

Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy

Kamal D. Srivastava, PhD,^{a,b} Alyssa Siefert, PhD,^c Tarek M. Fahmy, PhD,^{c,d} Michael J. Caplan, MD,^e Xiu-Min Li, MD,^{a,b} and Hugh A. Sampson, MD^{a,b}
New York, NY, and New Haven, Conn

Background: Treatments to reverse peanut allergy remain elusive. Current clinical approaches using peanut oral/sublingual immunotherapy are promising, but concerns about safety and long-term benefit remain a barrier to wide use. Improved methods of delivering peanut-specific immunotherapy are needed.

Objective: We sought to investigate the efficacy and safety of peanut oral immunotherapy using CpG-coated poly(lactic-co-glycolic acid) nanoparticles containing peanut extract (CpG/PN-NPs) in a murine model of peanut allergy.

Methods: C3H/HeJ mice were rendered peanut allergic by means of oral sensitization with peanut and cholera toxin. Mice were then subjected to 4 weekly gavages with CpG/PN-NPs, vehicle (PBS), nanoparticles alone, peanut alone, CpG nanoparticles, or peanut nanoparticles. Untreated mice served as naive controls. After completing therapy, mice underwent 5 monthly oral peanut challenges. Anaphylaxis was evaluated by means of visual assessment of symptom scores and measurement of body temperature and plasma histamine levels. Peanut-specific serum IgE, IgG₁, and IgG_{2a} levels were measured by using ELISA, as were cytokine recall responses in splenocyte cultures.

Results: Mice with peanut allergy treated with CpG/PN-NPs but not vehicle or other treatment components were significantly protected from anaphylaxis to all 5 oral peanut challenges, as indicated by lower symptom scores, less change in body temperature, and a lower increase of plasma histamine levels. Importantly, CpG/PN-NP treatment did not cause anaphylactic reactions. Treatment was associated with a sustained and

significant decrease in peanut-specific IgE/IgG₁ levels and an increase in peanut-specific IgG_{2a} levels. Compared with vehicle control animals, peanut recall responses in splenocyte cultures from nanoparticle-treated mice showed significantly decreased levels of T_H2 cytokines (IL-4, IL-5, and IL-13) but increased IFN- γ levels in cell supernatants.

Conclusions: Preclinical findings indicate that peanut oral immunotherapy with CpG/PN-NPs might be a valuable strategy for peanut-specific immunotherapy in human subjects. (J Allergy Clin Immunol 2016;■■■■:■■■■-■■■■.)

Key words: Peanut allergy, anaphylaxis, mice, IgE, immunotherapy, nanoparticle

Peanut allergy is a serious and often life-threatening condition for which there are no therapeutic options outside of careful avoidance of peanut and epinephrine injections administered as rescue medication.^{1,2} Because of frequent hidden peanut contamination of processed foods, the risk of peanut-induced anaphylaxis provokes great anxiety in patients and caregivers and negatively affects their quality of life.^{3,4} Despite some promising results from recent clinical trials of peanut oral immunotherapy (OIT)/sublingual immunotherapy (SLIT),⁵⁻⁸ these approaches still carry unacceptable risks for adverse events.⁹⁻¹¹ Accurate assessment of the efficacy and safety of these approaches is made difficult by the fact that such trials often exclude patients with a history of serious anaphylactic events, and additionally, the majority of patients appear to regain reactivity after cessation of therapy.^{12,13} For these reasons, peanut OIT and SLIT are not widely used in clinical practice, and their implementation is a matter of much debate.^{9,14} Thus a need remains for development of improved modalities of delivering peanut-specific immunotherapy.

Poly(lactic-co-glycolic acid [PLGA]) nanoparticles, which are currently in clinical use, are biodegradable particles suitable for oral delivery of therapeutic agents because they are stable in the gastrointestinal environment.^{15,16} PLGA nanoparticles sized between 200 and 400 nm have been shown to be retained in lymphoid organs.¹⁷

In the current study we sought to investigate the efficacy of peanut OIT by using CpG-coated poly(lactic-co-glycolic acid) nanoparticles containing peanut extract (CpG/PN-NPs) in a murine model of peanut allergy. We found that 4 weekly gavages of CpG/PN-NPs to mice with peanut allergy provided significant and long-lasting protection from anaphylaxis to repeated peanut challenges extending over a period of 4 months after therapy. Protection was associated with decreased postchallenge histamine levels and reduced hypothermia. Peanut-specific T_H2 immune responses, such as peanut-specific IgE, IL-4, IL-5, and IL-13, were significantly reduced in CpG/PN-NP-treated mice, whereas

From ^aPediatric Allergy and Immunology, Icahn School of Medicine at Mount Sinai, New York; ^bthe Jaffe Food Allergy Institute, New York; ^cthe Department of Biomedical Engineering, Yale University, New Haven; and the Departments of ^dImmunobiology and ^eCellular and Molecular Physiology, Yale University School of Medicine, New Haven.

Supported by a grant from Allertein Therapeutics (to X.-M.L.). K.D.S. was partially supported by a KL2 Faculty Scholar Award KL2TR000069 from Mount Sinai CTSA.

Disclosure of potential conflict of interest: K. D. Srivastava and X.-M. Li have received research support from Allertein Therapeutics. A. Siefert and T. M. Fahmy have received research support from Yale University. M. J. Caplan has received consultancy fees from, has a patent with, and has stock/stock options in Allertein Therapeutics. H. A. Sampson has received consultancy fees from and has stock/stock options in Allertein Therapeutics and is employed by the Icahn School of Medicine at Mount Sinai.

Received for publication August 12, 2013; revised December 14, 2015; accepted for publication January 21, 2016.

Corresponding author: Hugh A. Sampson, MD, Kurt Hirschhorn Professor of Pediatrics, Director, Jaffe Food Allergy Institute, Department of Pediatrics, Box 1198, Icahn School of Medicine at Mount Sinai, New York, NY 10029. E-mail: hugh.sampson@mssm.edu.

0091-6749/\$36.00

© 2016 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2016.01.047>

Abbreviations used

CpG/PN-NP:	CpG-coated poly (lactic-co-glycolic acid) nanoparticles containing peanut extract
OIT:	Oral immunotherapy
PLGA:	Poly(lactic-co-glycolic acid)
PN/OEE/DNA-NP:	Organic <i>E coli</i> membrane nanoparticles containing peanut extract and sheared <i>E coli</i> DNA
rWNVE:	Recombinant West Nile virus envelope antigen
SLIT:	Sublingual immunotherapy
TLR:	Toll-like receptor

peanut-specific T_H1 responses, such as IgG_{2a} and IFN- γ , were increased. Importantly, administration of CpG/PN-NPs to mice with peanut allergy was not associated with allergic symptoms. This is the first report describing a PLGA nanoparticle-based peanut immunotherapy approach in mice with established peanut allergy, and our findings suggest that this approach merits further research for applicability in human subjects.

METHODS**Mice**

Five-week-old female C3H/HeJ mice purchased from the Jackson Laboratory (Bar Harbor, Me) were maintained in pathogen-free facilities at the Mount Sinai vivarium according to standard guidelines for the care and use of animals.¹⁸

Sensitization and challenge of peanut-allergic mice

As described previously,¹⁹ mice were intragastrically sensitized with 10 mg of homogenized peanut (whole roasted peanuts with skins but shells removed and blended in PBS) in 0.5 mL of PBS containing 75 mg of sodium bicarbonate (Fisher Scientific, Bridgewater, NJ), 20 μ g of the mucosal adjuvant cholera toxin (List Laboratories, Campbell, Calif), and 16.5 μ L (1.1 μ L/g body weight) of 80-proof Stolichnaya Vodka (a source of food-grade ethanol) to neutralize stomach pH and increase gastrointestinal permeability²⁰ at the times indicated in Fig 1. A boosting dose of 50 mg of peanut was given at weeks 6 and 8 using the same gavage solution as above. Naive mice were not sensitized. Oral challenge with 200 mg of peanut commenced at week 14, and subsequent challenges were given at weeks 18, 22, 26, and 30. Mice were given a sensitization dose of peanut and cholera toxin 1 day after all but the final challenge to maintain reactivity.

