

Single-tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts

Mike Kulis, PhD, Yifan Li, BS, Hannah Lane, BS, Laurent Pons, PhD, and Wesley Burks, MD *Durham, NC*

Background: Allergic reactions to tree nuts are often severe and are outgrown in less than 10% of diagnosed patients.

Objectives: To determine whether treatment of underlying tree nut sensitization will prevent allergic reactions to cross-reacting tree nuts and to determine the effects of single-tree nut immunotherapy on true multi-tree nut sensitization.

Methods: Cross-reactivity model: Cashew-sensitized mice underwent immunotherapy with cashew and were subsequently challenged with cashew and pistachio. Multisensitization model: Cashew plus walnut-sensitized mice were treated with cashew alone, walnut alone, or both cashew and walnut and then underwent challenges to cashew and walnut. Challenges were assessed on the basis of symptoms, changes in body temperature, and mouse mast cell protease-1 release.

Results: In the cross-reactivity model, cashew immunotherapy completely prevented allergic reactions on challenges with cashew or the cross-reactive pistachio. In the multisensitization model, mice with cashew plus walnut allergy were significantly protected from anaphylactic reactions on cashew challenge in both the cashew-alone and walnut-alone immunotherapy groups. Results from the walnut challenge demonstrated significantly decreased allergic responses in the walnut immunotherapy group, whereas mice in the cashew immunotherapy group experienced significantly lower symptoms. In the cross-reactivity model, immunotherapy effectively decreased IL-4 and IL-5 production and increased IL-12 relative to placebo while also inducing a 5-fold increase in specific IgG₁.

Conclusion: Single-tree nut immunotherapy can effectively decrease allergic responses in both the cross-reactivity and

multisensitization mouse models. Further studies are needed to determine which single-tree nut immunotherapies will be most effective for specific multi-tree nut allergy profiles. (*J Allergy Clin Immunol* 2010;■■■■:■■■■-■■■■.)

Key words: Food allergy, tree nut allergy, immunotherapy, cross-reactivity, cashew, walnut, pistachio

Allergies to tree nuts affect approximately 0.5% of children under 18 years old and 0.9% of adults in the United States.¹ Unlike egg and milk allergies that are outgrown by a vast majority of allergic children, nut allergies are outgrown by only an estimated 9% of patients.² Among tree nuts, walnut, cashew, almond, pecan, and pistachio appear to be most problematic, which may be a result of these being the most consumed.^{3,4} Sensitization to multiple tree nuts is common once diagnosis to a tree nut or peanut is made, with greater than 80% of patients having IgE for several nuts by age 14 years.⁵ Tree nuts can induce severe, and even fatal, reactions.⁶ Recently, a study from the United Kingdom (UK) provided evidence that cashew may induce allergic reactions more severe than peanut,⁷ which is often thought of as the most severe form of food allergy.

The major allergenic proteins in tree nuts, like peanut and other seeds, have been identified as seed storage proteins. The vicilin (7S globulin), legumin (11S globulin), and 2S albumins from cashew, walnut, and pistachio, along with other nuts, have been sequenced and demonstrated to be allergenic.^{3,8-10} Cross-reactivity among the allergenic proteins in tree nuts has been demonstrated *in vitro*.^{9,11-13} In particular, combinations that are highly cross-reactive are cashew and pistachio (Anacardiaceae family), and walnut and pecan (Juglandaceae family).¹¹ For instance, correlation coefficients of 0.95 and 0.96 were found for cashew and pistachio, and walnut and pecan serum IgE levels, respectively.¹⁴ Clinically, cashew and pistachio cross-reactivity appears to be relevant. A recent report stated that in 22 patients with documented reactions on first known exposure to pistachio, all 22 had IgE to cashew.⁸ The cross-reactivity among tree nut allergens typically leads to the recommendation to patients with nut allergy that all nuts should be avoided.

Currently it is not possible to determine whether patients with allergies to multiple nuts have been initially sensitized to several nuts, or whether 1 sensitization has occurred and the other sensitizations are a result of cross-reactivity. Both possibilities are conceivable; from a therapeutic perspective, it is worth investigating immunotherapy for both scenarios. We are particularly interested in determining whether a single-nut immunotherapy approach can induce tolerance to cross-reacting nuts and/or downregulate hypersensitivities for truly multisensitized patients. This idea may have broad-reaching implications for both pediatric and adult populations with allergies to tree nuts, fish, or shellfish, in which patients typically have multiple triggers from

From the Department of Pediatrics, Division of Pediatric Allergy and Immunology, Duke University Medical Center.

