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Immunopotentiation of Polysaccharides of *Atractylodes macrocephala* Koidz-loaded nanostructured lipid carriers as an adjuvant

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ABSTRACT

The immunoregulation and immunopotentiation of Polysaccharides of *Atractylodes macrocephala* Koidz (PAMK) have been widely demonstrated. Nanostructured lipid carriers (NLC) have high drug loading capacity for lipophilic and hydrophilic drugs, and have good biocompatibility and high bioavailability. In this study, the effect of PAKM-NLC on the surface molecule expression of bone marrow-derived dendritic cells (BMDCs) in vitro was investigated by flow cytometry, and the cytokines secreted by dendritic cell supernatants were detected by ELISA. The results showed that compared with other control groups, PAMK-NLC could significantly increase the expression of CD80 and CD86 and promote the secretion of IL-1β, IL-12, TNF-α and IFN-γ, indicating that PAMK-NLC have a more pronounced effect on the maturation and differentiation of BMDCs. In addition, effects of PAMK-NLC nanoparticles on OVA-immunized mice were explored. Compared with other control groups, PAMK-NLC-OVA can significantly promote the production of OVA-specific antibodies in serum, stimulate the secretion of cytokines, increase the proliferation rate of spleen lymphocytes after OVA re-stimulation, and induce stronger activation of $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes. As an adjuvant of OVA, PAMK-NLC has a better immunological enhancement effect than PAMK or blank NLC, and has good adjuvant activity.

1. Introduction

Plant polysaccharides are considered to be a low-toxic and effective biological response modifier [1], which is also considered as an important component of traditional Chinese medicine. These components not only have antiviral effects, but also can exert immunopotentiating effects by modulating cytokines, lymphocytes, antibody levels, and the neuroendocrine immune system [2]. Polysaccharides of *Atractylodes macrocephala* Koidz (PAMK) is one of the main active ingredients isolated from the roots of traditional Chinese medicine *Atractylodes macrocephala*. It has an average molecular weight of 19.6×10^3 Da and consists of rhamnose, xylose, arabinose, glucose, mannose, and galactose in a ratio of 1:1.3:1.5:1.8:2.1:3.2 [3]. Studies have shown that PAMK, as an oral adjuvant, can significantly increase the secretion of IgG, IgG subclasses, IFN and IL-5, and promote the proliferation of spleen lymphocytes, thereby improving the body's cellular and humoral immune responses [4,5]. In addition, in a pharmacological study, it was found that PAKM can enhance serum IgG responses and intestinal mucosal immunity, increase the immune response of foot-and-mouth disease vaccines, and can also be used as a potential oral adjuvant to improve the efficacy of vaccination [6].

Pharmacological studies and clinical studies have confirmed that nanoparticle as a drug carrier can improve the role of drugs in vivo [7]. As one of the important carriers in nanoparticles, Nanostructured lipid carriers (NLC) have the advantages of low toxicity, good targeting, and high bioavailability [8]. The related researches in the field of pharmaceutical preparations have become increasingly active. Since NLC has a targeting effect, it can be taken up by antigen presenting cells (APCs). Dendritic cells (DCs) are unique APCs that have the ability to induce primary immune responses. They can directly activate naive T cells in vitro and in vivo, and can significantly stimulate T cell proliferation [9,10]. The ability of dendritic cells to capture and transmit information from the outside to cells in the adaptive immune system is critical not only for inducing primary immune responses, but also for inducing immune tolerance and modulating T cell-mediated immune responses [11]. For the above reasons, DCs were selected as models for studying the immunological function of polysaccharides of *Atractylodes macrocephala* Koidz-loaded nanostructured lipid carriers (PAKM-NLC).

Adjuvants are non-specific immunopotentiators that enhance the body's immune response to antigens, but do not induce immune responses themselves [12]. Aluminum compounds such as aluminum

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hydroxide (alum) have been widely used as human adjuvants to enhance humoral immune responses [13]. However, in some cases, alum is thought to cause IgE-mediated immediate-type hypersensitivity, although this is rare [14]. Therefore, there is an urgent need to develop new adjuvants on the market to enhance the immune response to various antigens. As a special drug delivery system, diagnostic tool and immune adjuvant, nanoparticles (NPs) have attracted extensive attention in the medical field in recent years. Their potential as immunological adjuvants depends on their structural properties, including size, hydrophobicity, and particle surface charge [15,16]. Compared with conventional carriers, nanoparticles can encapsulate more antigens, enhance antigen stability, promote the phagocytic processing of antigens by APCs, regulate cross-presentation of antigens, and generate stronger cellular and humoral immune responses [17].

In our previous researches, the results showed that PAMK-NLC could significantly stimulate lymphocyte proliferation and had a significant immunopotentiating activity in vitro [18]. To further explore the mechanism of action of PAKM-NLC in enhancing immune activity, we selected BMDCs as a cell model in this study. The effect of PAKM-NLC on the surface molecule expression of BMDC in vitro was examined by flow cytometry, and the level of cytokines secreted by dendritic cells was detected by ELISA. In addition, in order to further explore the adjuvant activity of the PAKM-NLC, OVA was used as a model antigen in this study. The effects of PAMK-NLC on the specific immune response of OVA were investigated by measuring the concentration of OVA specific antibody, the level of cytokine expression, the proliferation of spleen lymphocyte and the activation of lymphocyte.