Nanoparticle preparation and characterization

Nanoparticles composed of 50:50 PLGA (Durect, Pelham, Ala) were synthesized by using an endotoxin-free double-emulsion technique, as previously described.²¹ Briefly, PLGA was dissolved in methylene chloride (Thermo Fisher, Waltham, Mass) at room temperature. Roasted peanuts were ground to a paste and washed with acetone (Fisher Scientific) to remove fat and dried to powder. Defatted peanut extract dissolved in PBS solution was added dropwise to vortexing polymer. Polymer/peanut solution was sonicated for 30 seconds at 38% amplitude with a tekmar Sonic Distributor (tekmar Control Systems, Vernon, British Columbia, Canada) fitted with a CD26 sonicator. The emulsion was then added dropwise to a vortexing solution of avidin-palmitate, prepared as previously described,²¹ and 1.25% poly(vinyl alcohol) (Sigma-Aldrich, St Louis, Mo) and then ultrasonicated for 30 seconds. The resulting emulsion was added to a vigorously stirring solution of 0.3% poly(vinyl alcohol), allowing methylene chloride to evaporate while nanoparticles hardened at room temperature. Particles were collected by means of centrifugation at 18,500g, washed 3 times with deionized water, flash-frozen with liquid nitrogen, lyophilized, and stored at -20°C before analysis and use. Particle size was measured by using the liquid-phase Stokes diffusion

particle-tracking system (NanoSight, Wiltshire, United Kingdom), and spherical morphology was verified with scanning electron microscopy. Particles had an average diameter of 214 $\pm$ 39 nm. Total protein levels were measured by dissolving the nanoparticles in 0.2 N NaOH at room temperature overnight and assaying with a micro BCA protein assay (Pierce, Boston, Mass). Biotinylated type B CpG-ODN 1826 (5'-TCC ATG ACG TTC CTG ACG TT-3') with a phosphorothioate backbone (Keck Oligonucleotide Synthesis Resource, Yale University, New Haven, Conn) was added to peanut-loaded nanoparticles on the day of each dose administration. The nanoparticles and CpG-biotin were suspended in sterile PBS, pH 7.4, and incubated for 15 minutes at room temperature to allow for labeling and used within 30 minutes of preparation. For organic *E coli* membrane nanoparticles containing peanut extract and sheared *E coli* DNA (PN/OEE/DNA-NPs) (data shown in Fig E1 in this article's Online Repository at www.jacionline.org), PLGA particle assembly was essentially as described above but without avidin-palmitate and included *Escherichia coli* membrane extract.²² Particles were loaded with sheared *E coli* DNA, bacterial intracellular proteins, and 200 μ g of peanut extract.

Nanoparticle therapy

Nanoparticle therapy commenced at week 11 of the protocol shown in Fig 1. Mice were fasted for 2 hours and administered either 0.5 mL of PBS (treatment vehicle), 200 μ g of peanut extract, PLGA nanoparticles alone, CpG-PLGA nanoparticles (1.8 μ g of CpG per dose), peanut nanoparticles (200 μ g of peanut per dose), CpG/PN-NPs, or PN/OEE/DNA-NPs by means of oral gavage once a week for 4 consecutive weeks. Naive mice were not treated.

Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30 to 40 minutes after oral peanut challenge, as described previously.¹⁹ Visually observed symptoms were scored by using the scoring system described previously²³: 0, no symptoms; 1, scratching and rubbing around the snout and head; 2, puffiness around the eyes and snout, pilar erection, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; and 5, death. Cage identities were concealed during visual assessment of anaphylactic symptoms. Rectal temperatures were measured with a rectal probe (Harvard Apparatus, Holliston, Mass).

Measurement of plasma histamine levels

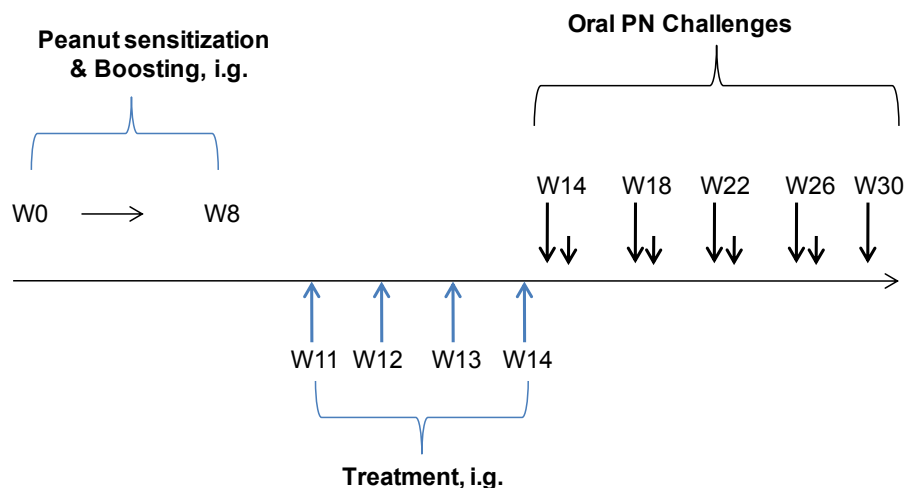
Thirty minutes after scoring and measurement of body temperature, blood was collected by means of submandibular bleeding, and plasma was harvested within 20 minutes after blood collection by means of submandibular bleeding at challenge and stored at -80°C until use.²⁴ Histamine levels were measured with an enzyme immunoassay kit (Fisher Scientific), as described by the manufacturer.

Measurement of food specific-IgE, IgG_{2a}, and IgG₁ levels

Peanut-specific IgE, IgG_{2a}, and IgG₁ levels in serum were measured, as reported previously.²⁵ Briefly, microtiter plates were coated with defatted peanut extract (sample wells), anti-mouse IgE (BD Biosciences, San Jose, Calif, for IgE reference wells), or DNP-HSA (Sigma-Aldrich for IgG_{2a} and IgG₁ reference wells) and held overnight at 4°C. After washing, plates were blocked with 2% BSA-PBS. Washed plates were incubated with diluted serum samples, mouse IgE (BD Biosciences), anti-DNP-IgG_{2a}, or anti-DNP-IgG₁ (Accurate Antibodies, Westbury, NY) overnight at 4°C and developed by using biotinylated anti-IgE IgG_{2a} or IgG₁ detection antibodies (BD Biosciences), avidin-peroxidase, and ABTS substrate (KPL, St Paul, Minn).

Cell culture and cytokine measurements

After final challenge at week 30, splenocytes from individual animals were prepared, as previously described, and cultured in the presence or absence of 200 μ g/mL defatted peanut extract.²⁵ Culture supernatants were collected



Treatment Groups (from 2 separate experiments):

1. Sham (PBS), N=13
2. PN (peanut alone), N=10
3. NP (nanoparticle alone), N=10
4. PN-NP, N=10
5. CpG-NP, N=10
6. PN/CpG-NP, N=22
7. Naïve, N=15

FIG 1. Experimental design. Protocol for sensitization, treatment, and challenge: 6-week-old C3H/HEJ mice were orally sensitized with peanut (PN) and cholera toxin at weeks 0 through 5. Mice were boosted with peanut and cholera toxin at weeks 6 and 8. Weekly oral treatment started at week 11 (blue upward arrows) and continued through week 14. Mice underwent oral peanut challenge 5 days later and then at 4-week intervals for another 4 challenges (weeks 14, 18, 22, 26, and 30, as indicated by tall black arrows). Short black arrows indicate oral gavage with sensitization dose of peanut plus cholera toxin given 1 day after each challenge, except the final challenge at week 30. N = 10-22 mice per group from 2 separate experiments. *i.g.*, Intragastric; *NP*, nanoparticles.

after 72 hours. Cytokine levels were determined by means of ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn, for IL-13 and BD Biosciences for all others), and reported after subtraction of basal cytokine levels of cultures stimulated with medium alone.

Statistical analysis

Data were analyzed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). Plasma histamine and body temperature data were normally distributed, and differences between groups were analyzed by means of 1-way ANOVA, followed by the Bonferroni posttest. For all other data, Kruskal-Wallis ANOVA on ranks was performed, followed by the Dunn posttest. *P* values of less than .05 were considered significant.