Supported by a grant from the Food Allergy Initiative to W.B. and M.K.

Disclosure of potential conflict of interest: M. Kulis receives research support from the Food Allergy Initiative and the National Institutes of Health. W. Burks is a consultant for ActoGeniX NV, Intelliject, McNeil Nutritionals, and Novartis; is on the advisory board for Dannon Co Probiotics; is on the expert panel for Nutricia; is a minority stockholder in Allertein and MastCell, Inc; receives research support from the National Institutes of Health, the Food Allergy and Anaphylaxis Network, and the Wallace Research Foundation; has provided expert witness testimony/legal consultation services in cases related to food allergy; is on the Medical Board of Directors for the Food Allergy and Anaphylaxis Network; is a Dermatological Allergy Committee member for the American College of Allergy, Asthma, and Immunology; is a study section member for the NIH HAI; is on the review board for the Journal of Allergy & Clinical Immunology; and is a member of the FDA. The rest of the authors have declared that they have no conflict of interest.

Received for publication April 19, 2010; revised September 15, 2010; accepted for publication September 16, 2010.

Reprint requests: Mike Kulis, PhD, Duke University Medical Center, Box 2644, Durham, NC 27710. E-mail: michael.kulis@duke.edu.

0091-6749/\$36.00

© 2010 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2010.09.014

Abbreviations used

HRP: Horseradish peroxidase
 MMCP-1: Mouse mast cell protease 1
 UK: United Kingdom

an allergenic food group because of the homology among the offending protein allergens.

Our approach to study cross-reactivity and multisensitization to tree nuts *in vivo* is to use a mouse model in which we control the sensitizing antigens and can easily administer multiple food challenges.¹⁵ In this report, we addressed 2 hypotheses by using our tree nut mouse models: (1) specific immunotherapy for cashew allergy will effectively prevent allergic reactions to the highly cross-reactive pistachio, and (2) single-tree nut immunotherapy with either of 2 sensitizing tree nuts (cashew and walnut) will lead to decreased allergic reactions to both nuts.

METHODS

Food extract preparations

Cashew, walnut, pistachio, peanut, and egg extracts were prepared as previously reported.¹⁵ Walnut seeds were extracted in a phosphate buffer with 1 mol/L NaCl to increase protein solubility.¹⁶ Briefly, ground seeds were mixed in a 1:5 (wt:vol) ratio in 2x PBS and homogenized for 30 minutes while maintaining an alkaline pH (8.5). Supernatants were clarified by centrifugation at 30,000g for 30 minutes at 4°C. Ammonium sulfate precipitation at 90% saturation was used to concentrate the proteins. After dialysis into PBS, all extracts were filter-sterilized, and protein concentration was determined via bicinchoninic acid assay (Pierce, Rockford, Ill).

Mouse model of immunotherapy for tree nut cross-reactivity

Five-week-old female C3H/HeJ mice purchased from Jackson Laboratory (Bar Harbor, Me) were maintained on tree nut-free, peanut-free, and egg-free food (PMI Nutrition International, St Louis, Mo) under pathogen-free conditions following standard guidelines for care and use. The sensitization, immunotherapy, and challenge protocols were based on our previous work.¹⁷ Mice were divided into 5 groups: (1) naive, (2) cashew-sensitized treated with cashew immunotherapy, (3) cashew-sensitized treated with placebo (PBS), (4) egg-sensitized treated with cashew immunotherapy, and (5) egg-sensitized treated with placebo. All groups consisted of 5 mice, except groups 4 and 5 contained 3 mice each. Mice were sensitized intraperitoneally with 0.5 mg appropriate food extract plus 2 mg alum (Imject Alum; Thermo Scientific, Rockford, Ill) with 1 injection during weeks 1, 2, and 4. The immunotherapy regimen began in week 7 and lasted 4 weeks with 3 intraperitoneal injections given per week (ie, Monday, Wednesday, and Friday). The doses followed a build-up and maintenance schedule intended to mimic clinical allergy immunotherapy. The doses were as follows: week 7, mice received 0.1 mg cashew extract or PBS (placebo); week 8, 0.25 mg cashew or PBS; weeks 9 and 10, 0.5 mg cashew or PBS. Submandibular facial sampling was used to collect sera from individual mice during week 6 (postsensitization) and week 13 (postimmunotherapy) for measuring antibody levels. Mice were challenged at week 14 via intraperitoneal injection of 1.0 mg of the food used for sensitization (cashew or egg). An additional challenge was performed at week 17 by using 1.0 mg pistachio extract in mice that were sensitized to cashew (ie, groups 2 and 3). Anaphylactic reactions were assessed by measuring core body temperatures with a rectal probe at 15-minute intervals for 45 minutes postinjection. Symptoms were scored after 30 minutes using a pre-established scale: 0, no symptoms; 1, scratching around the nose and head; 2, puffiness around the eyes and mouth with reduced activity; 3, labored respiration and/or cyanosis around the mouth and tail; 4, no activity after prodding, or tremor and convulsion; and 5, death.¹⁸ Blood was collected at 45 minutes to assess mouse mast cell protease 1 (MMCP-1) levels in select

