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An injectable, low-toxicity phospholipid-based phase separation gel that induces strong and persistent immune responses in mice

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ABSTRACT

Sustained antigen delivery using incomplete Freund's adjuvant (IFA) can induce strong, long-term immune response, but it can also cause severe side effects. Here we describe an injectable, phospholipid-based phase separation gel (PPSG) that readily transforms *in situ* into a drug depot. PPSG loaded with the model antigen ovalbumin (OVA) supported sustained OVA release in mice that lasted nearly one month. Immunizing mice with a single injection of PPSG/OVA elicited a strong and persistent increase in titers of OVA-specific IgG, IgG1 and IgG2a. Co-administering CpG-ODN further increased antibody titers. Such co-administration recruited dendritic cells to injection sites and activated dendritic cells in the draining lymph nodes. Moreover, immunization with PPSG/OVA/CpG resulted in potent memory antibody responses and high frequency of memory T cells. Remarkably, PPSG/OVA/CpG was associated with much lower toxicity at injection sites than IFA/OVA/CpG, and it showed no systemic toxicity such as to lymph nodes or spleen. These findings illustrate the potential of injectable PPSG for sustained, minimally toxic delivery of antigens and adjuvants.

1. Introduction

Vaccination is regarded as one of the greatest achievements in modern medicine, saving more than 3 million lives every year [1–3]. Vaccines based on protein subunits can avoid the safety risks associated with the use of attenuated or killed whole pathogens, but they usually induce poor immune response or even immune anergy. Co-delivering protein antigen and adjuvant can dramatically increase immunogenicity [3–5], which has spurred the development of numerous antigen delivery systems. These systems are based on microparticles that target immune cells, such as polymer nanoparticles [6], micelles [7], and inorganic metallic nanoparticles [8]; or they are based on depot-like delivery systems that can support sustained release, such as 3D scaffolds [9], hydrogels [10], and emulsions [11].

Sustained drug release systems can reduce dosing frequency, improve patient compliance, strengthen and prolong antibody immune responses, as well as favor immunological memory-based responses [9,12–15]. Incomplete Freund's adjuvant (IFA) has been used for more than 60 years as a water-in-oil emulsion for co-administration of antigens and pathogen-associated molecular pattern molecules [16]. Although IFA elicits strong, long-lasting IgG responses, it is not approved for human immunotherapy because of potentially severe side effects, including local irritation, inflammation, necrosis, persistent painful granulomas, sterile abscesses and cysts at the injection site [17,18]. This highlights the need for developing antigen-adjuvant delivery systems with IFA-like potency but much lower toxicity.

Work from our group has highlighted the potential of phospholipid-based phase separation gel (PPSG) for delivery of chemotherapeutic drugs and peptides [19,20]. Injected PPSG forms an implant *in situ* and supports sustained delivery of drugs and peptides, however, the effectiveness of PPSG in immunologic application has never been reported. PPSG features three biodegradable components: phospholipids, which are natural constituents of cells and act mainly as a drug depot; a small amount of ethanol, which acts as a solvent for phospholipids to ensure injectability; and either soybean oil or a medium-chain triglyceride, which reduces the amount of ethanol necessary and increases viscosity. The observed low toxicity of PPSG and its ability to support sustained release of cargo led us to ask whether it can be used for sustained antigen delivery and whether it is an effective adjuvant for immunological application.

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In this study, we loaded PPSG with the model antigen ovalbumin (OVA) and examined its ability to stimulate OVA-specific immune responses with minimal toxicity. We also coupled PPSG with CpG oligodeoxynucleotide (CpG-ODN or CpG), a ligand of Toll-like receptor 9, to enhance both humoral and cellular immune responses [21]. We measured sustained antigen release using *in vivo* fluorescence imaging, and we compared the levels of antibody and immunological memory T cells elicited by administration of OVA free in solution or formulated with PPSG or IFA. Local and systemic toxicity of the different OVA formulations were also compared.

2. Materials and methods

2.1. Materials

Phospholipid (E80) was purchased from Lipoid (Germany). Soybean oil was obtained from AVIC Pharmaceutical (Tieling, China). Ethanol (HPLC-grade) was purchased from Kemiou (Tianjin, China). OVA from chicken egg white and IFA were purchased from Sigma-Aldrich (MO, USA). CpG-ODN 1826, with the sequence 5′-tccatgacgttcctgacgtt-3′ and a phosphorothioate backbone, was synthesized by Sangon (Shanghai, China). The (5/6)-TAMRA-SE Dye Labeling Kit was obtained from G-Biosciences (MO, USA).

2.2. Animals

Healthy female C57BL/6 mice (6–8 weeks) and female athymic nude mice (6–8 weeks) were obtained from the Laboratory Animal Centre of Sichuan University (Chengdu, China), and housed in a specific pathogen-free, light-cycled and temperature-controlled facility. All animal experiments were approved by the Institutional Animal Care and Ethics Committee of Sichuan University.

