

ORIGINAL ARTICLE

Author Proof

Desensitizing mice to ovalbumin through subcutaneous microsphere immunotherapy (SMITH)

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Background: We investigated whether subcutaneously injected, biodegradable, poly (D,L-lactic-co-glycolic) acid (PLGA) microspheres loaded with ovalbumin (OVA) were able to down-regulate the T helper 2 (TH2) response in mice that were sensitized to this protein, and whether this response was dose-dependent.

Methods: PLGA microspheres were created using a double emulsion technique. Female BALB/c mice were sensitized to OVA and then assigned to receive subcutaneous microsphere immunotherapy (SMITH) using either blank microspheres (n = 4), low-dose OVA-loaded microspheres (n = 5), or high-dose OVA-loaded microspheres (n = 5). Antigen-specific immunoglobulin E (IgE) in serum was measured at day 0 (prior to sensitization), day 14 (prior to immunization), and days 28 and 42 (after immunization).

Results: All mice were successfully sensitized to OVA. In the group receiving blank microspheres, there was a continual rise in antigen-specific IgE from Day 14 to Day 42, which was statistically significant. In the group receiving low-dose OVA-loaded microspheres, there was a statistically significant drop in the antigen-specific IgE levels from Day 14 to

Day 28, but overall no significant change in IgE level when looking at the Day 14 to Day 42 interval. A similar pattern of antibody level changes was seen in the group receiving high-dose OVA-loaded microspheres, but the decrease from Day 14 to Day 28 was not statistically significant.

Conclusion: Our data suggest that SMITH has the ability to downregulate the production of antigen-specific IgE in a food allergy model, and this observation in our study was not dose-dependent. Its potential use in the treatment of IgE-mediated allergic disease warrants further investigation. © 2011 ARS-AAOA, LLC.

Key Words:

allergy; anaphylaxis; desensitization; IgE; ovalbumin; PLGA; subcutaneous immunotherapy

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It is estimated that allergic rhinitis affects up to 40% of people in the United States.¹ Antigen-specific immunotherapy, via the weekly, subcutaneous, bolus injection route (SCIT), represents the “gold standard” of treatment for immunoglobulin E (IgE)-mediated environmental

allergies. SCIT provides significant reduction in total symptom scores for seasonal allergic rhinitis, decreases the need for allergy medication use, and decreases the likelihood of developing comorbidities such as sinusitis and asthma. Specific scores for nasal, bronchial, and ocular symptoms are also improved along with quality of life scores.² However, barriers to the acceptance of this therapy include risk of anaphylaxis, inconvenience of weekly office visits, and aversion to repeated injections.

Alternate routes of administration have been sought, such as oral immunotherapy and sublingual immunotherapy (SLIT), but neither have gained widespread acceptance, in part due to the need for daily therapy, lack of U.S. Food and Drug Administration (FDA) approval, and lower efficacy.³ Pharmacokinetic and absorption data suggest that the efficacy of immunotherapy is related not to rapid absorption of large doses of antigen into the systemic circulation, but rather the retention of lower doses of antigen in the submucosal and subcutaneous spaces for an extended period of time where the antigen can interact with lymphoid tissue.⁴

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This information suggests that a method for administering SCIT using a slow-release system might provide an advantage over the traditional bolus method.

Injectable, protein-loaded, biodegradable poly (D,L-lactic-co-glycolic) acid (PLGA) microspheres were first introduced in the mid-1980s for the purpose of cancer therapy.⁵ Since then, they have been used in medicine for the delivery of chemotherapeutic drugs, immunosuppressants, anti-inflammatory agents, antibiotics, opioid antagonists, steroids, hormones, anesthetics, vaccines, and gene therapy.⁶ They have also recently been used to deliver a photosensitizer for photodynamic treatment of head and neck cancer.⁷ Microspheres made from PLGA are able to slowly release protein over time, in a controlled fashion, requiring less frequent therapy. By avoiding the bolus of antigen, the drug spends more time in the therapeutic window, which theoretically improves the efficacy of the treatment and avoids unwanted reactions associated with toxic drug levels.⁶

Q2 This pilot study is designed to assess the feasibility of subcutaneous microsphere immunotherapy (SMITH) to down-regulate the T helper 2 (Th2) response using PLGA microspheres containing a food allergen, ovalbumin (OVA), in an established mouse model for allergy. The primary hypothesis for this study is that the continual release of antigen from subcutaneous, biodegradable PLGA microspheres in sensitized mice will lead to a downregulation in production of antigen-specific IgE, which would suggest that desensitization is occurring. A secondary hypothesis is that the response seen from antigen-loaded microspheres will act in a dose-dependent fashion.