mice by using an ELISA kit (Moredun Scientific, Midlothian, Scotland). All procedures were approved by the Institutional Animal Care and Use Committee at Duke University.

Mouse model of immunotherapy for multi-tree nut sensitization

Five-week-old female C3H/HeJ mice were used and maintained as discussed. Mice were divided into 5 groups of 5 mice each. Groups 1 to 4 were sensitized to cashew plus walnut and treated as follows: (1) placebo, (2) walnut plus cashew immunotherapy, (3) cashew immunotherapy alone, and (4) walnut immunotherapy alone. Group 5 was a naive control. Mice were sensitized intraperitoneally during weeks 1, 2, and 4 with 0.5 mg tree nut extract plus 2 mg alum as previously described.¹⁵ Immunotherapy began in week 7 and was administered as discussed. Cashew immunotherapy alone and walnut immunotherapy alone were given in identical doses as described. The walnut plus cashew immunotherapy group received 3 injections of 0.1 mg cashew and 0.1 mg walnut extracts during week 7; 3 injections of 0.25 mg cashew and 0.25 mg walnut during week 8; and 3 injections per week for weeks 9 and 10 of 0.5 mg cashew and 0.5 mg walnut extracts. The cashew and walnut extracts were injected within 15 minutes of each other for each immunotherapy dose given. Mice were bled at week 12 for antibody level measurements. Mice were challenged at week 13 with 1.0 mg cashew extract and at week 15 with 1.0 mg walnut. Allergic reactions were assessed as discussed.

IgE, IgG₁, and IgG_{2a} measurements

Specific IgE, IgG₁, and IgG_{2a} were measured by ELISA using a reference curve. Plates were coated with appropriate food extracts (20 µg/mL) in carbonate-bicarbonate buffer at pH 9.6. Detection of IgE was performed with sheep antimouse IgE (0.5 µg/mL; Binding Site, Birmingham, UK), followed by biotinylated donkey antisheep IgG (0.5 µg/mL; Accurate Chemical, Westbury, NY) and neutravidin-horseradish peroxidase (HRP; 0.2 µg/mL; Pierce). IgG₁ was detected by HRP-conjugated goat antimouse IgG₁ (Southern Biotech, Birmingham, Ala) used at 1:40,000. IgG_{2a} was detected by HRP-conjugated goat antimouse IgG_{2a} (Southern Biotech). The HRP activity was measured with color development of 3,3',5,5'-tetramethylbenzidine substrate (KPL, Gaithersburg, Md). Inhibition ELISA experiments for cashew-sensitized mouse IgE were conducted with log-fold increases in protein extracts from 1 ng/mL to 1 mg/mL for pistachio, walnut, peanut, and egg.

Western blotting

Protein extracts, under denatured and reduced conditions, were separated on NuPAGE 4% to 12% Bis-Tris gels (Invitrogen, Carlsbad, Calif) and then transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, Mass). Blots were blocked by using 2% BSA in PBS containing 0.05% Tween-20. Sera from cashew-sensitized mice were pooled and diluted in 2% BSA in PBS containing 0.05% Tween-20 before incubation with the blot. IgG₁ was detected with HRP-conjugated goat antimouse IgG₁ (Southern Biotech). The blots were developed with a chemiluminescence detection kit (Super Signal West Pico Chemiluminescent Substrate; Pierce).

