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Immune responses to vaccines involving a combined antigen–nanoparticle mixture and nanoparticle-encapsulated antigen formulation

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ABSTRACT

Many physicochemical characteristics significantly influence the adjuvant effect of micro/nanoparticles; one critical factor is the kinetics of antigen exposure to the immune system by particle-adjuvanted vaccines. Here, we investigated how various antigen–nanoparticle formulations impacted antigen exposure to the immune system and the resultant antigen-specific immune responses. We formulated antigen with poly(lactic-co-glycolic acid) (PLGA) nanoparticles by encapsulating antigen within nanoparticles or by simply mixing soluble antigen with the nanoparticles. Our results indicated that the combined formulation (composed of antigen encapsulated in nanoparticles and antigen mixed with nanoparticles) induced more powerful antigen-specific immune responses than each single-component formulation. Mice immunized with the combined vaccine formulation displayed enhanced induction of antigen-specific IgG antibodies with high avidity, increased cytokine secretion by splenocytes, and improved generation of memory T cell. Enhanced immune responses elicited by the combined vaccine formulation might be attributed to the antigen-depot effect at the injection site, effective provision of both adequate initial antigen exposure and long-term antigen persistence, and efficient induction of dendritic cell (DC) activation and follicular helper T cell differentiation in draining lymph nodes. Understanding the effect of antigen–nanoparticle formulations on the resultant immune responses might have significant implications for rational vaccine design.

1. Introduction

Vaccination, considered as one of the most significant achievements in medicine, plays an important role in preventing infectious diseases, and saves more than 3 million people every year [1–3]. However, inadequate immunogenicity and/or safety concerns are still significant obstacles to developing ideal vaccines. While endogenous adjuvants endow traditional vaccines based on attenuated or inactivated pathogens with sufficient immunogenicity, but the side effects and safety concerns limit the extent to which they can be used against various pathogens, such as HIV and Hepatitis C. Although subunit vaccines based on protein antigens are usually better tolerated and regarded as safer alternatives to traditional vaccines, they are usually poorly immunogenic when used alone and therefore require exogenous adjuvants to augment resultant immune responses [3–5]. Alum is a conventional adjuvant and the only one widely licensed for human use. Despite being used for over 80 years in vaccines [6], alum has some disadvantages, including side effects and safety concerns [7], contributing little to or even suppressing cell-mediated immunity and subsequent CTL responses [8,9], and providing poor adjuvanticity for recombinant protein vaccines [9]. These disadvantages necessitate the development of new adjuvants for subunit vaccines.

As one potential alternative, particulate-based adjuvants can act as antigen delivery systems that facilitate the access of antigen

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to antigen presenting cells (APCs) and regulate the antigen presentation pathway, or as immune potentiators that enhance the subsequent antigen-specific immune responses [3,10,11]. Ever since Kreuter et al. evaluated the adjuvanticity of polymethylmethacrylate nanoparticles for the influenza virus in 1976 [12], biodegradable polymer-based micro/nanoparticles have been extensively investigated as adjuvants for subunit-based vaccines. The efficacy of particle vaccines are significantly influenced by various physicochemical characteristics of micro/nanoparticles (such as particle size [11,13–15], surface charge [14–16], hydrophobicity [17,18]), administration route [19,20], and antigen release kinetics [13,21–23]. Antigen release kinetics affects the efficiency of particulate-based vaccines by regulating antigen exposure to the immune system. For example, Kanchan et al. reported that slow and continuous antigen release from polymer-based particles played a critical role in eliciting memory antibody responses after a single immunization [21]. Demento et al. reported that sustained antigen release from poly(lactic-co-glycolic acid) (PLGA) nanoparticles favored long-term effector-memory cellular responses [22], and Johansen et al. demonstrated that antigenic stimulation increasing exponentially over days induced more potent $CD8^+$ T cell responses and antiviral immunity than a single dose or multiple doses (equivalent doses, administered daily) [23].

