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Immunomodulatory effects of sulfated polysaccharides of pine pollen on mouse macrophages

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ABSTRACT

This study was undertaken to explore the effects of sulfated polysaccharide (SPPM60-D) from masson pine pollen on $[Ca^{2+}]_i$ and immune function of RAW264.7 macrophages. SPPM60-D was obtained by subjecting Masson pine pollen to boiling water and alcohol precipitation, 60% ethanol grading precipitation, Sephacryl S-400HR purification, and chlorosulfonic acid-pyridine method sulfation. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the effect of SPPM60-D on relative activity and proliferation of RAW264.7 cells, and a fluorescence spectrophotometer was used to determine $[Ca^{2+}]_i$. Phagocytosis of neutral red was used to determine phagocytosis capacity. Adherence, scratch healing, and transwell assays were used to assess migration and adhesion abilities of macrophages. An enzyme-linked immuno sorbent assay (ELISA) assay was used to assess the secretion of cytokines and inflammatory mediators. A dexamethasone (DEX) inhibition method was used to measure the recovery of RAW264.7 immune activity by SPPM60-D. SPPM60-D significantly increased relative activity, proliferation, and $[Ca^{2+}]_i$ levels of mouse RAW264.7 cells. It also significantly enhanced the immune function of macrophages from normal and immune-suppressed mouse. The results showed that SPPM60-D mainly bound to TLR4 on macrophages. This activated the TLR4-PI3K-PLC-IP3R signaling pathway, leading to the opening of calcium release-activated calcium channels (CRAC), increasing $[Ca^{2+}]_i$ and activating the macrophages, and thus improving immunity.

Plant polysaccharides show promise in a variety of medicinal applications, including anti-tumor activity, anti-clotting activity, anti-mutagenic activity, lipid- lowering therapy, anti-aging, anti-viral activity and treatment for other incurable diseases [1]. After sulfation, polysaccharides have varying degrees of enhanced activity. For example, polysaccharides of mushrooms *(Lentinus edodes)* [2] and of achyranthes *(Achyranthes bidentata)* [2,3] have no antiviral activity, but gain anti-viral activity after sulfation. However, naturally sulfated polysaccharides are relatively scarce, so the sulfation of polysaccharides is gaining more attention. Many studies have shown that sulfated polysaccharides and polysaccharides play an important role in regulation of the immune system. They can stimulate immune cell proliferation and production of immune-related molecules. For example, sulfated polysaccharides extracted from marine algae can bind to receptors on macrophages and lymphocytes to activate the cells through the recognition and transfer of a series of biological information, thus regulating immune functions [4]. Changes in intracellular calcium ion concentration is an important factor affecting cell signal transduction and other biological functions and is often accompanied by a series of biochemical reactions in the cell [4–6]. Currently, most studies are focused on related signaling pathways such as the PLC-IP3/Ca^{2+} and DAG/PKC transmembrane signal transduction pathways. CRAC (Ca^{2+} release-activated Ca^{2+} channel) is the main channel for intracellular calcium influx in macrophages, T-cells, B-cells and other non-excitatory cells and $[Ca^{2+}]_i$ increase is mainly caused by the entry of Ca^{2+} into the cell through CRAC channel l [7,8].

Our previous work has demonstrated that masson pine pollen polysaccharides (MPPP) and its esterified product can stimulate the proliferation of spleen lymphocytes and can significantly increase $[Ca^{2+}]_i$ of spleen lymphocytes [9,10], as well as activating T and B lymphocytes isolated from the spleen [11,12]. It remains unclear whether MPPP has any effect on immunomodulatory functions of macrophages, which play an important role in the innate immune system. In this study, we investigated the effects of sulfated polysaccharides of masson pollen on murine macrophage proliferation, $[Ca^{2+}]_i$, possible calcium signaling pathways, cytokine

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secretion, and production of inflammatory mediators, to determine the immune regulation of mouse macrophages.

1. Materials and methods

1.1. Materials and reagents

Broken masson pine (*Pinus massonian*) pollen was provided by Yantai New Era Health Industry Group, with a broken rate of more than 95%. Polysaccharide (PPM60) was extracted by hot water, classified by 60% ethanol and PPM60-D was separated and purified from PPM60-D by Sephacryl S-400HR. It was composed of mannose, galactose, fucose and an unknown monosaccharide at the molar ratios 2.37:0.75:1:0.68. Sulfated polysaccharide D (SPPM60-D), with a degree of substitution of 1.87, was made by chlorosulfonic acid–pyridine method.