Proliferation assay and quantification of secreted cytokines

Splenocytes were isolated from individual mice at least 2 weeks after completion of challenges. Cells were plated at a density of 2.5×10^6 /mL in complete medium (RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine). Antigens, also in complete medium, were used at 100 µg/mL for cashew, pistachio, walnut, peanut, and egg. All cultures were carried out in a 37°C, 5% CO₂, humidified incubator. Proliferation assays were carried out in triplicate in 96-well round-bottom plates with cells pulsed with 0.5 µCi [³H]-thymidine/well at 78 hours, harvested 18 hours later, and counted for β-radioactivity (Wallac, Turku, Finland). Cultures for cytokine assays were performed in 24-well plates with supernatant collection at 96 hours. Cytokines secreted into the culture medium were quantified by

ELISA, using antibodies from Biolegend (San Diego, Calif) for IL-4, IL-5, and IFN- γ ¹⁵ or R&D Systems (Minneapolis, Minn) for IL-12 p40.

Statistical analyses

Statistical analyses were performed by using SPSS 15.0 for Windows (SPSS Inc, Chicago, Ill). Differences in symptom scores were assessed with the nonparametric Mann-Whitney test. Antibody levels postsensitization and postimmunotherapy were compared by using the paired samples *t* test. All other statistical comparisons were made with the independent sample *t* test, not assuming equal variances. Differences were considered statistically significant with a *P* value of less than .05.

RESULTS

Pistachio is more cross-reactive with IgE and IgG₁ from cashew-sensitized mice than either walnut or peanut

Previously we showed cross-reactivity in cashew-sensitized mice with walnut and peanut.¹⁵ Here we further characterized cross-reactivity with pistachio, which is known to be strongly cross-reactive in human beings.^{11,14} ELISA for specific-IgE from cashew-sensitized mice demonstrated the highest cross-reactivity with pistachio, whereas walnut and peanut both showed some degree of cross-reactivity, and egg showed none (Fig 1, A). Pistachio cross-reactivity with cashew-sensitized mouse IgE was significantly higher than either walnut ($P < .01$) or peanut ($P < .01$) cross-reactivity. Inhibition ELISA experiments confirmed the relative cross-reactivities of these food extracts (data not shown).

Immunoblotting with IgG₁ from cashew-sensitized mouse sera was used to determine which proteins were responsible for cross-reactivity (Fig 1, B). Cashew and pistachio proteins showed a nearly identical banding pattern. Walnut vicilin, Jug r 2 (75, 68, and 42 kd), and the peanut vicilin, Ara h 1 (66 kd), also showed binding. Vicilin proteins among the 3 tree nuts and peanut all were recognized by IgG₁ and appear to account for the broad cross-reactivity of these nuts in mice. Interestingly, the cashew and pistachio extracts showed bands at 30 and 21 kd that bound IgG₁, which correspond to the acidic and basic subunits of legumin allergens, Ana o 2 and Pis v 2, in other literature reports.^{8,10,19}

Cashew immunotherapy prevents allergic reactions to cashew and the cross-reacting pistachio but has no effect on egg sensitization

Mice were sensitized to either cashew or egg, and then underwent an immunotherapy protocol with cashew extract including build-up and maintenance phases. After treatment, mice sensitized to cashew were challenged with cashew (week 14) and pistachio (week 17), whereas egg-sensitized mice were challenged with egg (week 14). The results from the cashew challenge demonstrated that cashew immunotherapy was effective in protecting the mice from allergic reactions to cashew (Fig 2, A). A significant difference in body temperature was found between the cashew immunotherapy and placebo groups at 15 minutes ($P < .05$), which became highly significant ($P < .01$) at 30 and 45 minutes. Symptom scores also showed a highly significant difference between placebo and cashew immunotherapy groups, because the cashew immunotherapy mice were all symptom-free but the placebo mice experienced allergic symptoms with a median of 3 ($P < .01$). In addition, MMCP-1, an indicator of

