

Finally, we estimated the ability of each biomarker to assess the effects of treatment with topical corticosteroids (see Fig E7 in this article's Online Repository at www.jacionline.org). O-SCORAD completely decreased according to the treatment (39.4 vs 1.8). Accordingly, both SCCA2 (7.4 ng/mL vs 1.5 ng/mL; $P < .001$) and TARC (2555 pg/mL vs 771 pg/mL; $P < .001$) significantly decreased, whereas the change in IgE was very slight (711.4 IU/mL vs 643 IU/mL). The change of O-SCORAD in TARC was almost the same as that in SCCA2, suggesting that TARC and SCCA2 have almost the same ability to estimate the effects of treatments.

In this study, we showed that SCCA2 is a more reliable biomarker than TARC to correspond to diagnosis of AD and to estimate the clinical severity of AD in children, TARC being the biomarker now assumed to be the most reliable. It was reported that combination of IL-17 and IL-22 upregulated in AD patients, particularly in Asian countries,⁹ enhances SCCA2 expression in keratinocytes,⁷ which may explain why SCCA2 is more reliable than TARC. It remains open how the cutoff values should be set for SCCA2; however, setting only 1 or 2 cutoff values depending on ages would be convenient for clinicians. In a simple way, only 1 cutoff value, 1.6 ng/mL, provided an acceptable result for corresponding to diagnosis of AD (79.5% of sensitivity and 95% of specificity across all ages) (Fig 1) and the difference of AUCs between 1 cutoff value and the cutoff values depending on age was not significant in SCCA2 compared with TARC (SCCA2: 0.042; TARC: 0.064) (see Table E3 in this article's Online Repository at www.jacionline.org), which suggests that the difference among ages is not big in SCCA2 compared with those in TARC. Please see the Online Repository for Tables E1 to E3 and Figs E1 to E7.

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Reprogramming immunity to food allergens



To the Editor:

Allergen immunotherapy is used to treat hypersensitivities to environmental or food allergens. It involves administering repeated and increasing doses of a soluble allergen until a maintenance dose is reached, at which symptoms are improved on allergen exposure.¹ Although widely used, several limitations exist, including the risk of severe adverse events (eg, fatal anaphylaxis), high costs of antigens and clinical monitoring, and requirement of many years of treatment.² Many patients remain refractory to treatment, and for peanut allergy, this strategy has a high risk of adverse reactions.³ Thus current immunotherapy protocols have distinct limitations, and there is a major need for improved approaches, particularly for food allergies.

When effective, the mechanisms of allergen immunotherapy are not well understood, although it is thought to involve a combination of antigen-induced desensitization of effector immune cells and development of tolerance. Skewing the T_H1/T_H2 profile toward a T_H1 direction could markedly reduce T_H2 -mediated pathologic responses to allergens. Thus attempts have been made to block production of T_H2 cytokines, to directly inhibit their action, or to favor T_H1 immunity by administering exogenous T_H1 -type cytokines, such as IL-12. For asthma treatment, exogenous IL-12 delivery was not effective, which