RESULTS

Peanut OIT delivered through PLGA nanoparticles expressing CpG oligonucleotide protects mice with peanut allergy from anaphylactic symptoms to oral peanut challenge

Mice with peanut allergy were generated by using the oral sensitization and boosting protocol shown in Fig 1. Starting at

week 11 of the protocol, mice were given 4 weekly oral treatments with agents listed in Fig 1 and described in the Methods section. Mice were observed for 30 minutes after receiving treatment. No visual signs of anaphylaxis were observed in the treatment group, and their behavior was indistinguishable from that of vehicle (PBS)-treated control mice. Consistent with our previous studies using this model, vehicle-treated mice showed observable signs of anaphylaxis, with 18 of 20 mice reacting at the first peanut challenge and 100% reacting at the following 4 challenges given monthly (Fig 2). Median symptom scores (indicated by bars in Fig 2) in the vehicle treatment group ranged from 2 to 3 on a 5-point scale. In comparison, more than 70% of mice treated with CpG/PN-NPs were protected completely from anaphylaxis at almost all monthly challenges, and median symptom scores in these groups were significantly lower ($P < .01$ vs .001 vs sham). Mice receiving peanut alone, nanoparticles alone, or CpG nanoparticles had reaction rates of 100% at all 5 monthly challenges. Three of 10 mice given peanut loaded within nanoparticles did not react to the first challenge; however, all mice in this group reacted to subsequent challenges.

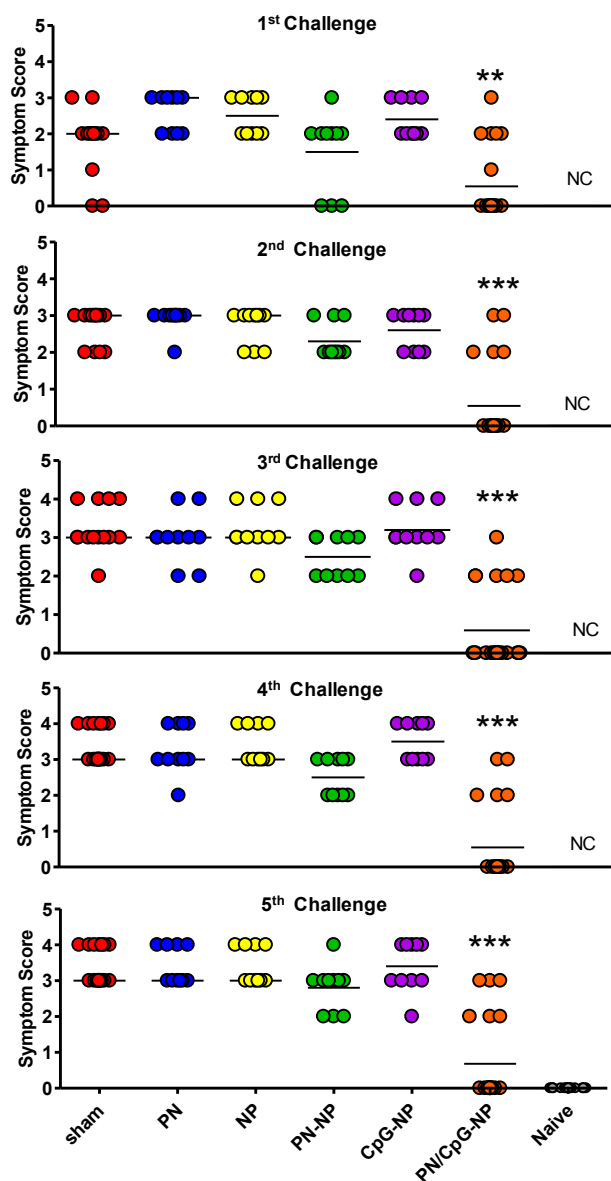


FIG 2. Anaphylaxis symptom scores. Thirty minutes after oral peanut (PN) challenge, mice were visually assessed for symptoms of anaphylaxis and assigned symptom scores using the scoring system described in the Methods section. Naive mice were challenged only at the fifth challenge. Bars represent group medians. ** $P < .01$ and *** $P < .001$ versus sham. N = 10-22 mice per group from 2 separate experiments. NC, Not challenged; NP, nanoparticles.

Mice treated with CpG/PN-NPs are protected from anaphylaxis-associated hypothermia

Hypothermia is associated with systemic anaphylaxis in mice.²⁶ After each oral peanut challenge, the vehicle control group had mean body temperatures that were consistently lower than those of the naive control mice, indicating systemic anaphylaxis (Fig 3). Postchallenge body temperatures in CpG/PN-NP-treated mice were significantly higher than those in vehicle control mice at all challenges except the first challenge at week 14 ($P < .05$ -.001 for CpG/PN-NPs vs sham treatment).

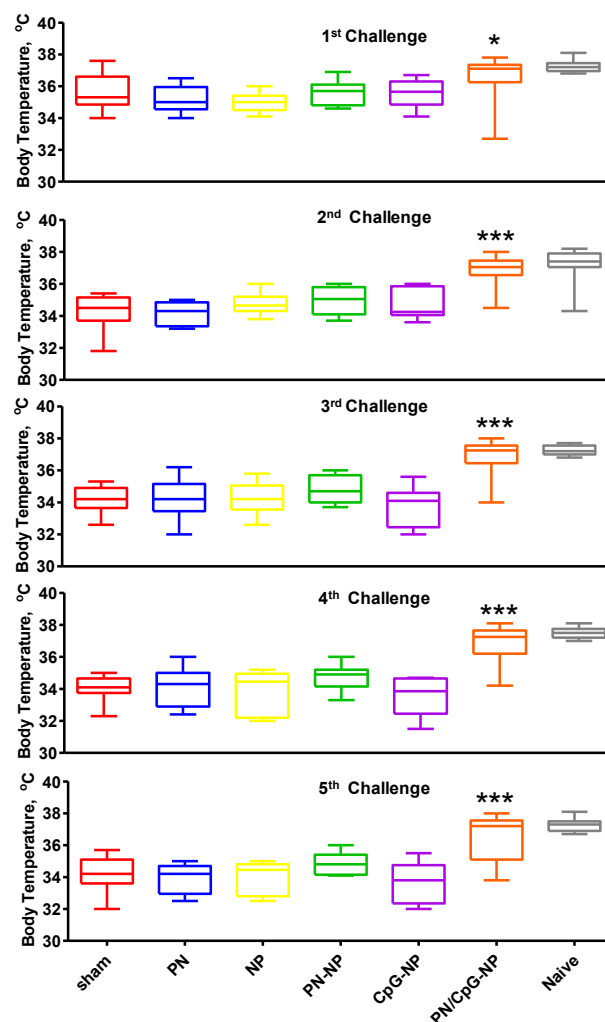


FIG 3. Body temperature after oral peanut (PN) challenges. After recording symptom scores, the core body temperature of each animal was measured with a rectal thermometer. Data show group medians and interquartile range. * $P < .05$ and *** $P < .001$ versus sham. N = 10-22 mice per group from 2 separate experiments. NP, Nanoparticles.

Postchallenge plasma histamine levels are significantly reduced in mice treated with CpG/PN-NPs

Consistent with protection from anaphylaxis, mean plasma histamine levels in blood drawn 30 minutes after each oral peanut challenge were markedly lower in CpG/PN-NP-treated mice compared with those in the sham group. Differences in group means between the treatment and vehicle control groups were observed at all 5 monthly challenges ($P < .05$ -.001), as shown in Fig 4. Mean postchallenge histamine levels in groups receiving nanoparticles alone, CpG nanoparticles, or peanut alone were indistinguishable from sham treatment for all challenges. Mean histamine levels in mice given peanut nanoparticles were modestly reduced only at the first monthly challenge, although without statistical significance.