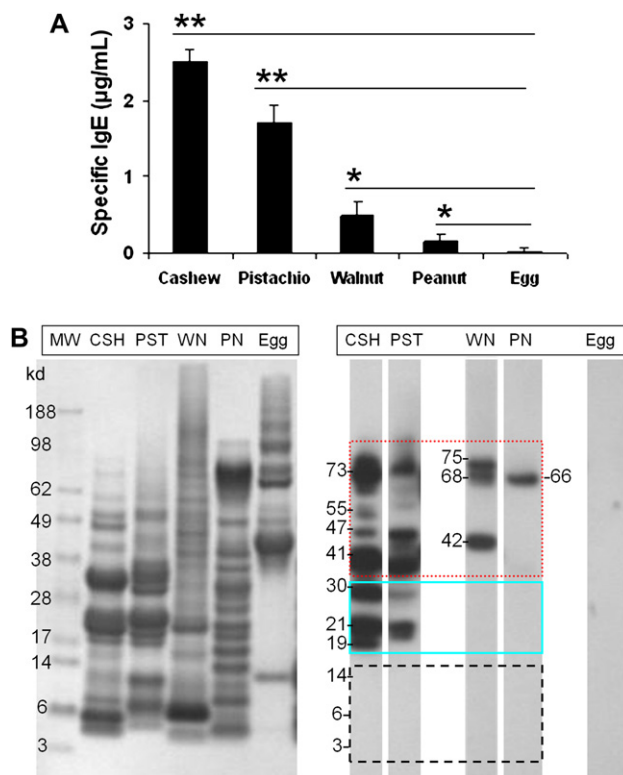


FIG 1. Cross-reactive IgE and IgG₁ in the serum of mice after sensitization with cashew proteins. **A**, Food-specific IgE demonstrating cross-reactivity are shown as averages with standard deviation. Significant differences ($*P < .05$; $**P < .01$) between specified foods; lines above bars indicate significant difference from all foods under the line. **B**, Left panel, Coomassie blue-stained SDS-PAGE gel. Right panel, Western blot showing proteins recognized by IgG₁. Red box (...) indicates vicilin proteins; cyan box indicates legumin proteins; black box (—) indicates 2S albumin proteins. CSH, Cashew; MW, molecular weight standards; PN, peanut; PST, pistachio; WN, walnut.

mast cell degranulation, was significantly higher in placebo mouse sera compared with cashew immunotherapy ($P < .01$).

The pistachio challenge yielded very similar results as the cashew challenge (Fig 2, B). Body temperatures for the cashew immunotherapy group did not go below the physiologic temperature of 37.0°C, whereas the placebo group experienced significantly lower body temperatures at 30 minutes ($P < .05$) and 45 minutes ($P < .01$). The symptoms experienced by the placebo mice on challenge with pistachio were the same as with cashew, experiencing a median score of 3, whereas the cashew immunotherapy group had a median score of 0 ($P < .01$).

The egg challenge demonstrated that cashew immunotherapy has no effect on a nonrelated food allergy. In egg-sensitized mice, the placebo-treated and cashew immunotherapy-treated groups both experienced symptoms with a median score of 4. Similarly, the body temperatures after egg challenge were not different between treatment groups (data not shown).

Single-tree nut immunotherapy has protective effects on multisensitized mice

Mice were sensitized to both cashew and walnut, then treated with single-tree nut immunotherapy, placebo, or both cashew and walnut as immunotherapy. After treatment, mice were administered