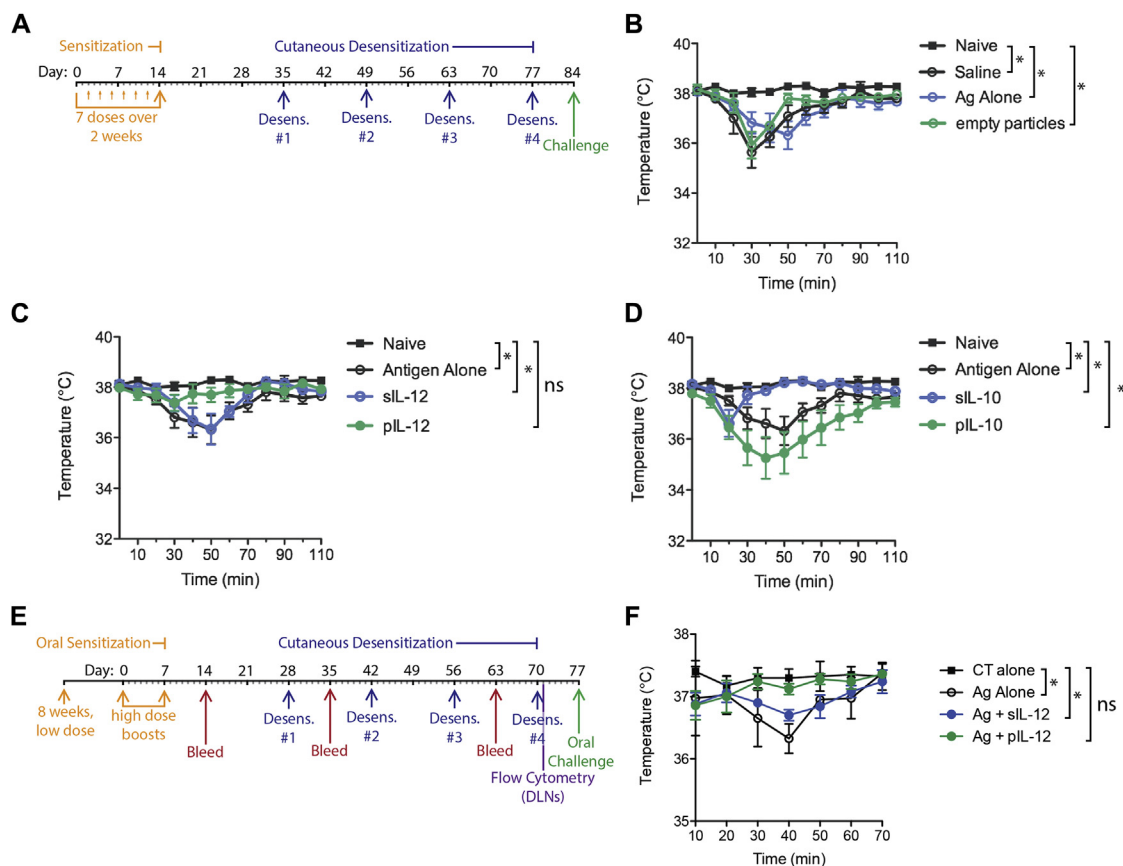


FIG 1. Nanoparticle-based IL-12 desensitization therapy protects against peanut-induced anaphylaxis. **A**, Experimental time course for mouse sensitization to peanut, desensitization, and peanut challenge. For sensitization, mice were given 7 repeated low-dose intraperitoneal injections of peanut antigen. Desensitization therapy involved coadministration of 1 μ g of peanut antigen with or without 1 ng of soluble or particulate cytokines (IL-12 or IL-10), treatment with peanut antigen alone without any cytokine-containing particles, or treatment with saline alone as a control. Mice were challenged by means of intraperitoneal injection with peanut antigen to induce anaphylaxis. **B-D**, Temperatures are shown after challenge for control animals and mice desensitized with empty particles (Fig 1, B), sIL-12 versus pIL-12 (Fig 1, C), or sIL-10 versus pIL-10 (Fig 1, D). For Fig 1, B-D, Naive indicates mice that were not peanut sensitized, and all other groups were sensitized. pIL-12 and, to a lesser extent, sIL-10 effectively prevented peanut-induced anaphylaxis. *Temperatures differ significantly between groups at more than 1 time point, as determined by using 2-way ANOVA. **E**, Time course of oral sensitization, cutaneous desensitization, and oral challenge. For 8 weeks, 50 μ g of peanut antigen was administered weekly by using oral gavage. Subsequently, 2 high-dose oral gavage boosts of 1 mg of peanut antigen containing cholera toxin were administered. For some mice, tissues were harvested for immune profiling, whereas others were challenged with 500 μ g of peanut antigen orally, and temperature was monitored. Control mice were given sensitization doses consisting of cholera toxin alone administered by means of oral gavage. **F**, Anaphylaxis was monitored after oral peanut challenge. Peanut-sensitized mice showed a significant decrease in temperature compared with control unsensitized mice that had received oral cholera toxin alone. Ag, Antigen; CT, cholera toxin; ns, Not significant.