CpG/PN-NP therapy reduces peanut-specific serum IgE and IgG₁ levels but increases peanut-specific IgG_{2a} levels

One day before commencement of treatment at week 11, we observed no differences in peanut-specific serum IgE levels

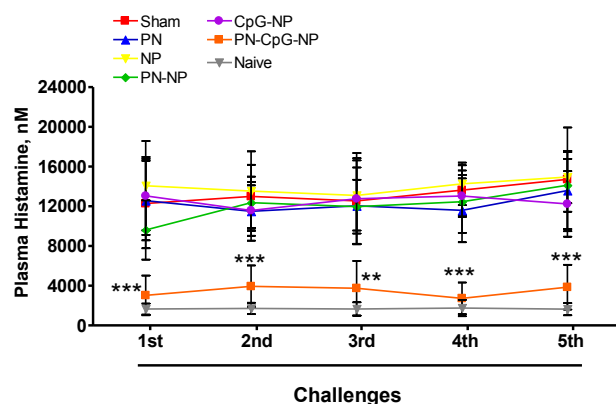


FIG 4. Plasma histamine levels after oral peanut (PN) challenge. Blood was drawn approximately 30 minutes after the oral peanut challenge, and plasma was harvested for measurement of histamine by using ELISA. Data are shown as means $\pm$ SDs at each of the 5 challenges. ** $P < .01$ and *** $P < .001$ versus sham. $N = 10$ –22 mice per group from 2 separate experiments. NP, Nanoparticles.

between mice assigned to treatment groups and mice assigned to the sham control group, as shown in Fig 5, A. After commencement of therapy, mean IgE levels in the CpG/PN-NP treatment group steadily decreased through the duration of the study, whereas IgE levels in sham-treated mice remained relatively unchanged. Significantly reduced peanut-specific IgE levels in CpG/PN-NP-treated mice were observed at all challenges except the first challenge at week 14 ($P < .05$ –.001). A similar trend of results was observed for peanut-specific IgG₁, a T_H2-associated immunoglobulin that has been implicated in anaphylactic reactions in mice (Fig 5, B).

IgG_{2a} is a T_H1-associated immunoglobulin in mice. Before starting treatment, group means of peanut-specific serum IgG_{2a} levels between the treatment and vehicle control groups were indistinguishable (CpG/PN-NPs: 339,592 $\pm$ 51,494 ng/mL vs vehicle control: 318,286 $\pm$ 32,586 ng/mL). Posttherapy peanut-specific IgG_{2a} levels were significantly increased in CpG/PN-NP-treated mice at all 5 challenge times ($P < .05$ –.01), as shown in Fig 5, C. Naive mice that were not sensitized or treated had undetectable peanut-specific IgE, IgG₁, and IgG_{2a} levels throughout the study.

Mice treated with CpG/PN-NPs show beneficial alterations in cytokine profiles in response to peanut restimulation

After the fifth and final peanut challenge, mice were killed, splenocyte cultures were restimulated with peanut, and levels of cytokines secreted in the cell supernatant were measured by using ELISA. As expected, high levels of T_H2 cytokines (IL-4, IL-5, and IL-13) were observed in cultures from sham-treated mice, whereas little to no peanut-restimulated cytokines were observed in cultures from naive control mice (Fig 6). Cell supernatant levels of IL-4, IL-5, and IL-13 (Fig 6, A–C) were significantly decreased in cultures from the CpG/PN-NP-treated groups ($P < .001$ for all). In contrast, IFN- γ levels in cultures in these 2 treatment groups were increased in comparison with levels in cultures from vehicle-treated animals ($P < .001$; Fig 6, D). No significant differences were observed in levels of

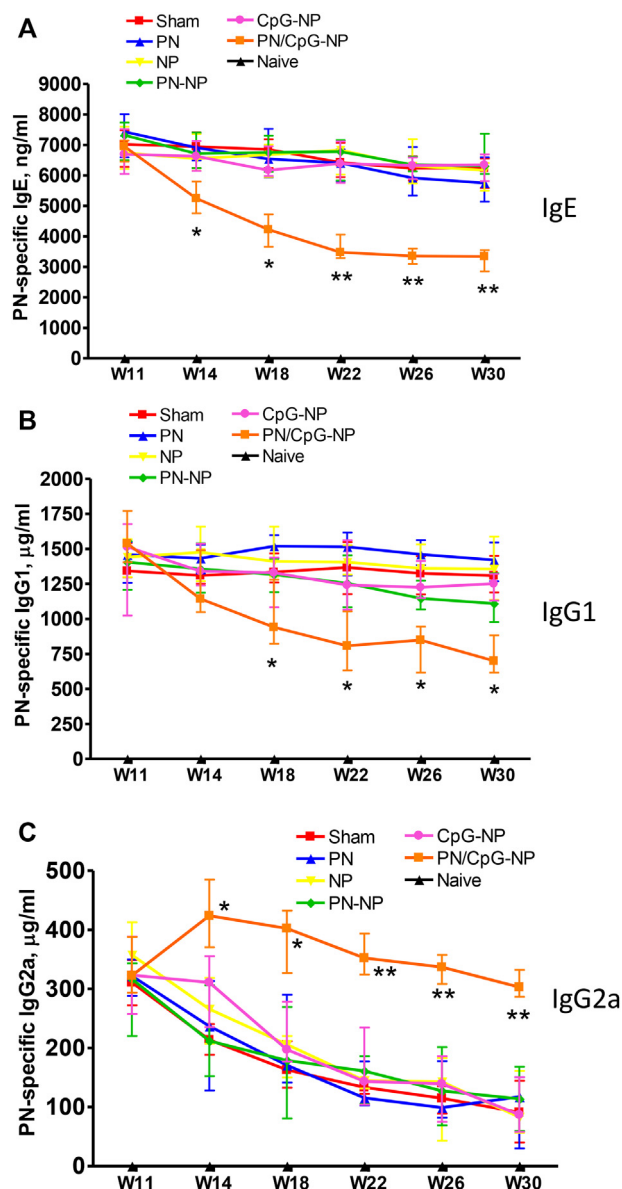


FIG 5. Peanut (PN)-specific immunoglobulins. Blood was drawn from mice at the times indicated. Peanut-specific serum IgE (A), IgG₁ (B), and IgG_{2a} (C) levels were measured by using ELISA. Data symbols indicate group medians, and bars indicate interquartile ranges. * $P < .05$ and ** $P < .01$ versus sham. $N = 10$ –22 mice per group from 2 separate experiments. NP, Nanoparticles.

peanut-restimulated IL-10 or TGF- β for any of the treated groups when compared with the sham group (Fig 6, E and F).

PLGA nanoparticles assembled with whole *E coli* extract and peanut reproduce therapeutic effects of CpG/PN-NPs

Although CpG/PN-NPs provide proof-of-concept data, the need to link CpG to the nanoparticle just before treatment undermines clinical practicality. In addition, the CpG sequences that are immunostimulatory in mice are not identical to those that are active in human subjects,²⁷ which could limit the potential translational utility of a CpG-based approach. To address these

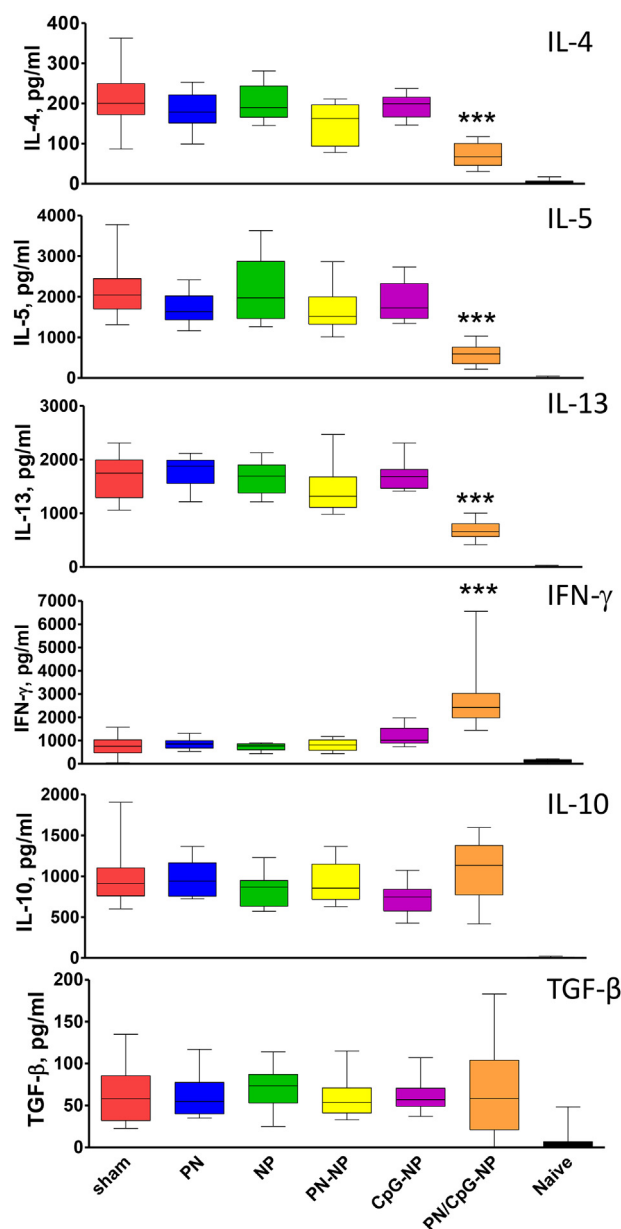


FIG 6. Peanut (PN)-restimulated splenocyte cytokine levels. Splenocyte cultures from individual mice were incubated in the presence of 200 μ g of peanut extract or medium alone for 72 hours under standard tissue culture conditions. Cytokine levels were measured by using ELISA. Data are displayed as group medians and interquartile ranges. *** $P < .001$ versus sham. $N = 10$ -22 mice per group from 2 separate experiments. NP, Nanoparticles.

