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Effects of *Pholiota nameko* polysaccharide on NF-κB pathway of murine bone marrow-derived dendritic cells

Haiping Li*, Yongqing Tao, Pei Zhao, Lihua Huai, Dexian Zhi, Jiangmei Liu, Guoliang Li, Chunlan Dang, Yufeng Xu

Tianjin Key Laboratory of Food Biotechnology, Faculty of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin 300134, PR China

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ABSTRACT

This study investigated the effect of a polysaccharide purified from *Pholiota nameko* (PNPS-1) on the NF-κB signaling pathway of murine bone marrow-derived dendritic cells (BMDCs) and relevant mechanisms. The results showed that PNPS-1 could decrease the expression of maturation markers CD40 and CD80 on BMDCs. PNPS-1 also could decrease the mRNA expression of Myd88, TRAF6, TIRAP, IRAKI, IKBKB, NFKB1, NFKB2 and RelA in immature BMDCs determined by RT-PCR, and decreased the IKKβ and P65 production in BMDCs determined by Western blot, and decreased the NF-κB P65 production determined by ELISA. In addition, the effects of PNPS-1 on BMDCs were significantly impaired by treating the cells with anti-TLR2 antibody prior to PNPS-1 treatment, implying direct interaction between PNPS-1 and TLR2 on cell surface. These results indicate that PNPS-1 regulates BMDCs through TLR2 and downstream NF-κB signalings.

1. Introduction

Pholiota nameko is a widely cultivated mushroom in China and Japan. We have 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. It has also been extensively studied for its effects of PNPS-1 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, PNPS-1 had been proved to inhibit the maturation of the murine bone marrow-derived dendritic cells (BMDCs) [4]. Recent experiment convictively demonstrated that PNPS-1 could bind in affinity with the receptor TLR2 on the BMDCs, which provided a supporting mechanism that PNPS could stimulate BMDCs by being bound with TLR2 receptors to transmit signals to themselves and other body cells, thus took their bioactive function [5]. PNPS-1 was composed mainly of Man, Glc, Gal, Ara and Xyl in a molar ration of 1:8.4:13.6:29.6:6.2 [6].

Many polysaccharides isolated from natural sources, which are known for their immunomodulatory effect, have been reported to be the primary factor for immunological cells stimulation through induction of the immune system of Toll-like receptors (TLRs) [7–12], mainly including TLR2 and TLR4. TLRs are one of the key receptor families forming an initial line of defence against invading pathogens. Like other pattern recognition receptors (PRRs), they are germline encoded, functioning to recognize and eliminate microbial non-self antigens. TLRs are widely expressed in the immune system and work cooperatively with associated proteins to generate diverse signaling responses as part of the innate immune response. TLRs are known to play a critical role in the early innate

Abbreviations: BMDCs, bone marrow dendritic cells; DCs, dendritic cells; ELISA, enzyme linked immunosorbent assay; FCM, flow cytometry; ICAM1, intercellular Adhesion Molecule 1; IKBKB, inhibitor of kappa light polypeptide gene enhancer in B-Cells, kinase beta; IKK, inhibitor of kappa B kinase; imBMDCs, immature BMDCs; IRAK, interleukin-1-receptor associated kinase; IRAK1, interleukin-1 receptor-associated kinase; IκB, inhibitor of kappa B; LPS, lipopolysaccharides; NF-κB, nuclear factor-kappaB; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa B; NFKB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; NFKB2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; Nfkbia, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; Nfkbib, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta; PAMPs, pathogen associated molecular patterns; PAMPs, pathogen-associated molecular patterns; p-IKKβ, phosphorylated IKKβ; PMSF, phenylmethyl sulphonyl fluoride; PNPS-1, *Pholiota nameko* polysaccharides; p-P65, phosphorylated P65; PRRs, pattern recognition receptors; RBC, red blood cell; RelA, v-rel avian reticuloendotheliosis viral oncogene homolog A; RT-PCR, real time PCR; TAL, tachypleus amebocyte lysate; TEM, transmission electron microscope; TIR, Toll/IL-1 receptor; TIRAP, TIR domain-containing adaptor protein; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF6, tumor necrosis factor receptor-associated factor 6; TRAF6, tumor necrosis factor-associated factor 6; ZA, Zymosan A.

* Corresponding author. Tel.: +86 22 26665930; fax: +86 22 26669795.
E-mail address: hhppli@163.com (H. Li).

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immune response [13]. All TLR signaling pathways culminate in activation of the transcription factor nuclear factor-kappaB (NF-κB), which controls the expression of an array of inflammatory cytokine genes. TLR2/4 recognizes various PAMPs, including peptidoglycan from Gram-positive bacteria, lipoarabinomannan from mycobacteria, hemagglutinin protein from measles virus and tGPI-mutin from Trypanosoma [14]. TLR2/4 utilizes MyD88 and TIRAP as adaptors [14]. TLR2/4 can be expressed on DCs [15].

We inferred that the affinity binding of PNPS with the TLR2 on DCs triggered NF-κB signaling pathway via MyD88 and TIRAP, two TIR (Toll/IL-1 receptor)-domain-containing adaptor proteins for TLR2. This is followed by the activation of tumor necrosis factor-associated factor 6 (TRAF6) and TRAF6 in turn activates inhibitor of kappa B(IκB) kinase (IKK) complex. In turn, the activation of IKK complex leads to the degradation of IκB and the activation of NF-κB, which regulates a wide spectrum of target genes [16]. Thus, this study aims to investigate the effect of PNPS-1 on the TLR2-NF-κB signaling pathway by analysis of ten NF-κB-related mRNA expression by RT-PCR, ICAM1 protein expression by flow cytometry, IKKβ and CCL2 protein excretion by western blot, and NF-κB P65 level by ELISA.