was attributed to the difficulty of administering soluble cytokine to the lungs in sufficient amounts to improve outcomes.⁴

Recently, we showed that a significant and long-lasting effect can be achieved if immunomodulatory cytokines are targeted to draining lymph nodes (DLNs), the command structures for the development and refinement of immunity.⁵ Cytokines were successfully targeted to DLNs by loading them into stable biodegradable nanoparticles (comprised of the carbohydrates heparin and chitosan).⁵ Injected at peripheral sites, these particles traffic through the lymphatics and, protected by the nanoparticle matrix, avoid degradation and dilution to reach DLNs in functionally relevant quantities. By subcutaneously injecting a vaccine

antigen along with nanoparticles containing TNF, rapid germinal center development and protective immunity were evoked.⁵ Furthermore, replacing TNF with IL-12 created particles that promoted interferon production by T cells in DLNs.⁵

Allergic immune responses are characterized by a T_H2 profile promoted by cytokines, such as IL-4 and IL-10, and by certain subclasses of antibodies, such as IgE and IgG₁.⁶ In contrast, T_H1 -polarized immunity involves IL-12 production by dendritic cells (DCs) that, in turn, promotes IFN- γ production by T cells.⁷

To test whether an immunotherapy involving the delivery of particulate IL-12 (pIL-12) along with peanut antigen can skew immunity toward a T_H1 profile and protect from anaphylaxis, we

