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The Use of Biodegradable Nanoparticles for Tolerogenic Therapy of Allergic Inflammation

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Abstract

Antigen-specific tolerance is the ultimate aim of treatment of allergic diseases. Here, we describe methods for the use of biodegradable nanoparticles to safely induce tolerance for the prevention and treatment of allergic inflammation in mice. Antigen is either conjugated to the surface of carboxylated poly(lactide-*co*-glycolide) (PLG) or encapsulated within PLG nanoparticles, and the resulting antigen-associated nanoparticles are then washed prior to intravenous injection to inhibit antigen-specific allergic immune responses.

Keywords

Tolerance; Nanoparticles; Allergy; Th2; Immunotherapy

1 Introduction

The induction of antigen-specific tolerance is a long-sought goal of immunotherapy for the treatment of allergic disease. Current specific immunotherapy for the treatment of allergy involves the administration of escalating doses of soluble antigen (Ag) delivered subcutaneously or mucosally. Although this approach has been successful in the clinic, it runs the risk of inducing adverse events which necessitate a prolonged dose escalation [1]. One approach to improving the induction of antigen-specific tolerance involves the delivery of antigen in the context of a noninflammatory carrier. In murine models of inflammation, these carriers have included, among others, apoptotic cells [2–4] and nanoparticles [5–9]. Due to relative ease of manufacture and control, biodegradable antigen-associated nanoparticles (Ag-NP) represent the more clinically feasible implementation of this concept for immunotherapy. In a model of OVA/alum-induced Th2-driven allergic airway inflammation, both surface antigen-conjugated PLG nanoparticles (Ag-PLG) and antigen-encapsulated PLG nanoparticles [PLG(Ag)] effectively induced prophylactic tolerance to inhibit Th2 sensitization [9]. Administered post-sensitization, Ag-NP safely inhibited existing Th2 responses and prevented development of airway inflammation upon challenge [9].

Here, we outline methods to conjugate protein or peptide antigens to carboxylated PLG nanoparticles using 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (EDCI) chemistry to

catalyze covalent peptide bonds between amino groups on antigen and carboxyl groups on nanoparticles to generate Ag-PLG. We then describe proper preparation and administration of PLG-Ag and PLG(Ag) to the tail veins of recipient mice for the induction of antigen-specific tolerance. This protocol does not concern the production of biodegradable PLG nanoparticles, which is described in detail elsewhere [7, 10]. While previously used for induction of tolerance against OVA in an OVA/Alum model of allergic airway inflammation, these methods of tolerance induction could potentially be applied to any allergic model using a protein or peptide-based antigen. Thus, these methods present a tool to induce immune tolerance in an antigen-specific manner to study regulatory and inflammatory mechanisms of Th2-induced allergic inflammation.

2 Materials

Use only sterile reagents throughout the protocol.

2.1 Common Reagents and Materials

1. Water: cell culture grade water.
2. PBS: Dulbecco's phosphate-buffered saline.
3. Microcentrifuge tubes: 1.5 mL polypropylene tubes.
4. Cryovials: 2 mL high-density polyethylene cryogenic storage vials.
5. Cell strainers: 40 μ m cell strainers.
6. Syringes: 1 mL tuberculin syringes.
7. Needles: 30 gauge 1/2 inch needles.
8. Thermal mixer with tube adapter.
9. Microcentrifuge.

2.2 Antigen-Conjugated PLG Nanoparticles and Antigen-Encapsulated PLG Nanoparticles

1. Carboxylated PLG nanoparticles: 500 nm carboxylated PLG nanoparticles. Nanoparticles can be prepared using a single emulsion technique as described in [7] or purchased from Phosphorex (see Notes 1 and 2).
2. ECDI: 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide.
3. Antigen: Either whole protein or peptide antigens are acceptable for conjugation to carboxylated nanoparticles for the induction of tolerance.
4. Antigen-encapsulated PLG nanoparticles: 500 nm PLG nanoparticles with encapsulated antigen can be generated through a double-emulsion process as described in [10] or custom-ordered.

3 Methods

Carry out all procedures at room temperature using sterile reagents and conditions unless otherwise noted.

3.1 Antigen-Conjugated PLG Nanoparticles (Ag-PLG)

1. Allow reagents to warm to room temperature.
2. Weigh out 1.25 mg of lyophilized carboxylated PLG nanoparticles per dose, and transfer to a 1.5 mL microcentrifuge tube (*see Note 3*).
3. Resuspend nanoparticles in 200 μ L of PBS (*see Note 4*). Add 1000 μ L of PBS, and pipette thoroughly to mix. Centrifuge nanoparticles for 5 min at $3000 \times g$ -force.
4. Repeat step 3 two additional times for a total of three washes.
5. Resuspend nanoparticles in 14.4 μ L of PBS per mg of nanoparticles, and vortex for 5 s.
6. Dissolve ECDI in PBS to a concentration of 200 mg/mL immediately before use. Add 1.6 μ L of ECDI solution per mg of nanoparticles to the suspension, and vortex for 5 s.
7. Add 4 μ L of a 10 mg/mL Ag solution per mg of nanoparticle suspension, and vortex for 5 s (*see Note 5*).
8. Incubate reaction mixture for 1 h at 25 °C with shaking at 750 rpm. Vortex for 5 s every 10 min during the incubation. After 1 h, centrifuge for 5 min at $3000 \times g$ -force.
9. Resuspend nanoparticles in 200 μ L of PBS (*see Note 6*). Add 1000 μ L of PBS, and pipette thoroughly to mix. Centrifuge nanoparticles for 5 min at $3000 \times g$ -force.
10. Repeat **step 9** two additional times for a total of three washes.
11. Resuspend nanoparticles to a final concentration of 6.25 mg/mL in PBS. Filter nanoparticle suspension through a 40 μ m cell strainer into a cryogenic vial to remove large aggregates (*see Note 7*). Antigen-conjugated nanoparticles are now ready for injection.
12. Vortex nanoparticles immediately before drawing for injection. Using a 1 mL syringe, draw up 240 μ L of nanoparticles, remove air bubbles, and fill the void volume of a 30 gauge $\frac{1}{2}$ in. needle. Inject 200 μ L into the tail vein, vortexing the vial of nanoparticles and filling the syringe between each injection (*see Notes 8–10*).