2.3. Preparation of PPSG and viscosity measurements

PPSG was prepared in two simple steps. First, E80 phospholipid, ethanol, and soybean oil were mixed together in the ratio 70:12:15 (w/w) in a clean, sterile tube, and vortexed vigorously for 1 h to dissolve phospholipids. Then water was added to a final concentration of 3% (w/w), and the mixture was vortexed for 30 min to generate empty PPSG. Air bubbles were eliminated using water bath sonication when necessary. Other batches of PPSG were prepared by adding OVA (10 μg, 20 μg, 50 μg or 100 μg) in the presence or absence of CpG (50 μg) to 100 mg PPSG instead of water. Good mixing was not obtained when all ingredients were added together at the same time. Therefore concentrated OVA and CpG solutions were added to empty PPSG to form a homogeneous solution. IFA formulations were prepared by mixing OVA (2 mg/mL) in the presence or absence of CpG (1 mg/mL) in PBS with IFA (v/v = 1:1), and vortexing for 30 min to form a water-in-oil emulsion.

Viscosity of empty PPSG and PPSG/OVA was measured at 25 °C using a digital viscometer (DV-C Brookfield Engineering Laboratories, Inc., USA). A dialysis system was set up to stimulate the gel-sol transition of PPSG. Briefly, PPSG was added to dialysis bags with a molecular weight cut-off 2 kDa, which were immersed in pre-warmed PBS (pH 7.2) with moderate agitation. After a few minutes, the PPSG transitioned from the sol-state to the gel-state, and the viscosity of gel-state PPSG was measured.

2.4. Determination of OVA release in vivo using fluorescence imaging

Female nude mice 6–8 weeks old were used for *in vivo* imaging, because their lack of hair reduced background fluorescence due to hair autoluminescence. OVA was labeled with (5/6)-TAMRA-SE dye (TS) according to the manufacturer's protocol. Mice (n = 5) were injected subcutaneously (s.c.) in the right flank with 100 μL PPSG/TS-OVA, IFA/TS-OVA, or TS-OVA in PBS. Concentrations were adjusted so that each mouse received the same amount of TS-OVA. At the indicated time points, mice were anesthetized using intraperitoneal 1% (w/v) sodium pentobarbital, and fluorescence images were collected using an IVIS® Spectrum system (Caliper, Hopkington, MA, USA). The excitation wavelength was 534 nm; the emission wavelength, 586 nm; and exposure time, 1 s. Background autofluorescence of whole-body images was measured by scanning naïve nude mice, and the results were subtracted from the images of the experimental mice using Living Image® software. Total radiant efficiency of fluorescent regions was quantitated, and fluorescence decay at each time point was calculated as the percentage of fluorescence intensity at 10 min.

2.5. Immunization of mice

Female C57BL/6 mice 6–8 weeks old were immunized with 100 μL PPSG containing 100 μg OVA with 50 μg CpG (PPSG/OVA/CpG) or without CpG (PPSG/OVA). Control groups received PBS, an equivalent amount of free OVA in PBS in the presence of free CpG (OVA/CpG) or in the absence of CpG (OVA), or an equivalent amount of free OVA in IFA in the presence of free CpG (IFA/OVA/CpG) or its absence (IFA/OVA). Each animal was inoculated s.c. with 100 μL of each formulation injected into the right flank, near the inguinal lymph node. Mice were sacrificed at scheduled times after immunization, and the formulation deposit as well as the following tissues were harvested: blood, spleen, lymph nodes, liver, and skin.

2.6. ELISA determination of OVA-specific antibodies in serum

ELISA was used to measure serum levels of OVA-specific IgG, IgG1, and IgG2a. Briefly, 96-well flat-bottom plates were coated overnight at 4 °C with 10 μg/well OVA in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). After washing with PBST (0.05% Tween 20 in PBS, pH 7.4), plates were blocked for 1 h at 37 °C with 200 μL 1% (w/v) BSA in PBS. Then plates were aspirated and incubated for 1 h at 37 °C with 100 μL of 10-fold serial dilutions of serum in PBST containing 1% BSA. Plates were washed five times and incubated for 1 h with 100 μL of a 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse IgG, IgG1, or IgG2a (Santa Cruz, USA). Plates were again washed five times and incubated at room temperature for 30 min with 100 μL TMB Substrate (Beyotime, Beijing, China), after which 50 μL of 2 M H_2SO_4 was added to stop the reaction. Finally, optical density (OD) at 450 nm was measured using a Varioskan Flash microplate reader (Thermo Fisher Scientific, MA, USA). Titers were calculated as the reciprocal of the dilution factor for the most diluted serum sample that still gave an OD 2-fold greater than the average OD of serum from naïve mice.

2.7. Expression of MHC II on dendritic cells (DCs) in draining lymph nodes

Flow cytometry was used to measure the levels of active signaling MHC II on DCs in draining lymph nodes. Female C57BL/6 mice were vaccinated s.c. with OVA formulated with PPSG or IFA in the presence or absence of CpG, or with OVA/CpG solution (n = 3–4 animals per condition). Mice were sacrificed on day 28, and the inguinal lymph nodes close to the injection site were harvested. Lymph nodes were disrupted to obtain single cells and stained with a mixture of anti-mouse FITC-CD11c and APC-MHC II antibodies. Cell suspensions were washed, and expression of MHC II on $CD11c^+$ DCs was measured using flow cytometry (Cytomics™ FC500, Beckman Coulter, Miami, FL, USA).