issues, we also tested the performance of PLGA nanoparticles assembled with total *E coli* membranes that were loaded with peanut protein and sheared *E coli* DNA (referred to as PN/OEE/DNA-NPs, see Results and Figs E1-E3 in this article's Online Repository at www.jacionline.org). These nanoparticles do not require postpreparation modification and might be expected to activate a broader spectrum of Toll-like receptor (TLR)-mediated signaling pathways in human subjects. Thus the PN/OEE/DNA-NP approach might represent a more feasible and effective therapeutic strategy. Using the sensitization and challenge protocol described in Fig 1, we evaluated the effect of treating

mice with peanut allergy with PLGA nanoparticles assembled in the presence of *E coli* membrane extracts and loaded with *E coli* sheared DNA and 200 μ g of peanut extract (PN/OEE/DNA-NP). Mice were given 4 weekly oral treatments (weeks 11-14). Subsequently, mice were orally challenged with peanut at 4-week intervals. We found that 75% to 80% of mice treated with PN/OEE/DNA-NPs were protected across 5 challenges ($P < .01$ vs sham; see Fig E1, A). Consistently, these mice better maintained normal body temperature ($P < .05$ -.001 vs sham; see Fig E1, B) and lower postchallenge histamine levels ($P < .001$ vs sham; Fig E1, C). As observed with PN/CpG-NP treatment, therapy with PN/OEE/DNA-NP resulted in a sustained decrease in peanut-specific IgE/IgG₁ levels ($P < .05$ -.001 vs sham; Fig E2, A and B) and increase in peanut-specific IgG_{2a} levels ($P < .001$ vs sham; see Fig E2, C). Evaluation of levels of peanut-restimulated cytokine levels in splenocyte cultures showed significant reductions in levels of T_H2 cytokines (IL-4/IL-5/IL-13; $P < .001$ vs sham; see Fig E3, A-C) and a significant increase in IFN- γ levels ($P < .001$ vs sham; see Fig E3, D). Modest increases in IL-10 and TGF- β levels (see Fig E3, E and F) in PN/OEE/DNA-NP-treated mice did not achieve statistical significance. As was the case with the CpG-PN-NPs, PN/OEE/DNA-NPs provided protection that persisted for several months after treatment was discontinued.

DISCUSSION

The current study addresses the need for development of improved peanut-specific immunotherapy with better safety profiles and longer-lasting benefits than the current approaches of peanut OIT and SLIT. We explored the concept of providing peanut OIT within PLGA nanoparticles that have surface-expressed CpG oligonucleotides. When given to mice with established peanut allergy, we found PN/CpG-NPs to be well tolerated, and this therapy conferred persistent protection from anaphylaxis and induced beneficial regulation of peanut-specific immune responses. This is the first report to describe a therapeutic application of nanoparticles in food allergy. A recent report by Pali-Scholl et al²⁸ described the results of protamine-based nanoparticle immunotherapy for peanut. Our study differs considerably in that we investigated therapeutic effects in mice with established peanut allergy, indicating the ability of our approach to alter entrenched proallergic immune responses. Furthermore, therapy in our study was provided orally, a preferred route of peanut immunotherapy protocols in human subjects. PLGA nanoparticles have been extensively investigated as vehicles for drug delivery in human subjects and have been shown to be safe and effective delivery agents in recent clinical trials.^{15,29} Nanoparticles have been reported to survive the gastrointestinal environment because they are stable across a wide pH range. Also, the size of the nanoparticles used in our study has been previously reported to be optimal for transport across the gut barrier and cellular uptake at mucosal sites. PLGA nanoparticles have been shown to be retained for considerable periods of time, allowing for gradual and extended release of payload before biodegradation.¹⁵ Using ovalbumin-loaded PLGA nanoparticles, our research group found that antigen persisted in cultured dendritic cells for 7 days, whereas naked antigen was cleared within 3 days (A. Seifert, manuscript in preparation). We believe this capacity to protect loaded proteins facilitates use of lower and less frequent doses of allergen

while reducing the risk of mast cell/basophil activation, which represents an improvement over conventional OIT with naked allergen and daily dosing.

The concept of including CpG oligonucleotides or TLR agonists as a component of allergen immunotherapy has been explored previously in murine models for food allergy and asthma³⁰⁻³³ and in human subjects for allergic rhinitis^{34,35}; however, these studies used exposed allergen and thus might be less suited for peanut OIT, especially in highly reactive patients.^{34,36} Increases in allergen-specific T_H1 responses have been shown to be associated with favorable outcomes of immunotherapy and spontaneous outgrowth of food allergy in human subjects.³⁷ Our observation of increased peanut-specific IgG_{2a} levels (a T_H1-associated immunoglobulin in mice) after starting therapy and significantly increased IFN- γ levels in response to peanut restimulation in splenocyte cultures indicate that inclusion of CpG residues (in CpG/PN-NPs) appeared to be successful in enhancing T_H1-skewed peanut-specific responses. These observations are consistent with our prior findings, where we evaluated CpG nanoparticles loaded with recombinant West Nile virus envelope antigen (rWNVE) in an infectious disease model.³⁸ CpG-rWNVE nanoparticles, but not antigen loaded in plain nanoparticles or rWNVE-alum complexes, elicited dendritic cell activation that was accompanied by significant increases in CD80/CD86 expression and increased IL-12 and IL-6 production. In addition, CpG nanoparticles were much more avidly endocytosed by dendritic cells than plain nanoparticles, suggesting that this increased uptake is likely to account mechanistically, at least in part, for their enhanced capacity to activate dendritic cells. Consistently, only mice exposed to CpG-rWNVE nanoparticles displayed increased rWNVE-specific IgG_{2a} and rWNVE-stimulated IFN- γ recall responses. It is highly likely that similar mechanisms are involved in peanut-specific T_H1 skewing observed in the current study. Regulatory cytokines were largely unaffected, as suggested by a lack of differences in IL-10 or TGF- β levels across treatment groups.

Importantly, we found that copresentation of peanut and CpG through nanoparticles was necessary for protection. Mice receiving only 200 μ g of peanut (naked or within nanoparticles), nanoparticles alone, or CpG nanoparticles were not protected. It must be noted that the dose (200 μ g) and frequency (weekly) of antigen used for treatment in our study is much lower than in other murine studies describing OIT (milligram amounts).³⁹⁻⁴¹ The CpG dose used in our study is also lower than in previous murine allergy/asthma studies. PLGA nanoparticles themselves have been shown to possess T_H1 adjuvant activity and have been demonstrated to enhance CpG adjuvant activity.^{42,43} However, in our model CpG nanoparticles or nanoparticles alone did not modify existing peanut-specific T_H2 responses or establish peanut-specific T_H1 responses. It is possible that although PLGA nanoparticles alone can skew naive T-cell responses, therapeutic alteration of antigen-specific responses requires simultaneous presence of antigen. Dendritic cells have emerged as a prime target for immunomodulatory nanoparticles because of their nature as professional antigen-presenting cells with the capacity to cross-present antigen.¹⁷ As reviewed by Silva et al,⁴⁴ nanoparticles that are approximately 200 nm or less can transit lymphatic barriers and be taken up by dendritic cells in lymph nodes, and when “decorated” with TLR agonists, such as CpG, such nanoparticles elicit robust T_H1 responses. Our study

also evaluated the performance of PN/OEE/DNA-NPs, a nanoparticle formulation that does not require postpreparation linkage of CpG and that might be expected to activate a broader range of TLRs, making it more feasible for clinical application. Therapeutic effects of PN/OEE/DNA-NPs were comparable with PN/CpG-NPs with regard to the magnitude and duration of protection from anaphylaxis and alteration of peanut-specific immune responses.