2. Materials and methods

2.1. Reagents

PAMK (80% UV) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). PAMK-NLC was synthesized based on the melt-emulsification and ultra-sonication technique according to a previous publication [18]. OVA, lipopolysaccharide (LPS), and phytohemagglutinin (PHA) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 were obtained from Gibco Invitrogen (Carlsbad, CA, USA). Red Cell Lysis Solution and MTT were the products of Biosharp. A mouse cytokine ELISA kit and an OVA-specific IgG ELISA kit were purchased from Hangzhou MultiSciences Biotechnology Co., Ltd. (Hangzhou, China). Antibodies for flow cytometry, including anti-CD86-FITC, anti-CD80-PE, anti-CD3e-FITC, anti-CD4-APC and anti-CD8a-PE antibodies, were purchased from ebioscience (San Diego, CA, USA). All other reagents were of analytical grade.

2.2. Animals

BALB/c mice (4-week-old) were purchased from the Comparative Medicine Center of Yangzhou University and allowed to acclimatize for 7 days before the experiments. All mice were kept at the Experimental Animal Center of Nanjing Agricultural University, and all agreements relating to animal subjects were approved by the College Committee for the Ethical Care and use of Animals. Each mouse was used once and treated in accordance with the National Institutes of Health's guidelines for laboratory animal care and use.

2.3. Preparation of PAMK-NLC loaded with OVA

The preparation of PAMK-NLC and OVA-PAMK-NLC was based on the melt-emulsification and ultra-sonication technique [18]. According to the response surface methodology, the optimal scheme was a mass ratio of stearic acid to Caprylic/capric triglyceride of 2:1, a mass ratio of Poloxamer 188 to soybean lecithin of 2:1 and ultrasound time of 12 min. To encapsulate OVA in the PAMK-NLC, OVA was added to the aqueous phase consisting of PAMK. The prepared nanoparticles were stored at 4 °C before use.

2.4. Bone marrow derived Dendritic cells (BMDCs) culture

BMDCs were generated as described in a previous study [19,20]. In brief, the muscle tissue and connective tissue on the femur and tibia were removed under aseptic conditions. Be careful not to break the bones to prevent contamination of the bone marrow cavity. Intact bones were soaked in 70% ethanol for 3–5 min and washed twice with PBS. BMDCs were collected and red blood cells were eliminated with Red Cell Lysis Solution. BMDCs were cultured with RPMI 1640 and supplements including 0.02 μg/L GM-CSF, 0.02 μg/L IL-4, 100 IU/mL streptomycin, 100 IU/ml penicillin and 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. The nutrient medium was replaced every two days and incubated for 7 days.

2.5. Analysis of BMDCs surface molecules by flow cytometry

10^6 cells were cultured in 6-well plates (Thermo Fisher Scientific, Inc., Waltham, MA). On the day 7, PAKM, blank NLC and three concentrations of PAKM-NLC were added to BMDCs, and LPS positive control group (50 μg/mL) and blank control group were additionally set. After culturing in a cell incubator for 44 h, the cell plates were removed and the supernatant was collected and stored at −20 °C for testing. The BMDCs from both testing group and control group were collected and washed twice with PBS, then stained with anti-CD80-PE and anti-CD86-FITC antibodies for 30 min. Flow cytometry (BD FACSCalibur, Biosciences, Bedford, MA) was conducted to analyze the surface molecules of BMDCs.

2.6. Detection of cytokines in serum of BMDCs by ELISA

The supernatants of BMDCs collected in 2.5 were collected and thawed at room temperature. The contents of IL-1β, IL-12, IFN-γ and TNF-α in the supernatants of BMDCs were determined according to the instructions of ELISA kit (MultiSciences Biotech Co., Ltd.).

2.7. In vivo immunization study

2.7.1. Immunization schemes

160 BALB/c mice (8 weeks of age, female) were randomly divided into 8 groups of 20 mice each. The specific groups were as follows: 3 concentrations of OVA-PAMK-NLC group, OVA-PAMK group, OVA-NLC group, OVA control group, OVA-FCA group, and saline group. 200 μL of various materials were injected subcutaneously into two sites on the back of the mice, and 50 μg of OVA was included per 200 μL of OVA-based materials. The first immunization was performed on day 0 and the second immunization was performed on day 14.

2.7.2. Detection of OVA-specific antibodies in serum

On days 21, 28, 35, and 42 after the first immunization, the blood samples were harvested by the method of eyeball extraction, and the sera were isolated and stored at −70 °C for subsequent analysis. The concentration of OVA-specific IgG antibody in the serum and the content of IgG1 and IgG2a were determined according to the instructions of the ELISA kit.

2.7.3. Detection of cytokines in serum

Serum samples from mice on the 14th day (D_{28}) after the second immunization were harvested as in Section 2.7.2,which was used to determine the expression levels of cytokines IL-4, IL-6, IFN-α and IFN-γ in serum according to the ELISA kit instructions (MultiSciences Biotech Co., Ltd.).