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Ganoderma lucidum polysaccharides encapsulated in liposome as an adjuvant to promote Th1-bias immune response

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ABSTRACT

Liposome-based vaccine delivery systems are known to enhance immune responses. *Ganoderma lucidum* polysaccharides (GLP) have been widely studied as immunomodulator and it could be as inducers of strong immune responses. In the research, GLP and ovalbumin (OVA) were encapsulated into liposome as vaccine and inoculated to mice. The magnitude and kinetics of the humoral and cellular immune responses were investigated. The results showed that GLP–OVA-loaded liposomes (GLPL/OVA) could induce more powerful antigen-specific immune responses than each single-component formulation. Mice immunized with GLPL/OVA displayed higher antigen-specific IgG antibodies, better splenocytes proliferation, higher cytokine secretion by splenocytes and significant activation of CD3 + CD4+ and CD3 + CD8+ T cells. Thus the GLPL/OVA formulation produced a heightened humoral and cellular immune response, with an overall Th1 bias. Enhanced immune responses elicited by the GLPL/OVA formulation might be attributed to effective activation and mature of DC in draining lymph nodes. Overall, these findings indicate that GLPL have the potential to enhance immune responses as vaccine delivery systems.

1. Instruction

The development of vaccines has achieved significant progress and breakthrough during the past several decades. New types of vaccine, such as DNA-based vaccines, recombinant vaccines and subunit vaccines, have been developed and marketed successfully (Brennan & Gordon, 2005). These vaccines have demonstrated outstanding superiorities over traditional inactivated and live attenuated vaccines. However, a major challenge for modern vaccine development is that new vaccines fail to induce sufficient immune protection because of that they based on highly purified antigen are often poorly immunogenic (Abdulhaqq & Weiner, 2008; Perrie, Mohammed, Kirby, McNeil, & Bramwell, 2008). Thus, it is necessary to enhance their immune responses by developing improved vaccine adjuvants (Pavot et al., 2013).

Particulate delivery systems offer the potential to act as adjuvants. They offer the ability to incorporate subunit antigens within pathogen-sized particles that protect antigens from degradation. In addition, they facilitate delivery to antigen presenting cells. Among the particulate drug delivery systems, liposomes were the first system described to offer adjuvant action with their immunological role and adjuvant properties being shown by Allison and Gregoriadis (1974). Liposomes has attracted extensive interest focusing on how to deliver antigen more efficiently to antigen presenting cells (APCs) and subsequently induce their maturation and activities in conditioning of the immune system for subsequent development of specific adaptive immune responses (Bachmann & Jennings, 2010; Hashimoto, Miller, & Merad, 2011; Reddy, Swartz, & Hubbell, 2006; Scheerlinck & Greenwood, 2008).

Ganoderma lucidum is an edible fungus with medicinal properties and has long been used as a pharmaceutical and nutraceutical agent in various Asian countries (Paterson, 2006). It has been applied for treatment of many diseases, including immunosuppression, hypertension, hypercholesterolemia and gastric cancer based on the role of several biologically active compounds. Polysaccharides are the main actives of these biological compounds, and their structures and pharmacological effects have been extensively studied (Harhaji Trajković et al., 2009; Huang et al., 2010). The composition of *G. lucidum* polysaccharide (GLP) is composed of D-glucose, D-galactose, D-mannose, D-xylose, l-fucose, l-rhamnose,

Abbreviations: GLPL, *Ganoderma lucidum* polysaccharide liposome; GLP, *Ganoderma lucidum* polysaccharide; OVA, ovalbumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDI, polydispersity index; PBS, phosphate buffered saline; OVA-EE, entrapment efficiency of OVA; DMSO, dimethyl sulfoxide; BL, blank liposome; MHC II, major histocompatibility complex class II molecule; FCA, Freund adjuvant; ELISA, enzyme linked immunosorbent assay; DC, dendritic cell; APC, antigen presenting cells.

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with M_W 3.7×10^4 D, and with 5.35:2.67:1:1.19:0.38:0.37 in molar ratio (Bao, Wang, Dong, Fang, & Li, 2002). Modern pharmaceutical research shows that GLP has several physiological and health effects, including immuno-modulating activities (Lin et al., 2006), strong antioxidant activities (Xu et al., 2009), and anti-tumor activities (Xu, Chen, Zhong, Chen, & Wang, 2011). However, the GLP also have some disadvantageous characteristics such as non-focused action scope, large-dose-dependent and a brief biological half-life, which can limit its application in the future.

In this study, GLP–OVA-loaded liposome (GLPL/OVA) was prepared and their characterization, antigen release, and the physicochemical stability were investigated. In addition, we investigated the magnitude and kinetics of humoral and cellular immune responses to GLP-Lip containing OVA protein and GLP (GLPL/OVA), in comparison to liposome containing OVA (BL/OVA), non-liposome OVA plus GLP (GLP/OVA), OVA alone or OVA formulated with Freund adjuvant (FCA/OVA). The immune response was characterized by measuring serum antibody titers and splenocytes cytokine expression from multiple cohorts of vaccinated mice, with a focus on markers of immune responses. The result showed that GLPL/OVA elicited a mixed Th1/Th2 immune response, with a greater bias toward Th1. The enhanced immune responses elicited by the GLPL/OVA might be attributed to efficient induction of DC activation in draining lymph nodes.