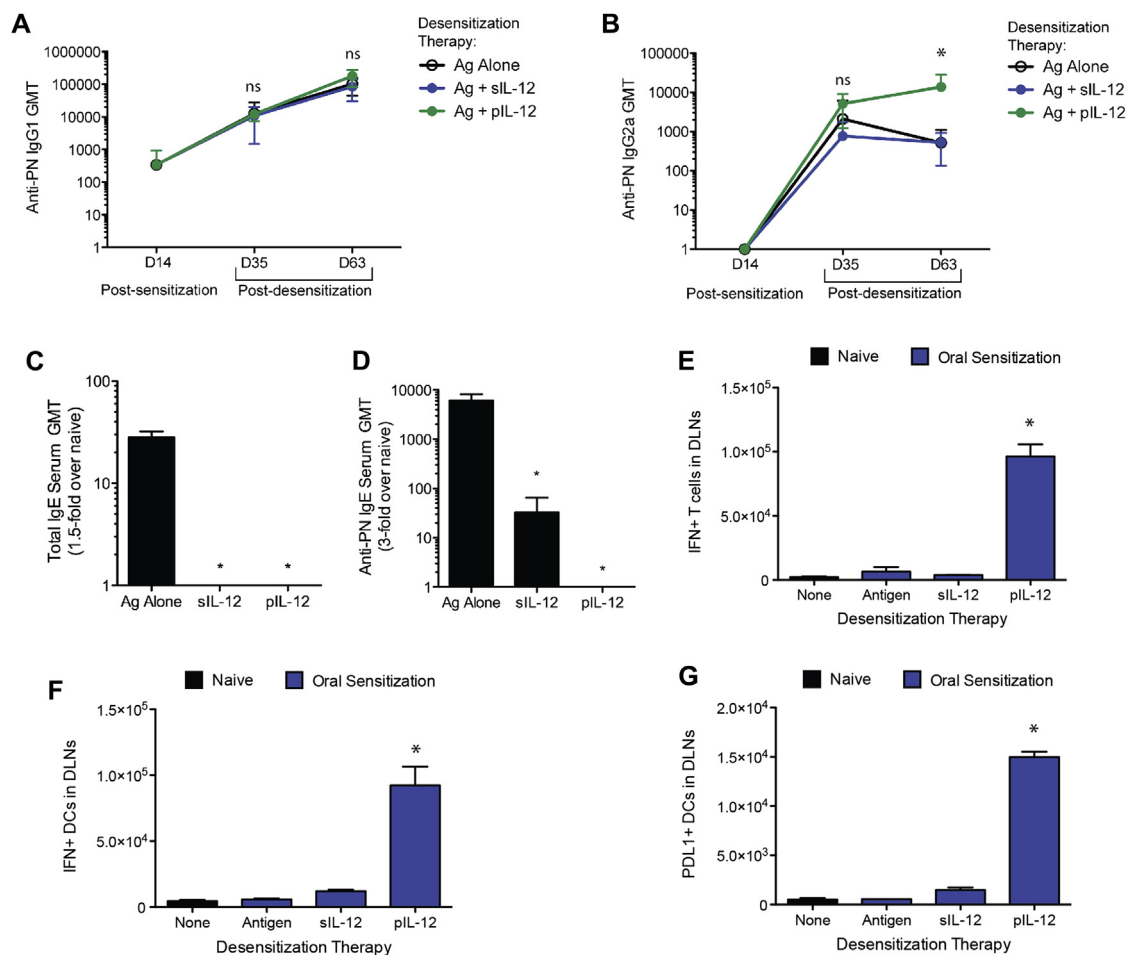


FIG 2. Desensitization is characterized by skewing of the T_H2 immune response toward a T_H1 immune response. **A** and **B**, Peanut-specific IgG₁ (Fig 2, A) and IgG_{2a} (Fig 2, B) antibody subclass titers were measured at the time of predesensitization and postdesensitization therapy (days 14, 35, and 63) and are represented as the geometric mean titer of at least 2-fold over naive control values. **C** and **D**, Total IgE (Fig 2, C) and peanut-specific IgG (Fig 2, D) levels on day 63 of the experimental protocol (after desensitization) are represented as geometric mean titer of at least 1.5-fold over naive control values. **E-G**, Cells isolated from the lymph nodes of the skin 24 hours after the final desensitization challenge (day 70) were stained for IFN- γ production by T cells (Fig 2, E) and DCs (Fig 2, F) and programmed death ligand 1 expression on DCs (Fig 2, G). * $P < .05$. GMT, Geometric mean titer; ns, not significant.

used an “active sensitization” model of peanut allergy (Fig 1, A). Peanut antigens were derived from crude peanut extracts and contained known allergens, including Ara h 1 to Ara h 8 (see Table E1 in this article’s Online Repository at www.jacionline.org). Mice were subsequently given one of several experimental desensitization therapies (or control treatments; Fig 1, A), including pIL-12 versus soluble IL-12 (sIL-12) and particulate IL-10 (pIL-10) versus soluble IL-10 (sIL-10), which were administered with soluble peanut antigen in 4 treatments (Fig 1, A). Although IL-12 was chosen because of its role in T_H1 skewing, IL-10 was selected based on its contributions to establishing tolerance. At week 12, mice were challenged with sufficient peanut antigen to induce anaphylaxis. Severity of anaphylaxis was determined by quantifying the decrease in temperature experienced after challenge, a standard measure of anaphylaxis in mice.⁸ Naive mice do not have anaphylaxis when given a peanut challenge; however, sensitized mice do (Fig 1, B). Neither the control treatment of peanut alone nor empty

particles plus peanut significantly influenced anaphylaxis in peanut-sensitized mice (Fig 1, B). In contrast, peanut-sensitized mice given desensitization therapy of peanut plus pIL-12 did not have anaphylaxis, whereas sIL-12 had no therapeutic effect (Fig 1, C). Surprisingly, the T_H2 -promoting cytokine IL-10 had the opposite effect in particulate form, resulting in more severe anaphylaxis when coadministered with peanut during desensitization (Fig 1, D), likely because of its role in DLNs as a T_H2 cytokine. In contrast, sIL-10 slightly improved the symptoms (Fig 1, D), potentially because of the immunosuppressive effect of IL-10 in peripheral tissue.⁹ These results demonstrate the plasticity of allergic responses and that they can be reprogrammed differentially through choice and delivery of cytokines.