Materials and methods

The animal use protocol was approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College (#0712-697A).

Animals

Female BALB/c mice, 6 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in the Research Animal Resource Center at Weill Cornell Medical College, New York City. They were fed standard diet and water ad libitum and treated according to the institutional rules of animal care.

Preparation of PLGA microspheres

Microspheres were created using a double emulsion technique as previously described.⁸ Briefly, the PLGA polymer (200 mg) (RG503, 50:50; Polysciences, Warrington, PA) was dissolved in methylene chloride (4 mL). OVA protein (20 mg) (EMD Biosciences, San Diego, CA) dissolved in phosphate buffered saline (PBS) (0.2 mL) was combined with the polymer solution in a polypropylene tube, and the 2-phase system was homogenized for 10 seconds. The

primary emulsion of protein surrounded by polymer was then sonified for 1 minute at 30% (Branson Sonifier 150, Branson, Danbury, CT) while kept on ice.

The primary emulsion was added to a 2.5% polyvinyl alcohol (PVA) solution (25 mL) and homogenized for 20 seconds using vortex to create the secondary emulsion, which was then quickly added to a 0.5% PVA solution (450 mL) and stirred on a magnetic stirrer at 800 revolutions per minute (rpm) for 3 hours at room temperature, allowing the methylene chloride to evaporate. The solid sphere suspension was divided into 10 polypropylene tubes and centrifuged at 4800 relative centrifugal force (RCF) for 20 minutes at 10°C (Beckman Coulter Allegra 25R centrifuge, Beckman Coulter, Brea, CA). The supernatant was removed and the pellets were washed 3 times with deionized (DI) water in order to remove any residual PVA and then combined.

The spheres were then suspended in DI water (10 mL) and lyophilized for a minimum of 12 hours (Labconco Freezone 4.5; Labconco, Kansas City, MO). The final products were fine powders with approximate yields of 40% for OVA-loaded particles (89 mg) and 53% for empty particles (117 mg). Last, particle morphology was studied at low voltage (5 kV) using scanning electron microscopy imaging (Leica 440; Leica, Wetzlar, Germany) after coating with palladium (Fig. 1).

Encapsulation and in vitro release studies

To measure the encapsulation of protein into the microspheres, 5 mg of protein-loaded microspheres were dissolved in 1 mL 1M NaOH, followed by neutralization with 1 mL 1M HCl. A similar procedure was carried out with 5 mg of blank microspheres in order to account for background absorbance from dissolved microspheres. The protein content was determined using a spectrophotometer (Ultraspec 3100 Pro; Amersham Biosciences, Piscataway,

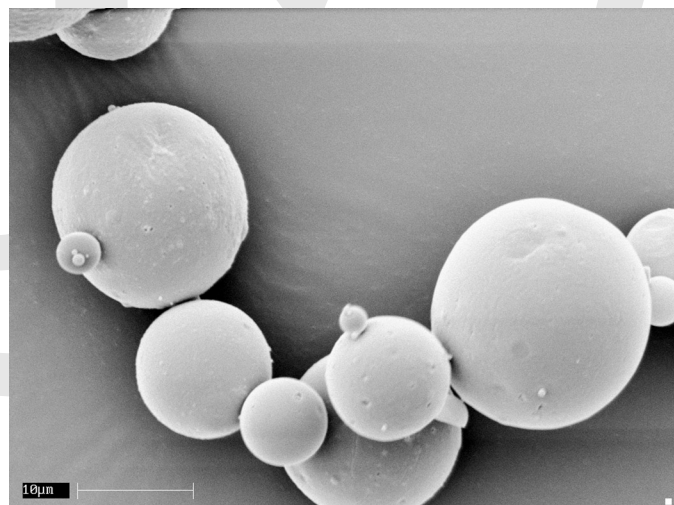


FIGURE 1. Protein-loaded microspheres as seen under the SEM. SEM = scanning electron microscope.