Although there have been few studies investigating OIT for food allergy in murine models, almost none have addressed long-term protection after stopping therapy.^{40,45-47} In a report by Leonard et al,³⁹ OIT of ovalbumin-sensitized mice resulted in protective desensitization when the antigen was presented through the gastrointestinal mucosa but not injected systemically, and protective effects were lost 2 weeks after therapy was discontinued. These results are consistent with recent clinical results in which the majority of patients undergoing peanut immunotherapy regained reactivity after discontinuing treatment.⁹ We believe factors such as extended retention of antigen through nanoparticles and establishment of peanut-specific T_H1 responses might be contributing to the persistent protection observed in our study. Overall, 16 of 22 mice retained protection despite continued periodic exposure to the peanut sensitization cocktail that was given to all mice to prevent spontaneous loss of reactivity over the course of the study. A recent study by Burton et al⁴¹ identified antigen-specific IgG as a mechanism of suppression of allergic responses through Fc γ R2B ligation after OIT in mice. We observed a persistent increase in peanut-specific IgG_{2a} (but not IgG₁) levels in our study, but further studies are needed to determine whether such mechanisms are operational in our system.

In summary, PLGA nanoparticles are well suited for providing safe peanut immunotherapy because they are readily taken up at mucosal sites after oral administration and persist for days, allowing for controlled and extended release of allergen. When applied to peanut immunotherapy in the clinic, this approach could greatly reduce the need for repeated daily dosing of peanut, as is required in current OIT protocols, potentially minimizing the risk of adverse reactions during therapy, enhancing compliance, and maximizing the opportunity to alter immune responses. Although our research findings might not directly correlate with findings in human subjects, long-term protection observed in our study, if reproducible in human subjects, suggests that peanut OIT with CpG/PN-NPs might be a valuable approach to safely induce long-term desensitization and possibly tolerance in human patients with peanut allergy.

Key messages

- OIT with peanut/CpG nanoparticles provided long-term protection in mice with established peanut allergy.
- Peanut/CpG nanoparticle therapy did not cause adverse reactions.
- Beneficial effects of peanut/CpG nanoparticle immunotherapy were associated with reduced peanut-specific T_H2 responses and increased T_H1 responses.

REFERENCES

1. Skripak JM, Wood RA. Peanut and tree nut allergy in childhood. *Pediatr Allergy Immunol* 2008;19:368-73.

2. Finkelman FD. Peanut allergy and anaphylaxis. *Curr Opin Immunol* 2010;22:783-8.
3. Primeau MN, Kagan R, Joseph L, Lim H, Dufresne C, Duffy C, et al. The psychological burden of peanut allergy as perceived by adults with peanut allergy and the parents of peanut-allergic children. *Clin Exp Allergy* 2000;30:1135-43.
4. Le TM, Lindner TM, Pasmans SG, Guikers CL, van HE, Bruijnzeel-Koomen CA, et al. Reported food allergy to peanut, tree nuts and fruit: comparison of clinical manifestations, prescription of medication and impact on daily life. *Allergy* 2008;63:910-6.
5. Varshney P, Jones SM, Scurlock AM, Perry TT, Kemper A, Steele P, et al. A randomized controlled study of peanut oral immunotherapy: clinical desensitization and modulation of the allergic response. *J Allergy Clin Immunol* 2011;127:654-60.
6. Chin SJ, Vickery BP, Kulis MD, Kim EH, Varshney P, Steele P, et al. Sublingual versus oral immunotherapy for peanut-allergic children: a retrospective comparison. *J Allergy Clin Immunol* 2013;132:476-8.e2.
7. Anagnostou K, Clark A, King Y, Islam S, Deighton J, Ewan P. Efficacy and safety of high-dose peanut oral immunotherapy with factors predicting outcome. *Clin Exp Allergy* 2011;41:1273-81.
8. Blumchen K, Ulbricht H, Staden U, Dobberstein K, Beschoner J, de Oliveira LC, et al. Oral peanut immunotherapy in children with peanut anaphylaxis. *J Allergy Clin Immunol* 2010;126:83-91.
9. Greenhawt MJ. Oral and sublingual peanut immunotherapy is not ready for general use. *Allergy Asthma Proc* 2013;34:197-204.
10. Hofmann AM, Scurlock AM, Jones SM, Palmer KP, Lokhnygina Y, Steele PH, et al. Safety of a peanut oral immunotherapy protocol in children with peanut allergy. *J Allergy Clin Immunol* 2009;124:286-91.
11. Varshney P, Steele PH, Vickery BP, Bird JA, Thyagarajan A, Scurlock AM, et al. Adverse reactions during peanut oral immunotherapy home dosing. *J Allergy Clin Immunol* 2009;124:1351-2.
12. Sheikh A, Nurmatov U, Venderbosch I, Bischoff E. Oral immunotherapy for the treatment of peanut allergy: systematic review of six case series studies. *Prim Care Respir J* 2012;21:41-9.
13. Nurmatov U, Venderbosch I, Devereux G, Simons FE, Sheikh A. Allergen-specific oral immunotherapy for peanut allergy. *Cochrane Database Syst Rev* 2012;9:CD009014.
14. Sampson HA. Is peanut oral immunotherapy ready for use in clinical practice? *J Allergy Clin Immunol Pract* 2013;1:15-21.
15. Kumar G, Shafiq N, Malhotra S. Drug-loaded PLGA nanoparticles for oral administration: fundamental issues and challenges ahead. *Crit Rev Ther Drug Carrier Syst* 2012;29:149-82.
16. Hunter Z, McCarthy DP, Yap WT, Harp CT, Getts DR, Shea LD, et al. A biodegradable nanoparticle platform for the induction of antigen-specific immune tolerance for treatment of autoimmune disease. *ACS Nano* 2014;8:2148-60.
17. Silva AL, Rosalia RA, Varypataki E, Sibuea S, Ossendorp F, Jiskoot W. Poly(lactic-co-glycolic-acid)-based particulate vaccines: particle uptake by dendritic cells is a key parameter for immune activation. *Vaccine* 2015;33:847-54.
18. Bayne K. Developing guidelines on the care and use of animals. *Ann N Y Acad Sci* 1998;862:105-10.
19. Srivastava KD, Bardina L, Sampson HA, Li XM. Efficacy and immunological actions of FAHF-2 in a murine model of multiple food allergies. *Ann Allergy Asthma Immunol* 2012;108:351-8.
20. Talbot RW, Foster JR, Hermon-Taylor J, Grant DA. Induced mucosal penetration and transfer to portal blood of luminal horseradish peroxidase after exposure of mucosa of guinea pig small intestine to ethanol and lysolecithin. *Dig Dis Sci* 1984;29:1015-22.
21. Fahmy TM, Samstein RM, Harness CC, Mark SW. Surface modification of biodegradable polyesters with fatty acid conjugates for improved drug targeting. *Biomaterials* 2005;26:5727-36.
22. De Castro C, Parrilli M, Holst O, Molinaro A. Microbe-associated molecular patterns in innate immunity: extraction and chemical analysis of gram-negative bacterial lipopolysaccharides. *Methods Enzymol* 2010;480:89-115.
23. Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000;106:150-8.
24. Srivastava KD, Kattan JD, Zou ZM, Li JH, Zhang L, Wallenstein S, et al. The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. *J Allergy Clin Immunol* 2005;115:171-8.
25. Qu C, Srivastava K, Ko J, Zhang TF, Sampson HA, Li XM. Induction of tolerance after establishment of peanut allergy by the food allergy herbal formula-2 is associated with up-regulation of interferon-gamma. *Clin Exp Allergy* 2007;37:846-55.
26. Smit JJ, Willemsen K, Hassing I, Fiechter D, Storm G, van Bloois L, et al. Contribution of classic and alternative effector pathways in peanut-induced anaphylactic responses. *PLoS One* 2011;6:e28917.
27. Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* 2001;98:9237-42.
28. Pali-Scholl I, Szollosi H, Starkl P, Scheicher B, Stremnitzer C, Hofmeister A, et al. Protamine nanoparticles with CpG-oligodeoxynucleotide prevent an allergen-induced Th2-response in BALB/c mice. *Eur J Pharm Biopharm* 2013;85:656-64.
29. Sah H, Thoma LA, Desu HR, Sah E, Wood GC. Concepts and practices used to develop functional PLGA-based nanoparticulate systems. *Int J Nanomedicine* 2013;8:747-65.
30. Kulis M, Gorenfla B, Burks AW, Zhong XP. Type B CpG oligodeoxynucleotides induce Th1 responses to peanut antigens: modulation of sensitization and utility in a truncated immunotherapy regimen in mice. *Mol Nutr Food Res* 2013;57:906-15.
31. Campbell JD, Kell SA, Kozy HM, Lum JA, Sweetwood R, Chu M, et al. A limited CpG-containing oligodeoxynucleotide therapy regimen induces sustained suppression of allergic airway inflammation in mice. *Thorax* 2014;69:565-73.
32. Horner AA, Takabayashi K, Beck L, Sharma B, Zubeldia J, Baird S, et al. Optimized conjugation ratios lead to allergen immunostimulatory oligodeoxynucleotide conjugates with retained immunogenicity and minimal anaphylactogenicity. *J Allergy Clin Immunol* 2002;110:413-20.
33. Horner AA, Nguyen MD, Ronaghy A, Cinman N, Verbeek S, Raz E. DNA-based vaccination reduces the risk of lethal anaphylactic hypersensitivity in mice. *J Allergy Clin Immunol* 2000;106:349-56.
34. Simons FE, Shikishima Y, Van NG, Eiden JJ, Hayglass KT. Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J Allergy Clin Immunol* 2004;113:1144-51.
35. Senti G, Johansen P, Haug S, Bull C, Gottschaller C, Muller P, et al. Use of A-type CpG oligodeoxynucleotides as an adjuvant in allergen-specific immunotherapy in humans: a phase I/IIa clinical trial. *Clin Exp Allergy* 2009;39:562-70.
36. Creticos PS, Schroeder JT, Hamilton RG, Balcer-Whaley SL, Khattignavong AP, Lindblad R, et al. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *N Engl J Med* 2006;355:1445-55.
37. Turcanu V, Maleki SJ, Lack G. Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J Clin Invest* 2003;111:1065-72.
38. Demento SL, Bonafe N, Cui W, Kaech SM, Caplan MJ, Fikrig E, et al. TLR9-targeted biodegradable nanoparticles as immunization vectors protect against West Nile encephalitis. *J Immunol* 2010;185:2989-97.
39. Leonard SA, Martos G, Wang W, Nowak-Wegrzyn A, Berin MC. Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa. *J Allergy Clin Immunol* 2012;129:1579-87.
40. Jimenez-Saiz R, Rupa P, Mine Y. Immunomodulatory effects of heated ovomucoid-depleted egg white in a BALB/c mouse model of egg allergy. *J Agric Food Chem* 2011;59:13195-202.
41. Burton OT, Logsdon SL, Zhou JS, Medina-Tamayo J, Abdel-Gadir A, Noval RM, et al. Oral immunotherapy induces IgG antibodies that act through FcγRIIb to suppress IgE-mediated hypersensitivity. *J Allergy Clin Immunol* 2014;134:1310-7.
42. Lutsiak ME, Kwon GS, Samuel J. Biodegradable nanoparticle delivery of a Th2-biased peptide for induction of Th1 immune responses. *J Pharm Pharmacol* 2006;58:739-47.
43. Diwan M, Elamanchili P, Cao M, Samuel J. Dose sparing of CpG oligodeoxynucleotide vaccine adjuvants by nanoparticle delivery. *Curr Drug Deliv* 2004;1:405-12.
44. Silva JM, Videira M, Gaspar R, Preat V, Florindo HF. Immune system targeting by biodegradable nanoparticles for cancer vaccines. *J Control Release* 2013;168:179-99.
45. Kawakita A, Shirasaki H, Yasutomi M, Tokuriki S, Mayumi M, Naiki H, et al. Immunotherapy with oligomannose-coated liposomes ameliorates allergic symptoms in a murine food allergy model. *Allergy* 2012;67:371-9.
46. Mondoulet L, Dioszeghy V, Larcher T, Ligouis M, Dhelft V, Puteaux E, et al. Epicutaneous immunotherapy (EPIT) blocks the allergic esophago-gastro-enteropathy induced by sustained oral exposure to peanuts in sensitized mice. *PLoS One* 2012;7:e31967.
47. Reisacher WR, Liotta D, Yazdi S, Putnam D. Desensitizing mice to ovalbumin through subcutaneous microsphere immunotherapy (SMITH). *Int Forum Allergy Rhinol* 2011;1:390-5.