In order to achieve these different antigen-exposure kinetics, most studies fabricated particulate-based vaccines with different antigen-release profiles by regulating particle structure [21], employing different types of particles [22,24], preparing particles with stimuli-responsive polymers [3], and so on. However, relatively little attention was focused on ways to optimally formulate antigens with the particles. Antigen can be formulated with particles through attachment (e.g., conjugation, encapsulation, adsorption) or simple mixing [25]. Although various antigen–particle formulations significantly affect the kinetics of antigen exposure to the immune system, how various antigen–particle formulations impact the resultant antigen-specific immune responses remains unknown.

We hypothesized that encapsulating antigen in PLGA nanoparticles would increase its antigen persistence *in vivo*, and combining soluble antigen with the nanoparticle vaccine would improve initial antigen exposure to immune system after vaccination, both together allowing for the generation of stronger and more prolonged adjuvant-induced, antigen-specific immune responses. In the present study, our objectives were therefore to determine the type and strength of the immune responses elicited by different PLGA nanoparticle-based vaccine formulations using ovalbumin (OVA) as the model antigen, and to elucidate the underlying mechanisms of action.

2. Materials and methods

2.1. Mice, reagents, and materials

Female Balb/c mice used in this study were purchased from Vital River Laboratories (Beijing, China). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, and were approved by the Experimental Animal Ethics Committee in Beijing. PLGA (75/25, Mw ≈ 13 kDa) was purchased from Lakeshore Biomaterials (Birmingham, AL, USA). Poly(vinyl alcohol) (PVA-217, degree of polymerization 1700, degree of hydrolysis 88.5%) was ordered from Kuraray (Tokyo, Japan). Ovalbumin (OVA) was supplied by Sigma–Aldrich (St. Louis, MO, USA). Premix membrane emulsification equipment (FMEM-500M) was provided by the National Engineering Research Center for Biotechnology (Beijing, China). Shirasu porous glass (SPG) membrane was provided by SPG Technology Co. Ltd. (Sadowara, Japan). The medium for splenocytes culture was RPMI 1640 (Gibco, Carlsbad, CA, USA) with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA). All mouse cytokines ELISA and fluorochrome-conjugated anti-mouse antibodies for flow cytometric use, were obtained from eBioscience (San Diego, CA, USA), unless otherwise indicated. ELISpot^{PLUS} kits were obtained from Mabtech AB (Nacka Strand, Sweden). All other reagents were of analytical grade.

2.2. Preparation and characterization of PLGA nanoparticles

PLGA nanoparticles were prepared using a two-step procedure by combining the solvent extraction method and the premix membrane emulsification technique, as described before with some modifications [26] (Fig. S1). Briefly, 1 mL deionized water containing 100 mg OVA (internal water phase) was added into 12 mL ethyl acetate containing 600 mg PLGA; the primary water in oil (W/O) emulsion was formed by sonication (120 W; Digital Sonifier 450, Branson Ultrasonics Corp., Danbury, CT, USA) in a tube over an ice bath for 1 min (4 s on and 2 s off). To prepare the double emulsion, the resulting primary emulsion was added into 65 mL external water phase containing 1.5% w/v PVA and 0.9% w/v NaCl, and magnetically stirred at 450 rpm for 90 s. Then, the resulting coarse double emulsion was extruded through the SPG membrane under a certain nitrogen pressure for 8 times and nanodroplets with narrow size distribution were obtained. The obtained double emulsion was poured into 800 mL deionized water containing 0.9% w/v NaCl (solidification solution) under magnetic stirring for 4 h to solidify the nanoparticles. The obtained PLGA nanoparticles encapsulating OVA were collected by centrifugation at 15,000 $\times g$ for 5 min, washed 3 times with deionized water to remove residual PVA, and then lyophilized and stored at 4 °C for later use. To prepare blank PLGA nanoparticles, 1 mL of deionized water without OVA was used as the internal water phase.