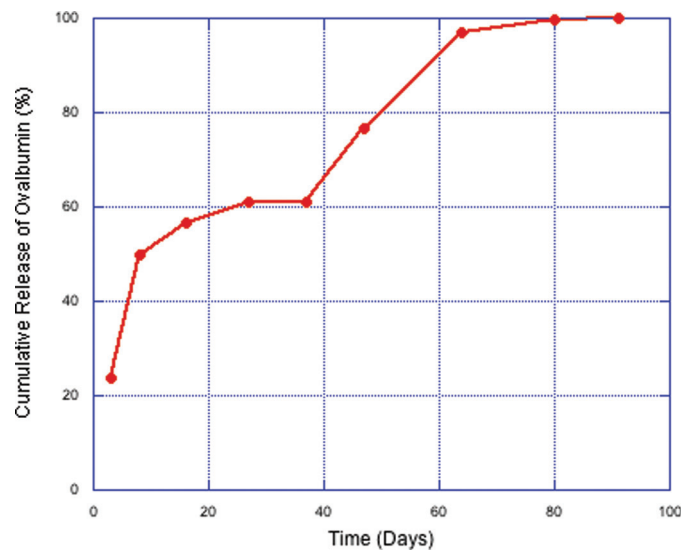


FIGURE 2. Cumulative release of OVA from loaded microspheres (as a percentage of the total amount released), suspended in PBS, plotted as a function of time (days). OVA = ovalbumin; PBS = phosphate buffered saline.

NJ) by measuring the absorbance of these solutions at 280 nm.

The release characteristics were examined by suspending 15 mg of protein-loaded microspheres in 1 mL PBS in a centrifuge tube that was incubated on a rocker at 37° C. On days 3, 7, 14, 21, 28, 35, and 42 the sample was centrifuged for 15 minutes at 8000 rpm. The supernatant was removed and the microspheres were resuspended with 1 mL of fresh buffer. The concentration of protein in the supernatant was estimated using a spectrophotometer by measuring the absorbance of this solution at 280 nm (Fig. 2).

Sensitization and immunization of mice

All mice ($n = 14$) received a 0.2-cc intraperitoneal injection of 20 μ g OVA in PBS adsorbed to 0.34 mg $\text{Al}(\text{OH})_3$ on day 0 with an identical booster given 7 days later (Fig. 3). On Day 14, the mice were randomly divided into 3 groups, and each group received a subcutaneous injection of microspheres suspended in 200 μ L PBS. The first group ($n = 5$) received blank microsphere containing no OVA. The second group ($n = 5$) received a low dose of OVA-loaded microspheres (130 μ g OVA) and the third group

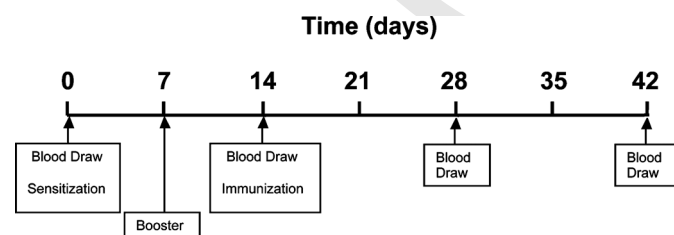


FIGURE 3. Schematic illustration of the experimental protocol.

($n = 5$) received a high dose of OVA-loaded microspheres (1.3 mg OVA).

Measurement of antigen-specific IgE

Serum was harvested from each mouse via the retro-orbital sinus prior to the first sensitization injection (Day 0), prior to immunization with microspheres (Day 14), and after immunization, on Days 28 and 42. Serum-specific OVA IgE levels were measured in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) kit with a sensitivity of 3.8 ng/mL according to the manufacturer's recommendations (MD Biosciences, St. Paul, MN). Briefly, 20 μ L of serum were mixed with 0.1% sodium azide solution (180 μ L) to create a 1/10 dilution. Samples and standards (100 μ L) were pipetted in duplicate into the wells of a polystyrene microtiter plate with anti-OVA-IgE monoclonal antibody adsorbed to the surface. After incubation at room temperature for 60 minutes on an orbital shaker (750 rpm), the contents of the wells were aspirated and the plate was washed four times with a 1 $\times$ solution of buffered surfactant. Biotinylated OVA in a stabilizing buffer (100 μ L) was placed in each well and the plate was then incubated for 30 minutes and washed in a similar fashion. The wells were aspirated and filled with 100 μ L of diluted streptavidin-horseradish peroxidase (HRP) in a stabilizing buffer and incubated in the dark without agitation for 30 minutes. After aspiration and washing, tetramethylbenzidine (TMB) substrate solution (3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in citric acid buffer, pH 3.3) was added to each well (100 μ L) and incubated in the dark for an additional 15 minutes without agitation. To stop the color reaction, 100 μ L of 0.3 M sulfuric acid was added and the optical density 450 nm (OD_{450}) was measured by a plate reader. A standard curve was created using known concentrations of OVA IgE and the OD_{450} of all samples were found to be within the working range of the assay.