2. Materials and methods

2.1. Preparation of P. nameko polysaccharides

Isolation and purification of the *P. nameko* polysaccharides (PNPS-1) were performed as reported by Li and Wang [6] in our laboratory, which were taken as the objective polysaccharides for further study of its activity on the TLR2-NF-κB signaling pathway of BMDCs. LPS were assayed for PNPS-1 under endotoxin-free experimental conditions by using endotoxin-specific tachypleus amebocyte lysate (TAL) (Xiamen Horseshoe Crab Reagent Manufactory, Co. Ltd., Xiamen, China) according to the manufacturer's protocol. To further ensure that the effects of PNPS-1 on DCs were not due to LPS contamination, PNPS-1 or LPS was incubated with 5 μg/ml polymyxin B (Sigma) for 30 min before treatment with DCs. Then expression on DCs was analyzed with PE-conjugated CD80 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) by flow cytometry using a FACSCalibur System (Becton Dickinson, San Jose, USA).

2.2. Generation of murine BMDCs

2.2.1. Animal

Four-week-old male C57BL/6 mouse with initial weights between 18 g and 20 g were purchased from Tianjin Huarong Experimental Animal Feeding Company (Tianjin, China). In the experiment, the rats 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 to 8 pm). The study protocol was approved by the Animal Ethics Committee of Tianjin University of Commerce, Tianjin.

2.2.2. Generation of murine BMDCs

BMDCs were prepared as described previously in detail by Inaba et al. [17] with minor modification. Briefly, bone marrow cells from the femurs and tibias of male C57BL/6 mice were flushed and depleted of red blood cell (RBC) by hypotonic lysis using RBC lysing buffer (Sigma). Cells were grown from precursors at a starting concentration of 1×10^6 cells/ml in RPMI 1640, supplemented with 20 ng/ml rmGM-CSF (ProSpec), 20 ng/ml rmIL-4 (ProSpec), 10% v/v heat-inactivated fetal bovine serum (FBS, Gibico), 2 ml penicillin-streptomycin (1×10^6 U penicillin and 1×10^6 U streptomycin in 100 ml sterilized PBS) (Sigma), β-mercaptoethanol (0.3 μl/100 ml) (Sigma) for 3 d and then non-adherent cells were washed out. Another 10 ml of fresh complete medium containing 20 ng/ml rmGM-CSF (ProSpec) and 20 ng/ml rmIL-4 (ProSpec), was added, and on day 6 half of the medium was replaced. On day 7, non-adherent and loosely adherent DCs were harvested by vigorous pipetting and used as immature BMDCs (imBMDCs). 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. The imBMDCs were grown 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), and 20 ng/ml rm IL-4 (ProSpec).

2.3. Grouping

On the seventh day, by gently pipetting with RPMI-1640 medium, the imBMDCs aggregates separated from the adherent stromal cells, and the imBMDCs separated cells was collected by being concentrated at 1500 r/min for 10 min. The cells were resuspended in RPMI-1640 medium with the maxmum concentration of 1×10^6 cells/ml. Then the 1×10^6 imBMDCs cells/ml were seeded in 100-mm^2 culture dishes, and the culture dishes were added 20 μg/ml PNPS-1 as the sample groups, 1 ml RPMI-1640 medium as the blank control group, and 0.1 μg/ml lipopolysaccharide (LPS, serotype 0111:24, Sigma) as the positive control group. All the culture dishes were incubated at 37 °C with 5% CO_2 for 12 h. Neutralization experiments were performed to recognize the TLR2/4 receptors. BMDCs were pre-incubated for 1 h with 0.1 μg/ml anti-mouse TLR2/4 monoclonal antibody free of azide (Lifespan Biosciences, Seattle, USA). PNPS-1 (20 μg/ml) was added and incubated for 24 h. Then cells or culture supernatants were collected and analyzed as the PNPS-1 (20 μg/ml) + anti-TLR2 (20 μg/ml) group and PNPS-1 (20 μg/ml) + anti-TLR4 (20 μg/ml) group.

2.4. Observation of cell morphology of BMDCs

The imBMDCs post treatment with 20 μg/ml PNPS-1 for 24 h, were collected and checked for morphological study by an inverted microscope (Nikon, Ti-U, Japan), and imBMDCs treated with 0.1 μg/ml LPS and untreated imBMDCs were taken as controls.

2.5. Observation of intracellular phagosomes inside the BMDCs with TEM

Another portion of BMDCs post treatment with 20 μg/ml PNPS-1 for 24 h, were collected and observed for intracellular phagosomes with Transmission electron microscope (TEM, JEOL-1200EX, Japan). The sample preparation steps included cultured cell digestion by trypsin, centrifugation at 1000 rpm for 10 min, glutaraldehyde fixation, slicing. Finally the prepared samples were analyzed by TEM.

2.6. mRNA expression analysis

Twelve NF-κB-related mRNA expression of TLR2, TLR4, Myd88, TRAF6, TIRAP, IRAKI, NFKBI, NFKB2, RelA, IKBKB, Nfkbia and Nfkbib (Table 1), were analyzed by RT-PCR after the stimulation of PNPS-1. Total mRNA was isolated from cultured BMDCs with Trizol Reagent (Invitrogen, Paisley, UK). Isolated RNA was reverse transcribed and then amplified with a commercial kit (All-in-One™ First-Strand cDNA Synthesis Kit, GeneCopoeia, Hanover, USA) according to the manufacturer's protocol. Subsequently, cDNAs were then subjected to 25 cycles of PCR amplification under the following conditions: an initial denaturation at 95 °C for 15 s, followed by annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The PCR was conducted with an ABI7500 Fast real-time PCR system (Applied Biosystems, Foster City, USA) following the instructions from Platinum® SYBR®