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Recognization of receptors on bone marrow-derived dendritic cells bound with *Pholiota nameko* polysaccharides

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ABSTRACT

Three major active polysaccharides isolated from *Pholiota nameko* (PNPS), including PNPS-1, PNPS-2 and PNPS-3, had been proved to inhibit the maturation of the murine bone marrow-derived dendritic cells (BMDCs). This paper recognized the affinity bind between PNPS and the five receptors (TLR2, TLR4, CD14, Dectin-1 and Mannose receptor) on BMDCs, using the bio-layer interferometry (BLI)-based biosensor technology developed by ForteBio on Octet RED system (Fortebio, Inc.). From the primary binding experiment, the gradient binding experiment and the inhibition binding experiment between the receptor proteins and PNPS, combined with the binding experiment between PNPS and the BMDCs membranes, we found that PNPS-1, PNPS-2 and PNPS-3 presented strong affinity bind with both TLR2 and Dectin-1 on BMDCs, only PNPS-3 with Mannose receptor. These data confirmed that PNPS could interact with TLR2, Dectin-1 and Mannose receptor that were very important for the affinity bind of these receptors and PNPS, which triggered the further stimulation on BMDCs.

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1. Introduction

Pholiota nameko is a widely cultivated mushroom in China and Japan. We had proved that one of the major polysaccharides isolated from *P. nameko* (PNPS-1) possessed both significant anti-inflammatory activities in different models of inflammation [1] and strong hypolipidemic effect [2] in our laboratory. PNPS-1 had also been extensively studied for its effects on human cytokine network in serum [3], which suggested that PNPS-1 could stimulate dendritic cells (DCs) to transmit signals to other body cells through producing relative cytokines. Further experiment had studied the inhibitory effects of PNPS-1 on the maturation of bone marrow dendritic cells (BMDCs) via concrete changes both inside and outside BMDCs. The results indicated that PNPS-1 could markedly inhibit the maturation of BMDCs and had potential significant down-regulation immunity [4].

In the gastrointestinal tract, where there is continuous exposure to food, bacterial, and environmental antigens, DCs play a key role in immune regulation, both at sites of antigen uptake and within inductive lymphoid tissues, and in direct sampling of commensals and pathogenic microorganisms. DCs percept and respond to extracellular signals including pathogens and pathogen-associated molecular patterns (PAMPs) through various receptors. Many bioactive substances have been proved to take action by being bound with these receptors on DCs. The polysaccharide-related receptors mainly include TLR2 (Toll-like receptor 2), TLR4, CD14, Dectin-1 and MR (Mannose receptor, CD206), and the two TLRs are the primary receptors among them. TLRs play an important role in the innate recognition of PAMPs and initiation of immune responses. The specific recognitions of different PAMPs are achieved by up to now 10 mammalian TLRs 1–10 [5]. Engel et al. found that the protein-bound polysaccharide-K could activate TLR2 on DCs [6]. Byun et al. reported that epigallocatechin-3-gallate, a major active polyphenol of green tea, could down-regulate the TLR4 signal transduction in DCs [7]. Chaung et al. reported that zymosan enhanced Dectin-1/TLR2/TLR4 expression in all of three types of cells, porcine alveolar macrophages, immature DCs and mature DCs [8]. Li et al. reported that a polysaccharide isolated from a Chinese medicinal herb, Zhu Ling (Sclerotium of Polyporus

Abbreviations: BLI, bio-layer interferometry; BMDCs, bone marrow dendritic cells; DCs, dendritic cells; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; imBMDCs, immature BMDCs; KD, affinity constant; kdis, dissociation constant; kon, association constant; LPS, lipopolysaccharides; MES, 2-N-morpholino-ethanesulfonic acid; MR, mannose receptor; PNPS, *Pholiota nameko* polysaccharides; PAMPs, pathogen-associated molecular patterns; SPR, surface plasmon resonance; TLR, toll-like receptor.

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umbellatus), was proved to promote the activation and maturation of murine BMDCs via TLR4 [9]. Presicce et al. reported that keyhole limpet hemocyanin, which was a xenoantigen largely used *in vitro* as an immunogen to study primary antigen-specific T cell responses and *in vivo* as a vaccine component with optimal carrier qualities, induced the activation and maturation of human DCs through the involvement of MR [10]. Asai et al. reported that the protein-bound polysaccharide isolated from basidiomycetes, had an ability to regulate LPS-binding protein and CD14 function [11]. Certainly, it is worthy of further studies to investigate the role of these receptors on DCs and the signaling pathways involved in the activities of PNPS.

The interaction between bioactive molecules and cell membranes plays a key role in functional factors development for food and pharmaceutical applications [12–15]. It is critical to develop new sensitive in vitro analytical techniques to investigate the interaction mechanisms explicitly, though many techniques, including microscopy, calorimetry, electrophoresis, analytical ultracentrifugation, circular dichroism, mass-spectroscopy and immobilized artificial membrane chromatography, have been used with the disadvantages of low throughput and low-resolution data [16]. Biosensor technologies, including Surface Plasmon Resonance (SPR) and Bio-Layer Interferometry (BLI), are used to determine kinetic and affinity constants for biomolecular interactions in real-time without labels [17]. However, BLI technology has the advantages of unique high-throughput, variety available biosensors selection, samples-recoverability, clogging prevention [18–20], compared with SPR.