RPMI-1640 and DMEM medium were obtained from HyClone (USA). DMSO and 0.1% crystal violet staining solution were obtained from Solarbio (Beijing). Penicillin, streptomycin and MTT were obtained from Klontech (USA). Fetal bovine serum was obtained from Evergreen (Hangzhou). LPS, U73122, 2-APB, and EGTA were purchased from Sigma (USA). LY294002 was obtained from Beyotime (Shanghai). DMSO was obtained from Solarbio. TAK-242 was obtained from Invitrogen (USA). Fura-2/AM and Pluronic F127 were obtained from Dojin (Japan). Triton X-100 was purchased from Amresco (USA). Verapamil (Verapamil, Ver) injection was obtained from Shanghai Harvest Pharmaceutical (Shanghai). Low molecular weight heparin (LMWH) sodium injection was purchased from Sanofi-aventis, France. Mouse IL-1, IL-6, and TNF-α detection kits were obtained from Bo Yun Biological Technology Co., Ltd. (Shanghai). NO and NO synthase detection kits were purchased from Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing). Other analytical reagents were made in China.

Kunming mice (KM mice) 18–20 g, were purchased from the Experimental Animal Center of Shandong University. This study was evaluated and approved by an Ethics Committee of Shandong Normal University. Mononuclear macrophage RAW264.7 was provided by Professor Zhang Wei, Shandong University.

1.2. Instruments

800 low-speed desktop centrifuge (Shanghai Surgical Instruments Factory, Shanghai); TU-1810PC UV–vis spectrophotometer (Persee General, Beijing); StatFax-2100 enzyme-linked immunosorbent assay (Awareness, United States); dual-wavelength fluorescence spectroscopic photometer (Cary Eclipse, USA); inverted microscope (Olympus, Japan).

1.3. Isolation and preparation of mouse peritoneal macrophages

18–20 g weighing mice were used to isolate peritoneal macrophages using standard procedure.

1.4. Effect of SPPM60-D on RAW264.7 macrophage proliferation

RAW264.7 cells were plated at a concentration of 2×10^6 cells/mL in a 96-well plate. 10 μg/mL of LPS was added as a positive control. PPM60-D and SPPM60-D were added at final concentrations of 25, 50, 100, 200, 400, 800, and 1600 μg/mL. After 20 h of culture, 20 μL of MTT was added to each well. After incubation for 4 h, the plate was centrifuged to remove the supernatants. An amount of 150 μL of DMSO was then added to each well. The absorbance values were measured at 490 nm.

1.5. Double-Wavelength fluorescence spectrophotometry to measure $[Ca^{2+}]_i$ in macrophage RAW264.7

Experimental methods and procedures were followed according to standard methods [11–13].

1.6. RAW264.7 macrophage adhesion experiment

Cultured murine macrophage RAW264.7 cells, after the cell density was adjusted to 2×10^6 mL, were plated into 6-well plates (2 mL each well). The treatments were a control group, with PPM60-D and SPPM60-D (800 μg/mL) as experimental groups. After cells were cultured for 1 h, cell adhesion in different groups was observed under an inverted microscope field of view (three wells per set).

1.7. RAW264.7 macrophage scratch healing assay

After the cells became adherent and the density was more than 95%, the cells were scratched by using small tips. The scratched cells were incubated with PPM60 and SPPM60 for 24 h, and at 0 h, 24 h pictures were taken. The degree of healing (three wells per set) in each cell treatment group was observed and recorded.

1.8. Macrophage RAW264.7 transwell migration assay

In the lower chamber of transwell culture wells, DMEM complete medium was added, which contained 10% FBS, together with PPM60-D (800 μg/mL), or SPPM60-D (800 μg/mL). RAW264.7 cells (1×10^5 mL) were added to the upper chamber of each group. After culturing for 24 h, the cells in the upper chamber were removed with a cotton swab, followed by washing with PBS to remove the residual medium. The opposite surface of the bottom of the upper chamber was fixed with 4% formaldehyde solution, stained with 0.1% crystal violet for 20 min, and washed 2–3 times with tap water. The migration of cells was observed under a microscope (three wells per group).

1.9. Neutral red phagocytosis experiments

RAW264.7 cell suspension was adjusted to 2×10^6 mL, and 100 μL was added to each well of a 96-well plate. After culturing for 2–4 h, the supernatant was discarded. The TLR4 inhibitor TAK242 was then added and after 6 h of incubation, the supernatants of the entire plate were discarded. The PPM60-D and SPPM60-D solutions were added to each well, and after 24 h of incubation, the supernatants were discarded. An amount of 100 μL of 0.09% neutral red solution was added to each well. After 1 h, the neutral red solution was discarded. After washing with pre-warmed PBS 3 times, the supernatants were discarded and 150 μL of cell-lysis solution was added. After standing at 4 °C for 10 min, the absorbance at 540 nm was measured (3 replicates in each experiment group).

1.10. Detection of cytokines and NO

The concentration of RAW264.7 cells was adjusted to 2×10^6 mL, and 1 mL was added to each well in a 24-well plate. There was a blank control group, an LPS positive control group, PPM60-D and SPPM60-D (800 μg/mL) experimental groups, and a TLR4 inhibitor (TAK-242) group. After culturing for 24 h, the supernatant was removed and centrifuged at 1000 rpm for 10 min and stored at −20 °C. The cytokines and inflammatory mediators were measured in accordance with kit instructions (3 replicates in each experiment group).