RESULTS

Using the sensitization and challenge protocol described in Fig 1, we evaluated the effect of treating mice with peanut allergy with PLGA nanoparticles assembled in the presence of *E coli* membrane extracts and loaded with *E coli* sheared DNA and 200 μ g of peanut extract (PN/OEE/DNA-NPs). Mice were given 4 weekly oral treatments (weeks 11-14). Subsequently, mice were challenged orally with peanut and then at 4-week intervals. We found that 75% to 80% of mice treated with PN/OEE/DNA-NPs were protected across 5 challenges ($P < .01$ vs sham; Fig E1, A). Consistently, these mice better maintained normal body temperature ($P < .05$ -.001 versus sham; Fig E1, B) and lower

postchallenge histamine levels ($P < .001$ versus sham; Fig E1, C). As observed with PN/CpG-NP treatment, therapy with PN/OEE/DNA-NPs resulted in a sustained decrease in peanut-specific IgE/IgG₁ ($P < .05$ -.001 versus sham; Fig E2, A and B) and increase in peanut-specific IgG_{2a} ($P < .001$ versus sham; Fig E2, C) levels. Evaluation of peanut-restimulated cytokine levels in splenocyte cultures showed significant reductions in T_H2 cytokines levels (IL-4/IL-5/IL-13: $P < .001$ versus sham; Fig E3, A-C) and a significant increase in IFN- γ levels ($P < .001$ versus sham; Fig E3, D). Modest increases observed for IL-10 and TGF- β (Fig E3, E and F) in PN/OEE/DNA-NP-treated mice did not achieve statistical significance.