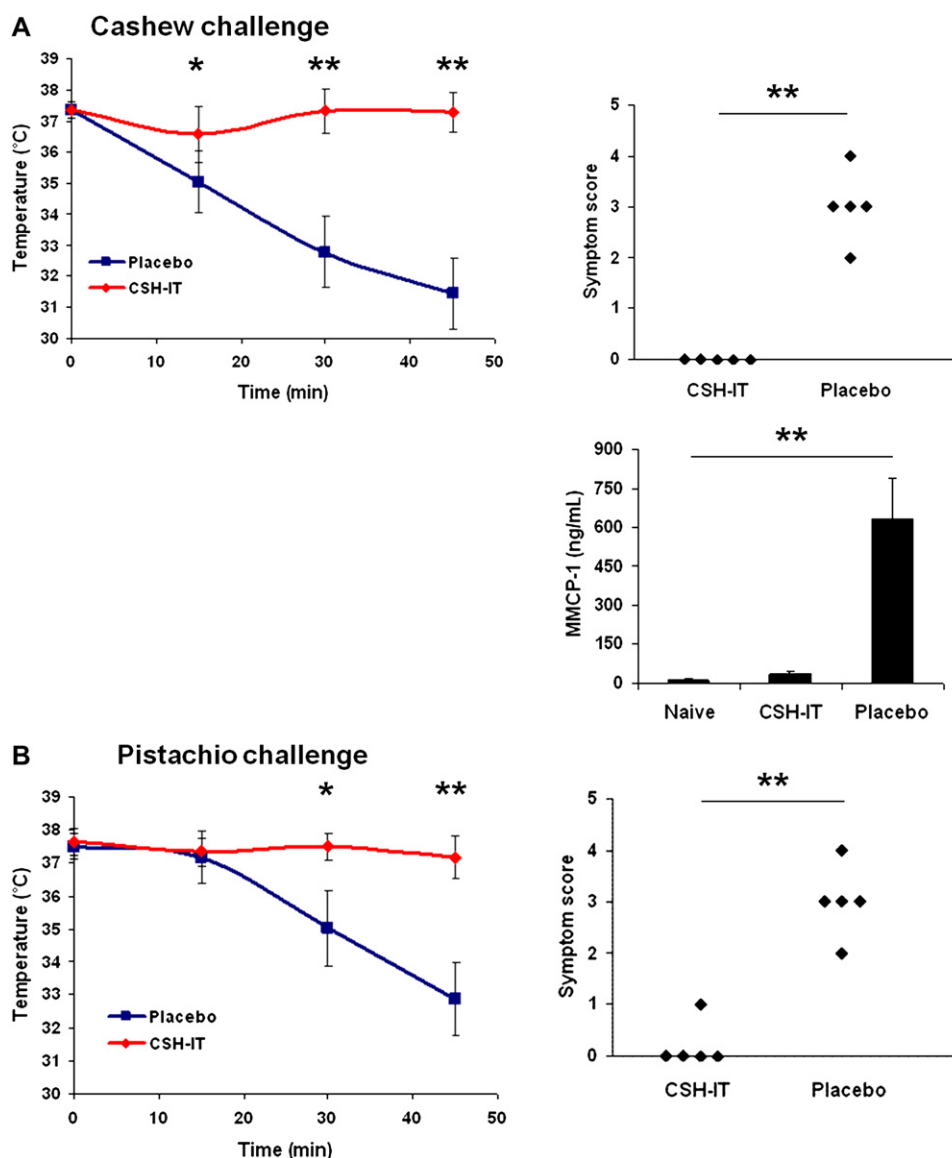


FIG 2. Food challenges to assess efficacy of cashew immunotherapy (CSH-IT) in cross-reactivity mouse model. **A**, Cashew-sensitized mice treated with placebo or CSH-IT underwent a cashew challenge. Body temperatures and MMCP-1 levels are shown as averages with SDs. Symptoms are shown for individual mice. **B**, Results from the pistachio challenge. Significant differences (* $P < .05$; ** $P < .01$).

2 separate challenges, first to cashew, and 2 weeks later to walnut. The cashew challenge results showed strong efficacy for both the cashew immunotherapy and the walnut plus cashew immunotherapy groups, because these mice behaved nearly identically with naive mice in terms of body temperature, symptom scores, and MMCP-1 release (Fig 3, A). Mice given walnut immunotherapy experienced only mild reactions on challenge with cashew. Importantly, the walnut immunotherapy protected mice from allergic reactions to cashew with highly significant differences relative to placebo for all 3 challenge parameters ($P < .01$).

The walnut challenge demonstrated strong efficacy for the walnut immunotherapy and the walnut plus cashew immunotherapy groups (Fig 3, B). Symptom scores did not go above 1 for either group, whereas the placebo mice had strong reactions with a median score of 3. Significant differences were also shown for

body temperature and MMCP-1 release in these immunotherapy groups relative to placebo. Cashew immunotherapy was effective in significantly decreasing allergic symptom scores relative to placebo ($P < .05$), although significant differences were not measured for MMCP-1 or body temperature.

Immunotherapy leads to increased specific IgG₁ with decreased T_H2-type cytokine production

For the cross-reactivity model, serum was collected postsensitization and postimmunotherapy to investigate changes in specific IgE, IgG₁, and IgG_{2a}. Cashew-specific IgE was not statistically different postsensitization or postimmunotherapy when comparing the cashew immunotherapy with the placebo group (data not shown). A highly significant increase ($P < .01$) was measured