To further prove the function of PNPS on the BMDCs *in vitro*, we developed a receptor-recognization method for recognizing the receptors on the BMDCs with the aid of bio-layer interferometry (BLI)-based biosensor technology developed by ForteBio on Octet RED system (Fortebio, Inc.). A layer of molecules attached to the tip of an optic fiber (Be called sensor) creates an interference pattern at the detector. Any change in the number of molecules bound causes a measured shift in the interferometric pattern, which is measured by interactions between waves. Interferometric pattern changes with increased molecular thickness and monitoring the interference pattern vs. time provides kinetic data on molecular interactions. ForteBio's Octet RED system is a multi-functional, label-free, real-time Dip & Read^{TM} analysis instrument for rapid protein quantitation and biomolecular binding kinetic analysis. Octet systems quantify protein concentrations and monitor protein–protein and other biomolecular interaction kinetics (kon, association constant; kdis, dissociation constant; KD, affinity constant), enabling informed research and development decisions earlier in the process, while their high throughput enables accelerated timelines.

The aim of this study was to investigate the bind affinity between PNPS and the receptors (TLR2, TLR4, CD14, Dectin-1, and MR), including the bind between PNPS and the receptor proteins and the bind between the PNPS and the BMDCs, to recognize the receptors on BMDCs bound with PNPS.

2. Materials and methods

2.1. Preparation of P. nameko polysaccharide

Isolation and purification of PNPS were performed as reported by Li and Wang [21] in our laboratory. PNPS-1, PNPS-2, and PNPS-3 were the main three polysaccharides isolated from *P. nameko* and selected as the objectives for further study of their affinity bind with the receptors on BMDCs, which were all proved chemically homogeneous by cellulose acetate electrophoresis. Monosaccharide composition, molecular size, physico-chemical characterization and morphology under SEM and AFM for PNPS-1, PNPS-2, and PNPS-3 PNPS-3 had been determined in our laboratory [1,3,4,21].

2.2. Preparation of murine BMDCs

2.2.1. Animal

Four-week-old male C57BL/6 mice with initial weights between 18 and 20 g were purchased from Tianjin Huarong Experimental Animal Feeding Company (Tianjin, China). In the experiment, the mice were fed a basal diet for more than a week with free access to water and feed to allow adjustment to their environment. The animal rooms were kept at 25 °C and 60% humidity with a 12 h light and dark cycle (8 am–8 pm). The study protocol was approved by the Animal Ethics Committee of Tianjin University of Commerce, Tianjin.

2.2.2. Generation and culture of murine BMDCs

BMDCs were prepared as described previously in detail by Inaba et al. [22] with minor modification [4]. The culture of immature bone marrow DCs (imBMDCs) were performed in RPMI 1640 complete medium, composed of 100 ml RPMI 1640 medium, 10 ml FBS, 2 ml penicillin–streptomycin, 20 ng/ml rmGM-CSF (ProSpec), 20 ng/ml rm IL-4 (ProSpec). Unless otherwise indicated, the imBMDCs were grown in RPMI 1640 complete medium and in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were plated and counted after 24 h to determine the seeding efficiency before experimentation.

To obtain highly purified imBMDCs populations (>90% cell purity) in the experiment, the imBMDCs were purified by paramagnetic MS columns (MiltenyiBiotec, BergischGladbach, Germany) labeled with bead-conjugated anti-CD11c mAb (MiltenyiBiotec, BergischGladbach, Germany), followed by positive selection according to the manufacturer's instructions.

2.3. Recognization of the receptors on BMDCs bound with PNPS

2.3.1. Recognization of the receptor proteins bound with PNPS

At first we performed a preliminary experiment to recognize the existence of the bind between the receptor proteins and PNPS, with the relatively high concentration of the receptor proteins and PNPS. If the preliminary experiment presented strong bind between the receptor proteins and PNPS, subsequently we performed the gradient binding experiment with gradient PNPS concentrations, to investigate the gradient binding changes with the gradient PNPS concentrations, and calculate the kinetic parameters, including kon, kdis and KD by data analysis. Further, we performed the inhibition experiment to prove the binding specificity of PNPS and the receptor proteins.

In the primary experiment, TLR4 (ENZO, ALX-522-073-C050), TLR2 (LSBio, LS-C148516), CD14 mouse recombinant (ProSpec, ALX-522-141-C050), Dectin-1 (ENZO, ALX-522-141-C050), and recombinant mouse MR (R&D, 2535-MM-050) were diluted with 50 ml double distilled water to the concentration of 1 mg/ml. Because these receptors were dissolved in PBS or lyophilized from a concentrated protein solution containing PBS, they were simply diluted with 50 ml double distilled water before use. PNPS was diluted with 0.01 M PBS (pH 7.2, with 0.14 M NaCl and 2.68 mM KCl contained) to the concentration of 1 mg/ml. LPS (1 mg/ml), β-glucan (1 mg/ml) and Mannan (1 mg/ml), dissolved with 0.01 M PBS, were set as the positive controls, while 0.01 M PBS was set as the blank control. TLR4, CD14 and MR were originally attached with His-tag, and TLR2 and Dectin-1 attached with Fc tag. Sensor tips which could link Fc-tag and His-tag were purchased from Fortebio Instrument Company (USA).

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