3.2 Antigen-Encapsulated PLG Nanoparticles [PLG(Ag)]

1. Rehydrate antigen-encapsulated nanoparticles with cell culture grade water at a concentration of 12.5 mg/mL. Centrifuge nanoparticles for 5 min at $3000 \times g$ -force (*see* Notes 3, 11, and 12).
2. Resuspend nanoparticles in 200 μ L of PBS. Add 1000 μ L of PBS, and pipette thoroughly to mix. Centrifuge nanoparticles for 5 min at $3000 \times g$ -force.
3. Repeat **step 2** two additional times for a total of three washes with PBS.
4. Resuspend antigen-encapsulated nanoparticles to a final concentration of 12.5 mg/mL in PBS. Filter nanoparticle suspension through a 40 μ m cell strainer into a cryogenic vial to remove large aggregates (*see* Note 7). Antigen-encapsulated nanoparticles are now ready for injection.
5. Vortex nanoparticles immediately before drawing for injection. Using a 1 mL syringe, draw up 240 μ L of nanoparticles, remove air bubbles, and fill the void volume of a 30 gauge $\frac{1}{2}$ in. needle. Inject 200 μ L into the tail vein, vortexing the vial of nanoparticles and filling the syringe between each injection (*see* Notes 8 and 9).

3.3 Timing of Ag-NP Administration

1. Prophylactic administration: For prophylactic tolerance, nanoparticles should be administered at least 1 week prior to immunization (*see* Note 13).
2. Post-sensitization administration: For induction of tolerance post-sensitization, Ag-NP should be administered at least 1 week after the final sensitization, with at least 1 week allowed between doses of Ag-NP (*see* Note 14).

4 Notes

1. 500 nm particles have been demonstrated to be an ideal size for the induction of tolerance in murine models of inflammation [6]. In our experience, carboxylated PLG nanoparticles purchased from vendors such as Phosphorex tend to be less efficient and more variable when used to induce tolerance than comparable particles manufactured using the methods outlined in Yap et al. (2014) [7].
2. Lyophilized nanoparticles are hygroscopic, and precautions should be taken to protect them from moisture. It is recommended to wrap nanoparticle storage containers in Parafilm and store in a desiccator under vacuum at room temperature.
3. Prepare an extra 20% of nanoparticles to account for void volume lost during injections.
4. Resuspension of pelleted nanoparticles can be difficult, requiring vigorous pipetting. Smaller volumes make this more aggressive resuspension easier.
5. If protein or peptide antigens are stored at a different concentration, simply adjust the volume of PBS used for nanoparticle resuspension accordingly. The

reaction volume should be 20 μ L per mg of nanoparticles with final concentrations of 50 mg/mL nanoparticles, 20 mg/mL ECDI, and 2 mg/mL antigen.

6. After antigen conjugation, nanoparticles will pellet in a streaky “comet” formation due to formation of particle-antigen-particle conjugates of varying sizes. Additional debris will coat the sides of the microcentrifuge tube. When resuspending, focus only on the pellet.
7. Nanoparticle suspensions can become stuck to the underside of the cell strainer. To maximize nanoparticle recovery, suction from the underside of the strainer using a fresh pipette tip, and add to the cryogenic vial.
8. Intravenous injection of nanoparticles is essential for efficient induction of tolerance as other administration routes such as mucosal or subcutaneous have been demonstrated to be either ineffective or pro-inflammatory [6, 7]. We have found that tail vein injection is the most consistent and efficient method of intravenous administration.
9. To dilate the tail vein and improve injection efficacy, dip tails in a beaker of warm water for a few seconds, and wipe dry immediately before injection.
10. Doing a single injection at a time is recommended to ensure even administration across multiple mice due to the quick rate at which nanoparticles settle out of suspension.
11. Thorough washing of PLG(Ag) prior to injection is critical to remove any non-encapsulated Ag remaining on the surface of lyophilized particles. Failure to wash particles may result in adverse reactions in sensitized recipient animals.
12. PLG(Ag) were successfully employed in prophylactic and postsensitization tolerance in a model of OVA-induced allergic airway inflammation [9]. In this model, tolerance was induced using a dose of 2.5 mg of PLG(OVA) delivering approximately 1 nmol of OVA. Varying encapsulation efficiencies of different antigens [8, 9] and the requirements of different models of inflammation will necessitate titration of doses in an antigen-and model-dependent fashion.
13. In our experience, prophylactic tolerance with Ag-NP works best by allowing at least 1 week for development of regulatory responses to tolerogen before sensitization. With repeated sensitizations in a model of OVA-/Alum-induced allergic airway inflammation, we have had the best success with administering Ag-NP before each immunization [9]. However, optimal dosage and timing of Ag-NP administration should be determined individually for each model.
14. In our experience in a model of OVA-/Alum-induced allergic airway inflammation, successful inhibition of inflammation was achieved with two doses of nanoparticles administered 1 and 2 weeks after the final sensitization, respectively [9]. Furthermore, superior inhibition, although not complete abrogation, of inflammation was achieved with a PLG(Ag) formulation.

However, optimal dosage and timing of Ag-NP administration should be determined individually for each model.

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