Subsequently, to test this strategy in a model that is more analogous to the natural route of peanut exposure in human subjects, animals were sensitized and challenged orally subsequently (Fig 1, E). Desensitization therapy involved

coadministration of peanut antigen with and without sIL-12 or pIL-12. Control mice maintained their body temperature after a peanut challenge, whereas peanut-sensitized mice given mock desensitization injections with only peanut antigen showed a modest but significant decrease in temperature after peanut challenge (Fig 1, F). Desensitization therapy with peanut plus sIL-12 also did not prevent anaphylaxis on peanut challenge (Fig 1, F). Mice given the desensitization therapy of peanut plus pIL-12 did not have anaphylaxis and maintained their temperature after challenge similar to the naive control group (Fig 1, F). Thus treatment of peanut-sensitized animals with small amounts of peanut combined with pIL-12 is an effective desensitization therapy to prevent oral peanut-induced anaphylaxis.

To verify that peanut desensitization was dependent on the effects of pIL-12 in skewing immunity toward a T_H1 profile, subclasses of peanut-specific antibodies were monitored throughout the protocol (Fig 2, A and B). Animals produced peanut-specific T_H2-associated IgG₁ (Fig 2, A) but not T_H1-associated IgG_{2a} (Fig 2, B) after oral sensitization. Consistent with T_H1 skewing, serum IgG_{2a} levels increased significantly after 3 doses of desensitization therapy in mice given peanut plus pIL-12 compared with mice given peanut antigen alone or peanut plus sIL-12 (Fig 2, B). IgG₁ levels did not differ among groups after desensitization (Fig 2, A). Thus pIL-12 desensitization increases the relative abundance of T_H1-associated IgG_{2a} in the serum (Fig 2, B). Because strong T_H2 responses and atopic conditions are associated with increased IgE levels and IgE also contributes to mast cell-mediated anaphylaxis, we expected IgE levels could be reduced by therapeutic T_H1 reprogramming. Indeed, after desensitization, total serum IgE levels were decreased in animals receiving both sIL-12 and pIL-12 desensitization therapies compared with those receiving control therapy of peanut antigen alone (day-77; Fig 2, C). Peanut-specific IgE levels were also reduced significantly by sIL-12 or pIL-12 desensitization therapies (Fig 2, D).

Moreover, a T_H1-associated activation profile is characterized by an abundance of IFN- γ -producing T cells and DCs. To investigate whether T_H1 immunity was enhanced by desensitization therapy, flow cytometry was performed 24 hours after administration of the final desensitization dose to determine the total number of IFN- γ ⁺ cells in the lymph nodes draining the subcutaneous injection site of the desensitization therapy. DLNs of mice given peanut plus pIL-12 showed significantly increased numbers of IFN- γ ⁺ T cells and DCs compared with control DLNs, whereas those given peanut antigen alone or peanut plus sIL-12 did not (Fig 2, E and F). Mice given peanut plus pIL-12 also had significantly increased numbers of DCs expressing programmed death ligand 1 (Fig 2, G), which is associated with development of tolerogenic immune responses.

Altogether, we demonstrated that by targeting selected immunomodulatory cytokines to DLNs while administering antigens at peripheral sites, it is possible to achieve rapid reprogramming of DLNs, shifting the profile of antigen-specific immunity away from a harmful allergy-associated T_H2 profile. Although effectiveness in human subjects has not yet been evaluated, compared with the existing analogous protocols for allergen immunotherapy in animal models, this is a dramatic reduction in time required for desensitization and can be

instructive to the immune system with smaller quantities of antigen without gradual ramping of the allergen desensitization dose. These studies demonstrate the effectiveness of a novel desensitization strategy with advantages over traditional allergen immunotherapy.

In conclusion, we show that by using our novel approach to deliver the T_H1 cytokine IL-12 to the DLNs, combined with soluble peanut antigen, we can effectively reprogram a T_H2-associated allergic response toward a T_H1 response and protect from peanut-induced anaphylaxis.