Statistical analysis

Data are presented as mean values $\pm$ standard error of means (SEM). Statistical analysis was performed using the paired Student t test to compare the mean values at each time point within each group (GraphPad Software, Inc.). P values <0.05 were considered statistically significant. One-way analysis of variance (ANOVA) was used to evaluate for statistically significant variance between the 3 groups (Excel 2004 for MacIntosh, version 11.5.5). F values >1 were considered statistically significant.

Results

Encapsulation and release of OVA from microspheres

Microspheres were approximately 10 μ m in diameter (Fig. 1). The encapsulation rate was 92%, which is similar

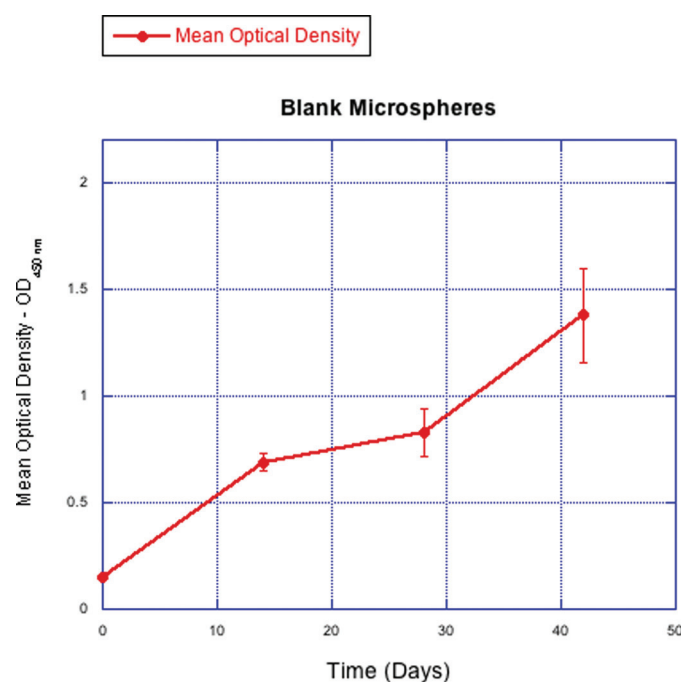


FIGURE 4. Humoral response after sensitization of mice with OVA, followed by subcutaneous immunotherapy with blank microspheres. The optical density (OD₄₅₀) is proportional to the IgE antibody binding capacity of OVA. IgE = immunoglobulin E; OD₄₅₀ = optical density 450 nm; OVA = ovalbumin.

to what has been reported previously.⁹ It was determined that 53 μ g of encapsulated protein were released per 1 mg dry weight of suspended microspheres during a 6-week period. An initial burst phase was seen, likely due to diffusion of protein from the surface of the microspheres, followed by a steady release of protein thereafter due to polymeric erosion (Fig. 2). This pattern was also similar to what has been described previously in the literature.¹⁰

Humoral response after immunization

All of the mice were successfully sensitized to OVA, as demonstrated by a statistically significant rise in OVA-IgE from Day 0 (baseline) to Day 14 in all 3 groups. In the group receiving blank microspheres, there was a continual rise in antigen-specific IgE levels from Day 14 to Day 42, which was statistically significant (Fig. 4). In the group receiving a low dose of antigen-loaded microspheres, there was no significant difference in IgE levels on Day 14 and Day 42, but there was a statistically significant decrease in the antigen-specific IgE level on Day 28 (Fig. 5). In the high dose group, there was no significant difference in IgE levels on Day 14, Day 28, or Day 42 (Fig. 6). One-way ANOVA detected no significant treatment variance between groups.