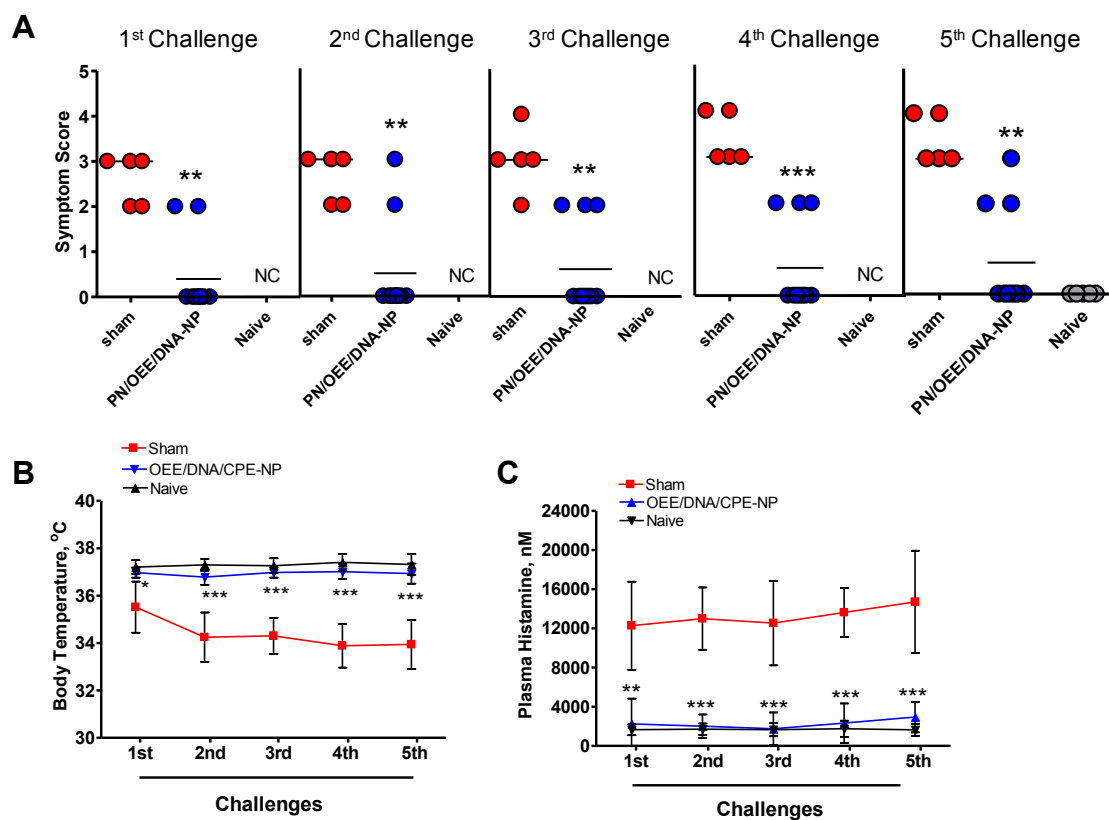


FIG E1. Anaphylactic reactions to oral peanut (PN) challenge. **A**, Mice were visually assessed for symptoms of anaphylaxis 30 minutes after challenge and assigned symptom scores by using the scoring system described in the Methods section. Naive mice were challenged only at the fifth challenge. Bars represent group medians. **B**, Body temperatures were measured with a rectal thermometer. **C**, Histamine levels in plasma harvested after challenge were measured by using ELISA. Data are shown as means $\pm$ SDs. Bars indicate group medians in Fig E1, A, and group means $\pm$ SDs for Fig E1, B and C. * $P < .05$, ** $P < .01$, and *** $P < .001$ versus sham. N = 5-10 mice per group. NP, Nanoparticles.

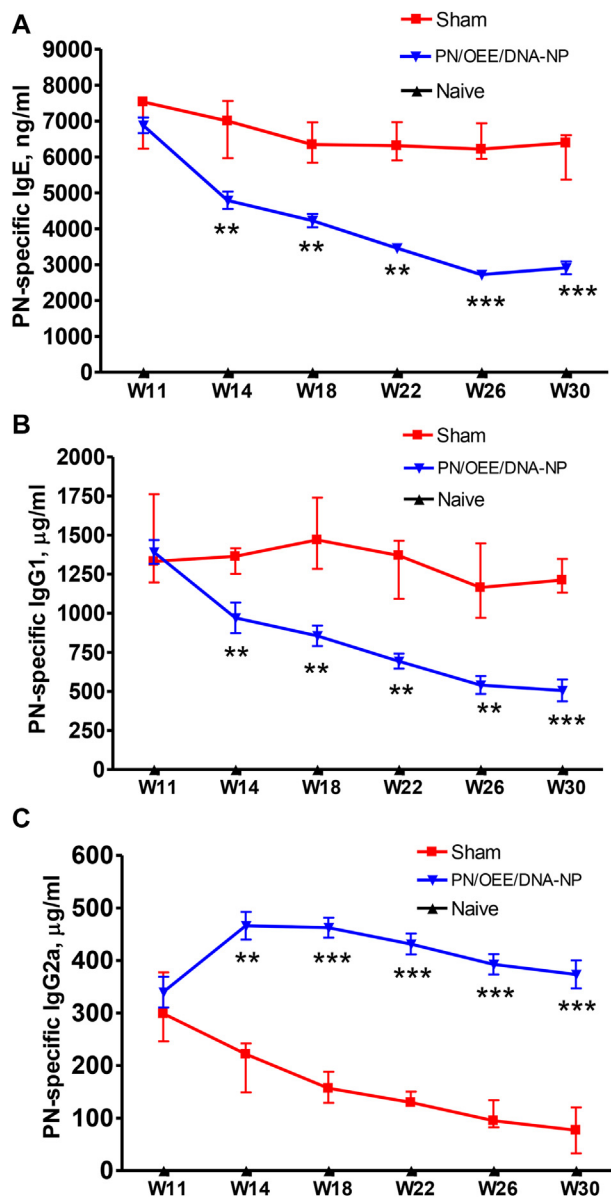


FIG E2. Peanut (PN)-specific immunoglobulins. Blood was drawn before treatment (week 11) and each of the challenges (weeks 14-30). Peanut-specific serum IgE (**A**), IgG₁ (**B**), and IgG_{2a} (**C**) levels were measured by using ELISA. Symbols indicate group medians, and bars indicate interquartile ranges. ** $P < .01$; *** $P < .001$ versus sham. $N = 5-10$ mice per group. NP, Nanoparticles.

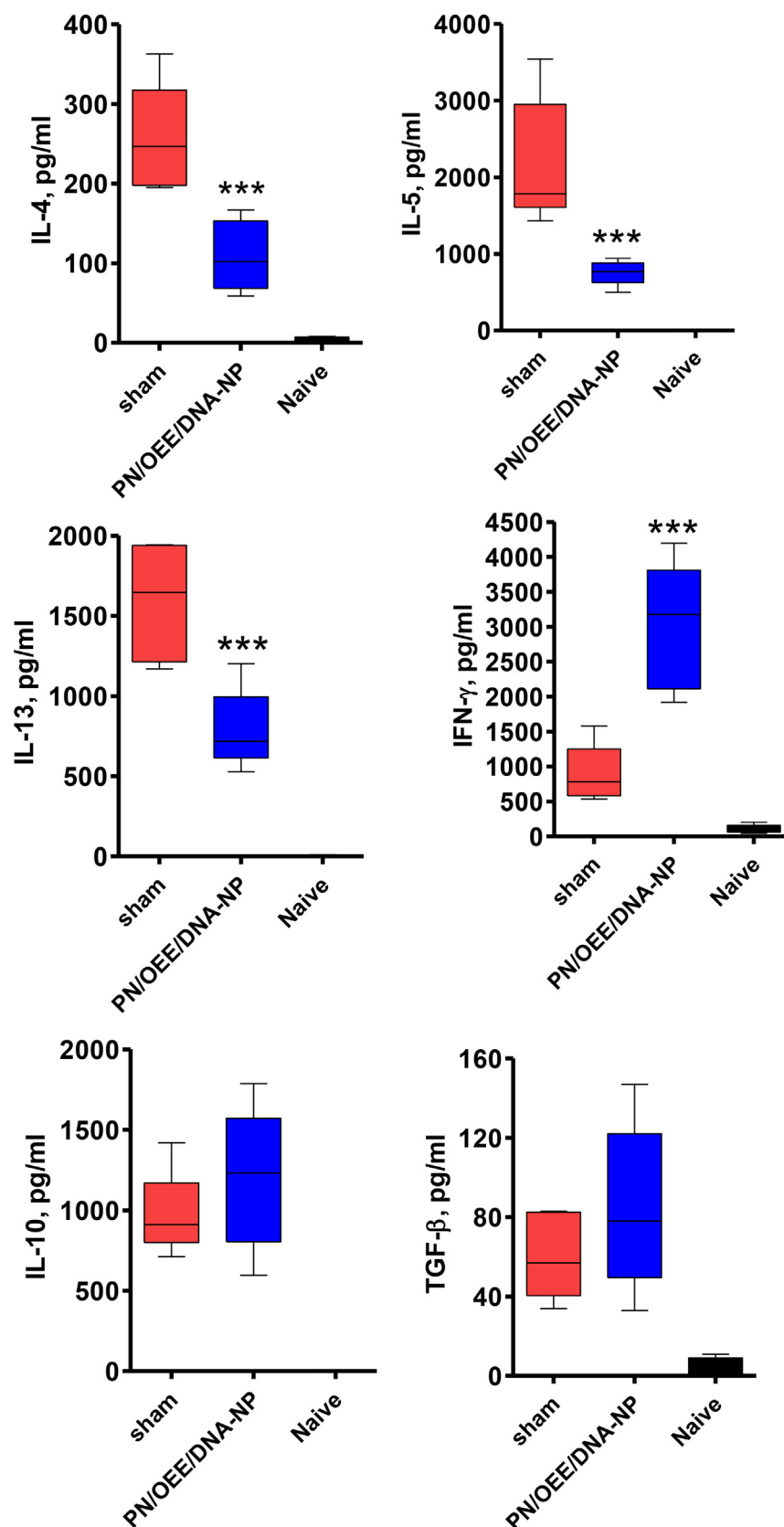


FIG E3. Peanut (PN)-restimulated splenocyte cytokine levels. Splenocyte cultures from individual mice were incubated in the presence of 200 μ g of peanut extract or medium alone for 72 hours under standard tissue culture conditions. Cytokine levels were measured by using ELISA. Data show group medians and interquartile ranges. *** $P < .001$ versus sham. N = 5-10 mice per group.