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GITR cosignal in ILC2s controls allergic lung inflammation



To the Editor:

Group 2 innate lymphoid cells (ILC2s) produce large amounts of type 2 effector cytokines such as IL-5 and IL-13, which cause allergy.¹⁻⁴ ILC2s in the lung highly express glucocorticoid-induced TNFR-related protein (GITR),^{5,6} and thus we hypothesized that GITR plays an important role in ILC2s.

In this study, we newly found GITR on common helper-like ILC progenitors (Lin⁻ CD127⁺ α 4 β 7⁺ Flt3⁻) (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Despite the expression of GITR on common helper-like ILC progenitors,

METHODS

Animal sources

Six-week-old female C57BL/6 mice were purchased from the National Cancer Institute. All animals were housed at the Duke University Vivarium, and all experimental protocols were approved by the Duke University Institutional Animal Care and Use Committee.

Nanoparticle construction

Heparin-chitosan nanoparticles were constructed based on a previously described protocol.^{E1} In brief, this involved gradually combining 0.1% heparin (Calbiochem, San Diego, Calif) and 0.1% chitosan (Vanson, Redmond, Wash), both in dH₂O, at approximately pH 4.5. The chitosan had been purified to drug grade, as previously described. One volume of 0.1% chitosan was added to 5 volumes of 0.1% heparin and vortexed for 30 seconds. Addition of chitosan was repeated until a final 1:1 ratio of 0.1% chitosan to 0.1% heparin was achieved. After 10 minutes at room temperature, the pH was then adjusted to neutrality to prevent further aggregation.

To load particles with cytokines, either rIL-12 or rIL-10 (both from R&D Systems, Minneapolis, Minn) was vortexed for 10 minutes in 0.1% heparin before addition of chitosan. Particles were centrifuged at 14,000g for 10 minutes at 4°C, washed with water once, and then resuspended in PBS for injections. Each injection contained 100 µg of heparin, 100 µg of chitosan, and 1 ng of cytokine in particle form.

Peanut antigens

Peanut antigens, which were previously characterized in detail,^{E2,E3} were provided by Dr Wesley Burkes.

Mass spectrometric identification of major allergens present in complex peanut antigens

Quantitative proteomic analysis by means of liquid chromatography–tandem mass spectrometry was performed on soluble peanut antigen at Duke University's Proteomics and Metabolomics facility. Acquired data were searched against the TrEMBL *Arachis hypogaea* database by using the Mascot search engine. Scaffold (version Scaffold_4.8.4; Proteome Software, Portland, Ore) was used to validate tandem mass spectrometry–based peptide and protein identifications. Peptide identifications at greater than 99.0% probability and protein identifications at greater than 99.0% probability were used to achieve a false discovery rate of less than 1.0% and contained at least 2 identified peptides. The data presented in Table E1 list the major proteins identified in our complex antigen sample based on their total exclusive spectral counts.

Sensitization to peanut allergens and intraperitoneal challenges

For active sensitization to peanut, mice were given 7 doses of peanut antigen every 48 hours. Beginning at week 5, desensitization therapy was initiated, which involved subcutaneous injection in the right rear footpad of soluble peanut antigen (1 µg) combined with nanoparticle-encapsulated cytokines (or appropriate controls) in a 20-µL volume of PBS. Injections were repeated every 2 weeks for a total of 4 desensitization treatments. At week 12, animals were challenged with 0.5 mg of peanut antigen by means of intraperitoneal injection. Anaphylaxis was monitored by measuring the animals' temperatures at regular intervals with a rectal thermometer probe.

Sensitization to peanut allergens and oral challenges

For oral sensitization, mice were sensitized for 8 weeks with a low dose (50 µg) of peanut antigen administered by means of oral gavage, followed by 2 weeks of high-dose oral gavage boosts (1 mg) containing cholera toxin based on a published protocol.^{E4} Control mice received cholera toxin alone in PBS. The cutaneous desensitization protocol was initiated 3 weeks after beginning the high-dose sensitizing boosts and followed the same procedure as described above, with 4 biweekly desensitization injections. Mice were challenged with

500 µg of peanut antigen in PBS administered by means of oral gavage, and anaphylaxis was monitored, as above, by using body temperature measurement.