Discussion

PLGA, which is a component of devices such as absorbable sutures and bone fixation materials, is well tolerated by the

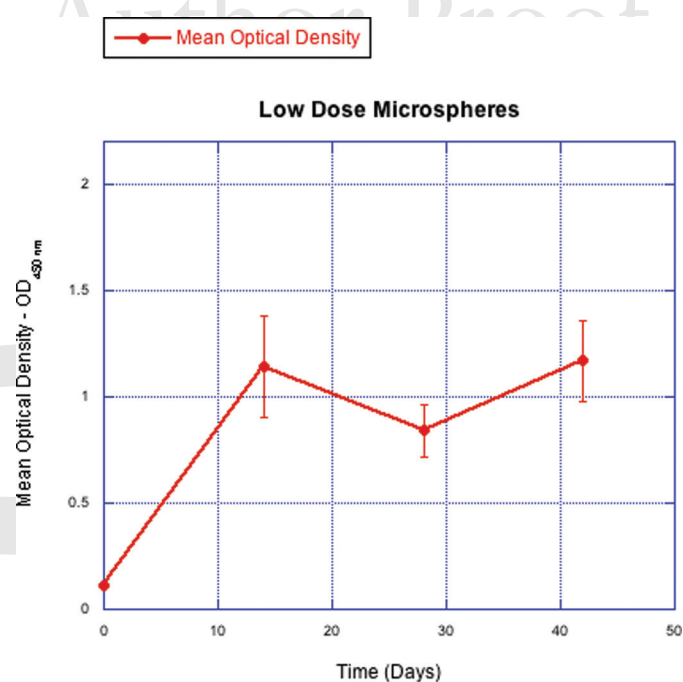


FIGURE 5. Humoral response after sensitization of mice with OVA, followed by subcutaneous immunotherapy with microspheres containing a low dose (130 μ g) of OVA. The optical density (OD₄₅₀) is proportional to the IgE antibody binding capacity of OVA. IgE = immunoglobulin E; OD₄₅₀ = optical density 450 nm; OVA = ovalbumin.

body. When coming in contact with artificial or biologic fluids, it is broken down by nonenzymatic hydrolytic cleavage into lactic and glycolic acids, which are easily metabolized into carbon dioxide and water.^{11,12} The release rate of

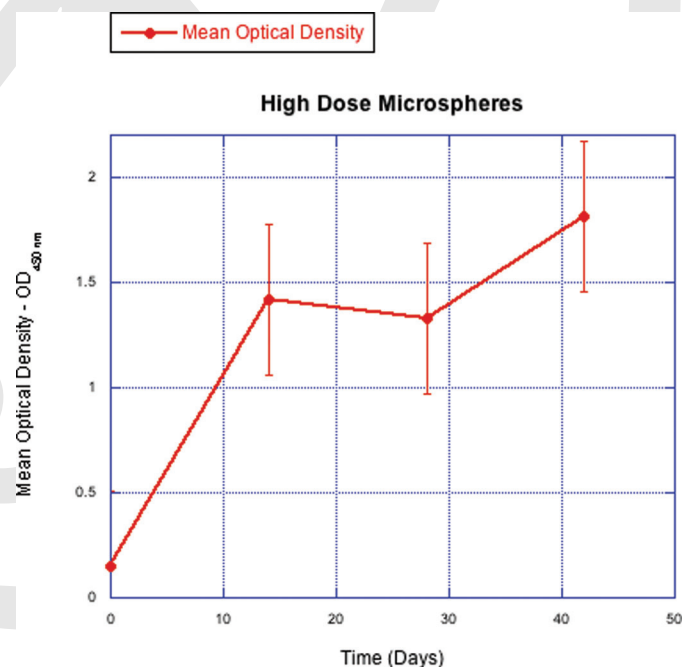


FIGURE 6. Humoral response after sensitization of mice with OVA, followed by subcutaneous immunotherapy with microspheres containing a high dose (1.3 mg) of OVA. The optical density (OD₄₅₀) is proportional to the IgE antibody binding capacity of OVA. IgE = immunoglobulin E; OD₄₅₀ = optical density 450 nm; OVA = ovalbumin.

encapsulated protein can be controlled by varying the particle size and polymer material.¹³ The literature suggests that a single immunization with particles as small as 1 μm may be sufficient to elicit an immune system response.¹⁴ PLGA microspheres less than 10 μm in diameter are phagocytosed by macrophages in the region, while microspheres greater than 10 μm remain at the injection site releasing protein.^{15,16} However, high-serum antibodies have been demonstrated to occur regardless of the pathway taken.¹⁷

In 1994, Sharif et al.^{18,19} first encapsulated dust mite antigen, Der p 1, into PLGA microspheres and discovered that the encapsulation and release processes did not significantly alter the IgE and immunoglobulin G (IgG)-binding capabilities to this antigen. Scholl et al.^{18,19} also demonstrated that encapsulated major birch pollen allergen, Bet v 1, retained its immunogenic potential after encapsulation.²⁰ In vitro studies have demonstrated that both subcutaneous and intraperitoneal administration of encapsulated olive tree pollen, Ole e 1, is able to elicit a specific Th1 immune response in mice, producing high levels of IgG2a antibodies.^{14,21}