The hydrodynamic size and zeta potential of PLGA nanoparticles were measured by a Nano-ZS Zeta Sizer (Malvern Instruments Ltd., Malvern, UK). Morphology of the PLGA nanoparticles was characterized by scanning electron microscopy (JEM-6700F, JEOL Ltd., Tokyo, Japan). Nano Measurer 1.2 software was employed to measure the size of PLGA nanoparticles according to the scanning electron micrographs.

The OVA content of PLGA nanoparticles was determined by incubating 3 mg of lyophilized PLGA nanoparticles in 1 mL of 0.1 M NaOH solution under gentle shaking overnight. Protein concentration in the solution was determined using Micro-BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. OVA dissolved in 0.1 M NaOH was used to establish a standard curve, and blank PLGA nanoparticles were used as the control.

2.3. Immunization studies

As shown in Table S1, 30 mice were randomly divided into 5 groups ($n = 6$) and intramuscularly immunized with 100 μL (50 μL/hind leg) of different vaccine formulations containing 25 μg of antigen (OVA) (Fig. 1E). Mice were immunized 3 times at 2-week intervals (Fig. S2). Blood samples were collected from the caudal vein before each immunization and 10 days after the third immunization. Sera was separated and stored at −70 °C for later analysis. At 10 days after the third immunization, splenocytes were collected for *in vitro* proliferation, cytokine response, and flow cytometric assays.

2.4. Determination of OVA-specific IgG and IgG subclasses

OVA-specific IgG, IgG1, and IgG2a in the serum were quantitatively determined by enzyme-linked immunosorbent assay (ELISA) in accordance with a protocol described previously [27]. Briefly, 96-well ELISA plates (Costar, Corning, New York, USA) were coated overnight at 4 °C with 2 μg of OVA per well in coating buffer (0.05 M CBS, pH 9.6). Plates were washed with PBST (0.01 M PBS containing 0.05% [m/v] Tween 20, pH 7.4) and blocked by incubating with 2% (m/v) BSA (Roche, Basel, Switzerland) in PBST for 60 min at 37 °C. After washes with PBST, 100 μL per well of appropriate sera dilutions were added to the plates, serially diluted two-fold in dilution buffer (PBST containing 0.1% [m/v] BSA), and incubated for 30 min at 37 °C. Plates were then washed and incubated with 100 μL horseradish peroxidase-conjugated goat antibodies against either mouse IgG (Sigma–Aldrich, St. Louis, MO, USA), IgG1, or IgG2a (Santa Cruz, CA, USA) (IgG diluted 1:20,000; IgG1 and IgG2a diluted 1:2000) for 30 min at 37 °C. Thereafter, the plates were washed again with PBST, and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 20 min at room temperature. After stopping the reaction by adding 50 μL of 2 M H_2SO_4 to each well, the optical density (OD, 450 nm) was measured by an Infinite M200 Microplate Spectrophotometer (Tecan, Männedorf, Switzerland). Titers were given as the reciprocal sample dilution corresponding to twice higher OD than that of the negative sera.

OVA-specific IgG avidity measurement of was carried out by ELISA with a urea-elution step [28]. The antigen–antibody complexes with low-affinity were dissociated by incubating the plates with urea (Sigma–Aldrich, St. Louis, MO, USA) at 20 °C for 10 min. Avidity index (AI) was calculated in accordance with the following formula: AI = (IgG titer incubated with urea/IgG titer incubated without urea) × 100.

2.5. Determination of cytokine levels by ELISA

Splenocytes were harvested from vaccinated mice 10 days after the third immunization and restimulated with OVA (50 μg/mL) for 60 h at 37 °C in a humid atmosphere with 5% CO_2, and the supernatant was collected. IL-4, IL-10, IL-12, and IFN-γ levels in the supernatant were measured by Ready-to-use Sandwich ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.