2. Materials and methods

2.1. Encapsulation of OVA and GLP in liposome (GLPL/OVA)

Preparation of liposome containing GLP (GLPL) was based upon the reverse-phase evaporation method (Liu et al., 2015). The optimized amount of soybean phospholipid (Shanghai Taiwei Pharmaceutical Co., Ltd.), cholesterol (Tianjin Bodi Chemical Technology Co., Ltd.) and tween-80 (Sinopharm Chemical Reagent Co., Ltd.) was dissolved by ethylether in a dry round bottom flask and the mixed materials were dissolved completely with the help of ultrasound. Then GLP (98%, No. CY140220, Shanxi Ciyuan Biotechnology Co., Ltd.) solution was added into the mixture. The resulting mixture was homogenized in ice water by using an ultrasonic cleaner to form a stabile W/O emulsion. The ethylether was removed by evaporating under vacuum and a colloid was formed. Then, PBS was added to hydrate for an additional 15 min. The resulting mixture was homogenized using an ultrasonic cell disintegrator (JY92-II DN, Xinzhi Bio-technology and Science Inc.) to form the uniform liposome. Ultimately, the solution was filtered using 0.8 μm, 0.45 μm and 0.22 μm millipore membrane.

OVA were encapsulated in GLPL microparticles using a repeating freeze–thaw method. In brief, GLPL were mixed with OVA solution at volume ratio of 10:1. The mixture was frozen for 12 h in −20 °C; Then they were transferred to a constant temperature oscillator for 30 min under the conditions of 37 °C and 80 rpm. This operation was repeated 3 times.

2.2. Determination of encapsulation efficiency of OVA (OVA-EE) and GLP

Encapsulation efficiency of OVA was determined by BCA Protein Assay Kit (Pierce Chemical Co., USA) with some modifications. Briefly, 1.5 mL GLPL/OVA was centrifuged at 12000pm for 30 min. The protein concentration in the supernatant (C_s) was measured. The total protein content (C_t) in 1.5 mL GLPL/OVA was measured by mixing with Triton X-100 and protein content was measured by BCA Protein Assay Kit (Vazyme Biotech Co., Ltd.). The encapsulation efficiency of antigen was calculated according to the formula:

$$EE = \frac{(C_t - C_s) \times 100\%}{C_t}$$

Encapsulation efficiency of GLP was determined as described previously (Liu et al., 2015).

2.3. Characteristics of GLPL

To study the stability of nanoparticles, the BL/OVA and GLPL/OVA dispersion was put into a tube and stored at two different temperatures (4 °C and 37 °C). At chosen time intervals, the tubes were centrifuged at 12,000 rpm for 30 min. The supernatant was collected and the OVA content was measured by BCA Protein Assay Kit. The encapsulation efficiency of OVA was determined as a measure of stability according to the method described in Section 2.2.

On days 0, 7, 14, and 28, an aliquot of the sample in 4 °C was taken out, and the particle size and polydispersity index (PDI) was determined as measures of physical stability of the GLPL dispersion. Particle size and PDI were determined using a high-performance dynamic light scattering device with Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at a fixed angle of 90°. The BL, GLPL, BL/OVA, GLPL/OVA were diluted with PBS (0.2 mL → 1.0 mL). All measurements were done in triplicate and the standard deviations were plotted as error bars.

2.4. Animals

BALB/c mice were obtained from Comparative Medicine Centre of Yangzhou University and acclimatized for 7 days prior to use. All mice were maintained under controlled conditions with at temperature of 25 ± 1 °C, humidity of 50 ± 10%, and a 12/12-h light–dark cycle with free access to food and water. Each mouse was used once and treated according to the National Institutes of Health guide lines for the care and use of laboratory animals.

2.5. Vaccinations and sample collection

BALB/c mice, age 6–8 weeks, were immunized with 50 μg OVA + 200 μg GLP in 0.2 mL GLPL/OVA. The mice of control groups were immunized with 50 μg OVA in 0.2 mL PBS, 50 μg OVA + 200 μg GLP in 0.2 mL PBS, 50 μg OVA in 0.2 mL BL, and 50 μg OVA formulated with Freund adjuvant (FCA). All injections were given subcutaneously and mice were inoculated 3 times at one-week intervals. Mice were sacrificed on days 21, 28 and 35 after first immunization, and blood was collected and splenocytes were harvested. Serum samples were separated and stored at −70 °C.

2.6. Determination of OVA-specific IgG and IgG subclasses

Blood samples were obtained as described above. Separated sera were subsequently collected at −70 °C until further analysis for the production of Ova-specific IgG antibody by ELISA. The serum was inactivated at 56 °C for 30 min before analysis for the production of Ova-specific IgG antibody by ELISA as described previously (Zhao et al., 2013). IgG1 and IgG2a in the serum were quantitatively determined by were measured by Ready-to-use Sandwich ELISA kits (R&D, Co., USA) according to the manufacturer's instructions.

2.7. Splenocytes proliferation assay

Based on previously described methods (Yuan, Wu, Chen, Wu, & Hu, 2010), the Splenocytes proliferation assay was performed to evaluate antigen-specific splenocytes activation. Splenocytes