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A polysaccharide isolated from *Pueraria lobata* enhances maturation of murine dendritic cells

Hyung Sook Kim[a,1], Bo Ram Shin[a,1], Hong Kyung Lee[a], Yeon Jin Kim[a], Mi Jung Park[a], Sung Yeon Kim[b], Mi Kyeong Lee[a], Jin Tae Hong[a], Youngsoo Kim[a], Sang-Bae Han[a,*]

[a] College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 361-763, South Korea
[b] College of Pharmacy, Wonkwang University, Chonbuk 570-749, South Korea

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ABSTRACT

Maturation of dendritic cells (DCs) is a critical factor for initiating the immune response. However, DC maturation is usually attenuated in the tumor microenvironment, which is an important immunological problem in DC-based immunotherapy against cancer. Here, we report the effect of a polysaccharide (PLP) isolated from *Pueraria lobata* on phenotypic and functional maturation of DCs. Phenotypic maturation was demonstrated by increased expression of CD40, CD86, and major histocompatibility complex I/II. PLP induced functional maturation of DCs, as shown by increased production of interleukin (IL)-12, IL-1β, and tumor necrosis factor-α, decreased antigen capture capacity, and enhanced allogenic T cell stimulation. In addition, PLP activated DCs generated from C3H/HeN mice with normal TLR4, but not DCs from C3H/HeJ mice with mutated TLR4, suggesting that the TLR4 is a membrane receptor of PLP. We showed that PLP increased ERK, JNK, and p38 mitogen-activated protein kinase phosphorylation, and nuclear translocation of the nuclear factor-kappaB p65 subunit, which are signaling molecules downstream of TLR4. These results indicate that PLP induced DC maturation through TLR4 signaling.

1. Introduction

Dendritic cells (DCs) are antigen-presenting cells with a unique ability to induce primary immune responses [1]. DC progenitors are seeded through the blood into nonlymphoid tissues, where they develop into immature DCs. These immature DCs are characterized by a high capability for antigen capture but low T cell stimulatory capability [2]. Upon receiving microenvironmental signals such as inflammatory mediators, viral products, bacterial-derived antigens, and toll-like receptor ligands, immature DCs mature and migrate to T cell areas in secondary lymphoid organs. Mature DCs downregulate antigen uptake, but they sensitize naïve T cells through co-stimulatory molecules and the major histocompatibility complex (MHC) [3]. However, cancer tissues and closely draining lymph nodes contain a small number of DCs, and most are immature DCs [4]. These immature DCs induce either T cell unresponsiveness or regulatory T cells [5].

DC maturation can be induced by many stimuli such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) [6,7]. Although they are potent DC maturation stimuli, they have limited clinical applications due to toxicity [8]. Thus, many studies have attempted to identify nontoxic stimulators that can induce DC maturation. In particular, plant-derived polysaccharides might be ideal candidates for inducing DC maturation, as they are nontoxic and do not cause significant side effects [9]. For example, the PL polysaccharide from *Phellinus linteus*, the protein-bound polysaccharide K from *Coriolus versicolor*, grifolan from *Grifola frondosa*, and sparan from *Sparassis crispa* induce DC maturation [10–13].

Pueraria lobata is widely distributed in temperate regions of Korea, Japan, and China and is one of the earliest and most important edible crude herbs in traditional oriental medicine [14]. *P. lobata* has been used traditionally as an herbal medicine to treat the common cold, headache, diarrhea, and hypertension [15]. *P. lobata* contains high levels of flavonoids, particularly puerarin and daidzein, which possess many biological activities, including antihypertensive, antioxidative, anti-inflammatory, and anticarcinogenic properties [16,17]. In addition, several saponins, triterpenoids, and phenolic compounds have been isolated from *P. lobata* roots [18] and exhibit anticomplementary and antihepatotoxic activities [19,20]. The ethanol extracts of *P. lobata* roots also have antioxidant, antiosteoporosis, and antiinflammatory activities [14,15,21]. However, the biological activity of polysaccharide (PLP) isolated from *P. lobata* is still unknown. Here, we investigated the effect of PLP on immune functions of DCs, including maturation,

* Corresponding author at: College of Pharmacy, Chungbuk National University, 52 Naesoodong, Heungduk, Cheongju, Chungbuk 361-763, South Korea.
Tel.: +82 43 261 2815; fax: +82 43 268 2732.
E-mail address: shan@chungbuk.ac.kr (S.-B. Han).
[1] These authors contributed equally to this study.

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cytokine production, and allo-T cell activation. We also examined the underlying mechanisms related with DC activation by PLP.

2. Materials and methods

2.1. Materials

Female C57BL/6, BALB/c, C3H/HeN and C3H/HeJ mice (6–8 weeks old) were obtained from Korea Research Institute of Bioscience and Biotechnology (Chungbuk, Korea). Mice were housed in specific pathogen-free conditions at 21–24 °C and 40–60% relative humidity under a 12 h light/dark cycle. All animals were acclimatized for at least 1 week prior to the experiments. All experimental procedures were approved by the Animal Experimentation Ethics Committee of Chungbuk National University. Anti-mouse antibodies against CD11c, CD40, CD86, and MHC-I/II were purchased from BD Pharmingen (San Diego, CA, USA) and those against extracellular signal-regulated kinase (ERK), C-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinases (MAPKs), NF-κB p65, and histone were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipopolysaccharide (LPS), polymyxin B (PMB), and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Polysaccharide (PLP) from *P. lobata* was prepared as described previously [3]. Briefly, the dried fruit was pulverized into powder. The sample was then successively extracted twice with hot water (65–70 °C) each time for 10 h. The extract was combined and concentrated under reduced pressure to small volumes. The crude polysaccharide was precipitated by adding 4 volumes of ethanol. The precipitate was collected by centrifugation and washed twice with ethanol. The precipitate was then suspended in water and lyophilized to yield polysaccharide, named as PLP. No endotoxin was detected at 100 μg/ml of PLP as determined by the LAL test (Wako Pure Chemicals, Osaka, Japan).