Measurement of T_H1/T_H2 immune responses

For mice receiving oral peanut sensitization and cutaneous desensitization therapy, blood was collected at the time points indicated in Fig 1, E, and serum was isolated. For ELISAs, peanut antigen was suspended in CBC buffer (15 mmol/L Na₂CO₃ and 35 mmol/L NaHCO₃, pH 9.6) at a concentration of 2 µg/mL, plated into 384-well microtiter plates, and incubated overnight at 4°C. CBC buffer containing 3% nonfat dry milk was used to block, followed by washing 4 times with ELISA wash buffer (PBS, 0.05% Tween 20, and 0.1% sodium azide). Serum samples were diluted 6-fold in serum diluent (PBS, 2.5% BSA, 2.5% nonfat dry milk, 5% goat serum, 0.1% sodium azide, and 0.05% Tween 20) and added to ELISA plates that were incubated overnight at 4°C. After washing, alkaline phosphatase-conjugated anti-mouse IgG₁, anti-mouse IgG_{2a}, or anti-mouse IgE was added to the plates (Southern Biotechnology, Birmingham, Ala) and diluted 1:8000 in buffer containing PBS, 0.05% Tween 20, and 0.5% BSA. Plates were incubated at room temperature for 2 hours, washed 4 times with ELISA wash buffer, and reacted with the alkaline phosphatase substrate Attophos (Roche Molecular Biochemicals, Mannheim, Germany). After incubation for 15 minutes, plates were read at 405 nm on a FluoroCount plate reader (Packard Instrument Company, Meriden, Conn). Samples were considered positive for antigen-specific antibody when the relative light unit value for the sample dilution was 2-fold higher than the relative light unit value for a naive sample at the same dilution.

To assess cellular responses after desensitization therapy, the popliteal lymph nodes (which drain the footpad site of desensitization) were isolated from mice at the time point indicated in Fig 1, E; minced in RPMI containing 10% FBS; and incubated for 30 minutes at 37°C with 100 U/mL collagenase A (Sigma, St Louis, Mo). Single-cell suspensions were produced by straining the disrupted lymph nodes through a 70-µm cell-straining filter (BD Biosciences, San Jose, Calif). After blocking with 1% BSA in PBS for 30 minutes, cells were stained with the following antibodies: anti-CD3–fluorescein isothiocyanate (Invitrogen, Carlsbad, Calif), anti-CD11c–allophycocyanin (BD Biosciences), and anti-programmed death ligand 1–phycoerythrin (eBioscience, San Diego, Calif). After surface stains were completed, cells were fixed with 4% paraformaldehyde and treated with 1% saponin in PBS containing 1% BSA for intracellular staining by using anti-interferon-γ–peridinin-chlorophyll-protein complex–Cy5.5 (BD Biosciences).

All data were acquired with a FACSCalibur (BD Biosciences) and analyzed with CellQuest software.

Statistical analysis

Prism software (GraphPad Software, La Jolla, Calif) was used for all statistical analyses. Flow cytometry and ELISA experiments were analyzed by using 1-way ANOVA, and temperature readings were analyzed by using 2-way ANOVA, with the Bonferroni multiple comparison test to determine *P* values. Unless otherwise noted, error bars throughout the figures represent the SEM and 5 animals per group. For all experiments, results were considered significant at a *P* value of greater than .05.

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TABLE E1. List of major allergens identified in the complex peanut antigen by using mass spectrometry

Proteins identified
Known allergens
Ara h 1
Ara h 3
Ara h 6
Ara h 2 (conglutin-7)
Ara h 8
Ara h 7
Other proteins
Arachin 6
Gly
Galactose-binding lectin
Conarachin
Arachin Ahy-4
Arachin Ahy-3
Arachin Ahy-2
Peanut agglutinin