There has also been interest in developing an oral vaccine for type I allergies to enhance patient compliance, safety, and comfort. Antigens that are encapsulated in PLGA microspheres are better protected against acidic and proteolytic degradation in the gastrointestinal (GI) tract.²² In a small double-blind placebo-controlled human study, Tepas et al.²³ used a microencapsulated form of timothy grass pollen for oral immunotherapy. One daily treatment for 10 weeks caused a decrease in combined medication and symptom scores in 11 of 12 patients vs placebo along with decreased in vitro lymphocyte proliferative response to antigen challenge in 9 of 12. However, there were no significant changes in IgG, IgE, or skin reactivity.

PLGA microspheres have also been proposed as a mechanism of oral desensitization for food allergies. Studies have demonstrated that mice fed encapsulated protein had greater capacity to suppress specific serum IgG than soluble protein, but if the encapsulated dose was too high, oral tolerance was broken and a strong, Th2 cell response was activated.^{24,25} In 2000, Pecquet et al.²⁶ demonstrated that the oral administration of PLGA microspheres loaded with beta-lactoglobulin were able to induce oral tolerance in mice at a dose 10,000 times less than required with soluble antigen.

However, studies have shown that the subcutaneous administration of protein-loaded microspheres produces a significantly higher immune response than oral administration, as measured by serum IgG levels, even if the protein dose is 10 times lower.^{14,20} It has also been determined that subcutaneous exposure to an encapsulated antigen does not lead to further sensitization to that antigen in mice.²⁰ When Scholl et al.²⁰ injected microspheres containing birch pollen antigen subcutaneously into previously sensitized mice, levels of IgG1 decreased and levels of IgG2a

increased along with interferon (IFN)- γ and interleukin (IL)-10, suggesting a downregulation of the Th2 response. However, antigen-specific IgE did not change in their study.²⁰

In theory, SMITH offers some potential advantages over traditional subcutaneous immunotherapy (SCIT) for airborne allergies, including reduced frequency of injections, more effective desensitization, and reduced risk of anaphylaxis. In 2007, Martinez et al.²⁷ demonstrated that antigen-specific immunotherapy, using subcutaneous injection of PLGA microspheres loaded with bee venom, phospholipase A2 (PLA2), was able to induce production of IgG2a in mice, and that higher titers of antibodies correlated with better protection against temperature drop when anaphylaxis was induced.

Previous attempts at using traditional SCIT for IgE-mediated food allergy demonstrated that tolerance was induced in individuals allergic to peanuts, but the rate of systemic reactions was unacceptably high.²⁸ For that reason, SCIT is currently not recommended for the treatment of food allergies. However, because of the slower, steady release of antigen, SMITH may also represent a new modality for delivering subcutaneous immunotherapy for food allergies in the future.

All the mice in this study tolerated SMITH well, both low-dose and high-dose, without any adverse events. The continual rise in IgE seen in the blank microsphere group was not seen in the low-dose or high-dose microsphere groups. Within the statistical limits of this small sample size, levels of antigen-specific IgE in both treatment groups stabilized after immunization at Day 14, suggesting a downregulation in the Th2 response to OVA. It is unclear whether the drop in IgE levels in the low treatment group from Day 14 to Day 28 represents a dose-dependent effect. Future animal studies will include larger group sizes and a longer observation period to measure changes in both IgE and IgG levels. Cytokine production of IFN- γ and IL-10 will also be used to study T cell proliferation in response to therapy.

Conclusion

To our knowledge, ours is the first study to examine the immunogenic effect of SMITH in the treatment of type I allergy, using a food allergen in an established mouse model. Previous studies have only examined antigen-loaded microspheres for the purposes of oral immunotherapy and subcutaneous airborne immunotherapy. Our data suggest that SMITH has the ability to downregulate the rise in antigen-specific IgE after sensitization, and its potential use in the treatment of IgE-mediated allergic disease warrants further investigation. There was no difference in effect between the 2 doses of SMITH chosen for this study. In the future, SMITH may represent a safer, more convenient and more effective way to treat airborne allergies, as well as a new method of inducing tolerance to food allergens via

the subcutaneous route. The next steps for SMITH involve determining the proper dosage of antigen, evaluating and comparing the long-term therapeutic effects of this therapy against currently available treatment modalities, and ultimately advancing this information toward human trials. 

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