2.2. Nitrite production assay

Cells were plated at 5×10^5 cells/ml in 96-well culture plates and stimulated with LPS of PLP for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethyl-enediamine dihydrochloride and 2% phosphoric acid) and incubated at room temperature for 10 min. Nitrite production was determined by measuring absorbance at 540 nm versus a $NaNO_2$-derived standard curve [13].

2.3. Lymphoproliferation assay

Spleen cells were obtained from a specific pathogen-free C57BL/6 mouse (female, 6–7 weeks old) and freed of red blood cells by treating with lysis buffer. Spleen cells were cultured in RPMI 1640 complete medium. Cells were seeded in a 96 well plate at a density of 2×10^5 cells/well and stimulated with LPS or PLP. Cell were pulsed with ^{3}H-thymidine (113 Ci/nmol, NEN, Boston, MA, USA) at a concentration of 1 μCi/well for the last 18 h and harvested on day 3 using an automated cell harvester (Inotech, Dottikon, Switzerland). The amount of ^{3}H-thymidine incorporated into the cells was measured using a Wallac Microbeta scintillation counter (Wallac, Turku, Finland) [13].

2.4. Generation of bone marrow-derived DCs

DCs were generated from bone marrow (BM) cells obtained from 6 to 7-week-old female mice [3]. Briefly, BM cells were flushed out from femurs and tibias. After lysing red blood cells, whole BM cells (2×10^5 cells/ml) were cultured in 100-mm^2 culture dishes in 10 ml/dish of complete medium containing 2 ng/ml GM-CSF. On culture day 3, another 10 ml of fresh complete medium containing 2 ng/ml GM-CSF was added, and on day 6 half of the medium was changed. On day 8 non-adherent and loosely adherent DCs were harvested by vigorous pipetting and used as immature DCs. Immature DCs recovered from these cultures were generally >85% CD11c$^+$, but not CD3$^+$ and B220$^+$.

2.5. Phenotype analysis

Phenotypic maturation of DCs was analyzed by flow cytometry. Cell staining was performed using a combination of FITC-conjugated anti-CD40, anti-CD86, or anti-MHC plus PE-conjugated CD11c antibodies. Cells were analyzed using a FACSCalibur flow cytometer and data were analyzed using CellQuest Pro (BD Biosciences, San Jose, CA, USA). Forward and side scatter parameters were used to gate live cells.

2.6. Endocytosis assay

To analyze the endocytosis of DCs, 5×10^5 DCs were incubated at 37 °C for 1 h with 0.4 mg/ml FITC-dextran (42,000 Da, Sigma–Aldrich, St. Louis, MO, USA). After incubation, cells were washed twice with cold washing buffer (PBS containing 0.5% BSA) and stained using PE-conjugated anti-CD11c antibody. Double stained DCs were analyzed by flow cytometry. In addition, parallel experiments were performed at 4 °C to determine the nonspecific biding of FITC-dextran to DCs [22].

2.7. Cytokine assay

Total RNA was isolated using TRIZOL^{TM} Reagent (Molecular Research Center, Cincinnati, OH, USA). For RT-PCR, single-strand cDNA was synthesized from 2 μg total RNA. The primer sequences used were as follows: IL-12, sense, 5′-AGA GGT GGA CTG GAC TCC CGA-3′, antisense, 5′-TTT GGT GCT TCA CAC TTC AG-3′; IL-1β, sense, 5′-ATG GCA ATG TTC CTG AAC TCA ACT-3′, antisense, 5′-CAG GAC AGG TAT AGA TTC TTT CCT TT-3′; TNF-α, sense, 5′-AGG TTC TGT CCC TTT CAC TCA CTG-3′, antisense, 5′-AGA GAA CCT GGG AGT CAA GGT A-3′; interferon (IFN)-β, sense, 5′-CCA CAG CCC TCT CCA TCA ACT ATA AGC-3′, antisense, 5′-AGC TCT TCA ACT GGA GAG CAG TTG AGG-3′; β-actin, sense, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, and antisense 5′-TAA AAC GCA GCT CAG TAACAG TCC G-3′. PCR products were fractionated on 1% agarose gels and stained with 5 μg/ml ethidium bromide. Cytokine levels of IL-1β, IL-12, TNF-α, IL-2, and IFN-γ in culture supernatants were measured using ELISA (R&D Systems, Minneapolis, MN, USA) [13].

2.8. Mixed leukocyte reaction (MLR)

Responder T cells were purified from the spleen of BALB/c mice by negative depletion using biotinylated antibodies for B220, GR-1, and CD11c (BD Pharmingen, San Diego, CA) and Dynabeads M-280 streptoavidin (Invitrogen, Dynal, Inc., Oslo, Norway), as previously described [13]. Purity was typically more than 90%. DCs were generated from the BM cells of C57BL/6 mice and were treated with 40 μg/ml mitomycin C (MMC) for 1 h. MMC-treated DCs were added to 1×10^5 T cells in U-bottom 96-well plates. Allogenic T cells were pulsed with ^{3}H-thymidine (113 Ci/nmol, NEN, Boston, MA) at a concentration of 1 μCi/well for the last 18 h and harvested on day 3 using an automated cell harvester (Innotech, Dottikon, Switzerland). The amount of ^{3}H-thymidine incorporated into cells was measured using a Wallac Microbeta scintillation counter (Wallac, Turku, Finland).