocyte/macrophage colony-stimulating factor (GM-CSF) combined with interleukin-4 (IL-4) (R&D Systems) [14]. Bone marrows from the femurs and tibias were flushed out with basic RPMI1640 (Beijing Solarbio Science & Technology Co., China). Red cells were lysed with Tris–NH_4Cl (16.96 mM Tris, 139.6 mM HCl, pH 7.2). The bone marrow cells were cultured with RPMI1640 for 3 h at a concentration of 2×10^6 cells/mL. Then the supernatant containing non-adherent cells was depleted, and fresh RPMI1640 supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone), 50 μM β-mercaptoethanol, 10 ng/mL recombinant mouse (rm) GM-CSF and 10 ng/mL rmIL-4 were fed back. On the 2nd day, all the non-adherent cells in the medium were removed by centrifugation. On the 3rd day, the concentration of rmGM-CSF was added up to 20 ng/mL. Approximately 50% of the medium was exchanged with fresh RPMI1640 every two days. On the 5th day, newly generated non-adherent and loosely adherent cells were harvested as immature DCs. These immature DCs were generally >75% CD11c$^+$.

Immature DCs were stimulated with 100 μg/mL CM-PLCPs, 100 μg/mL PLCP, 1 μg/mL LPS respectively in 6-well plates for 48 h. On the 7th day, DCs were collected by vigorous pipetting.

2.5. Fourier transform infrared (FT-IR) spectral analysis

The FT-IR analysis of CM-PLCPs was performed with a Nicolet 5700 FT-IR spectrophotometer (Thermo Electron, Madison, WI, USA) between 500 and 4000 cm^{-1} wavenumbers using the KBr disc method.

2.6. Flow cytometric analysis of cell surface molecules

The surface molecules of DCs were analyzed by flow cytometry using fluorescein-labeled monoclonal antibodies (mAbs) to major histocompatibility complex (MHC) II, CD80 (B7-1) and CD86 (B7-2) (eBioscience). Cells were collected, washed twice with an ice cold staining buffer (PBS containing 2% FBS and 0.1% NaN_3). Pellets were resuspended and blocked with 10% (v/v) goat serum for 10 min at 4 °C. Then the fluorescein-labeled mAbs were added and incubated at 4 °C for another 1 h. After incubation, cells were washed three times and resuspended in a 500 μL staining buffer and analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA).

2.7. Endocytosis assay

The endocytosis of DCs was analyzed by flow cytometry using FITC-dextran (40,000 Da, Sigma) and PE-conjugated anti-CD11c antibody (eBioscience). 1×10^6 DCs were first incubated with 1 mg/mL FITC-dextran at 37 °C for 40 min. After incubation, DCs were washed twice with ice cold staining buffer and then stained with PE-CD11c. In addition, parallel experiments were performed at 4 °C to show that the uptake of dextran by DCs was inhibited at low temperatures.

2.8. ELISA assay for IL-12p70

Levels of IL-12p70 were determined in the supernatant by ELISA using Mouse IL-12p70 enzyme immunoassay kits (Wuhan Boster Biological Technology Co., Hubei Province, China) according to the manufacturer's instructions. The absorbance at 450 nm was determined by Thermo Scientific Varioskan Flash (Thermo, USA).

2.9. Allogenic mixed lymphocyte reaction (MLR)

DCs treated with 20 μg/mL mitomycin C (Sigma) were used as the stimulator. Lymphocytes from the spleen of C57BL/6 (H-2K^d and I-A^d) mice were separated using a lymphocyte separation medium (Beijing Solarbio Science & Technology Co., China). Then, T cells were obtained using a Pan T cell isolation kit II (Miltenyi Biotec Inc., Auburn, USA) as the responder.

The DC stimulator (1×10^4 cells/well) and T cell responder (1×10^5 cells/well) were co-cultured in 96-well plates for 48 h. Cell proliferation was estimated using an MTT cell proliferation and cytotoxicity assay kit (Beyotime, Shanghai, China). The absorbance at 570 nm was determined by Thermo Scientific Varioskan Flash (Thermo, USA).

2.10. Reverse transcription polymerase chain reaction (RT-PCR) for chemokine receptors CCR7 and CXCR4

Total RNA from DCs was extracted using a TRIzol reagent (Invitrogen) and single-strand cDNA was synthesized by a RevertAid^{TM} First Strand cDNA synthesis kit (Thermo). Then, PCR was subjected to 25 cycles using primer pairs (Table 1) and Taq PCR MasterMix (KT201, TianGen Biotech, Beijing, China). PCR amplification conditions were: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. The PCR products were separated through 1.5% agarose gel, stained with GoldView

Table 1
Oligonucleotide primers for CCR7, CXCR4 and β-actin.

Gene	Primer sequence	
CCR7	Forward	5′-GCCTTCCTGTGTGATTTCTACAG-3′
	Reverse	5′-TCACCTTCTCTCCTTTCTGTCAC-3′
CXCR4	Forward	5′-TGTTGCCATGGAACCGATCA-3′
	Reverse	5′-GGATCCAGACGCCCACATAG-3′
β-Actin	Forward	5′-TGGCACCACACCTTCTACAATG-3′
	Reverse	5′-CCTGCTTGCTGATCCACATCTG-3′