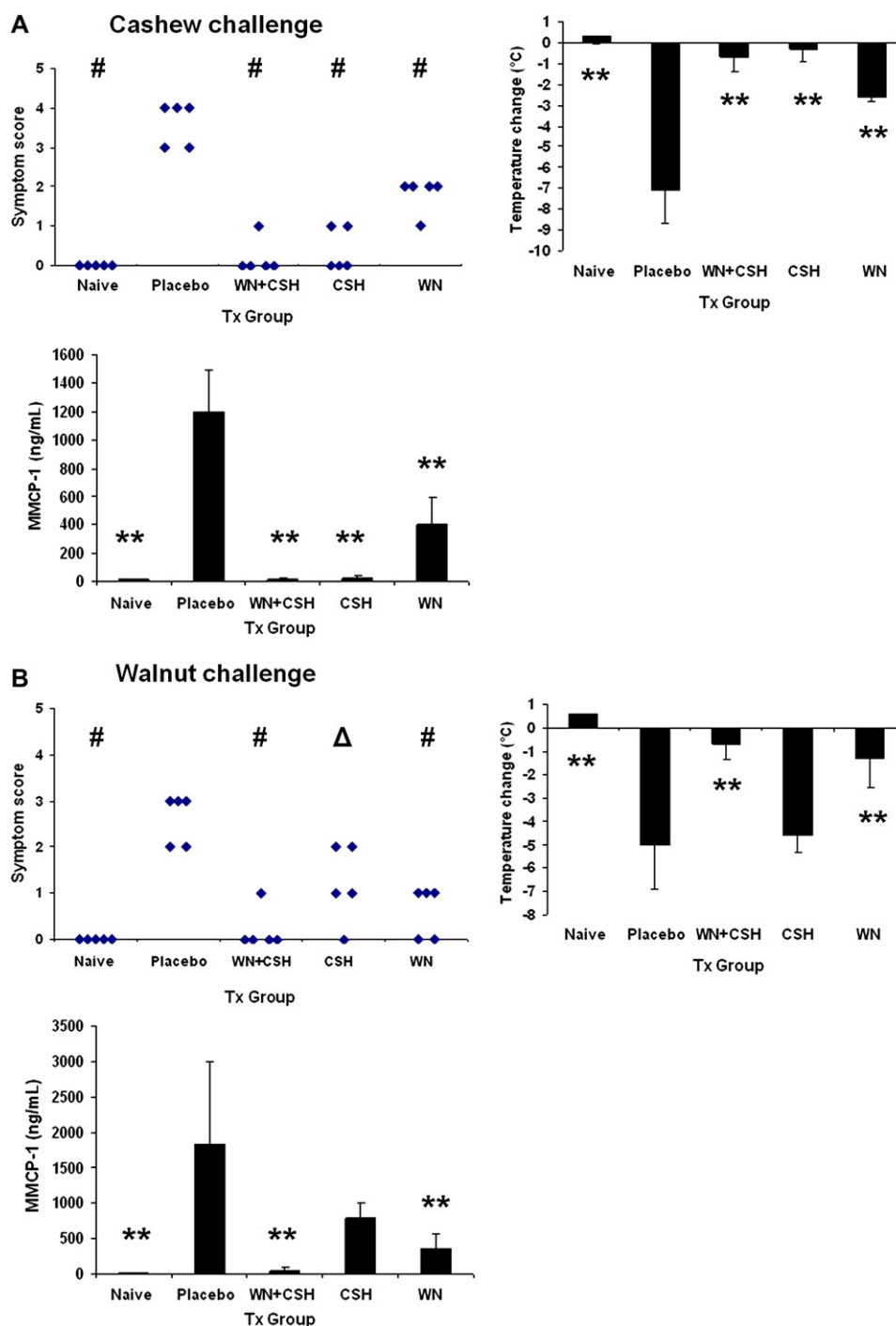


FIG 3. Food challenges to assess efficacy of immunotherapy in a multisensitization mouse model. **A**, Mice having undergone immunotherapy (treatment [Tx] groups shown on x-axis) were challenged with cashew (CSH). Body temperatures (45 minutes) and MMCP-1 levels are shown as averages with SDs. Symptoms are shown for individual mice. **B**, Mice having undergone immunotherapy were challenged with walnut (WN). ** or #, Significant differences ($P < .01$) between indicated group and placebo; Δ indicates significant differences ($P < .05$) between indicated group and placebo.

for cashew IgG₁ in cashew immunotherapy mice but not in placebo mice (Fig 4, A). Cashew IgG₁ increased greater than 5-fold, from a mean of 2.7 mg/mL to 13.8 mg/mL after immunotherapy. A statistically significant increase for cross-reacting IgG₁ was also noted for pistachio ($P = .015$) and walnut ($P < .05$) in the

cashew immunotherapy group (data not shown). No differences were found in cashew-specific IgG_{2a} between placebo and cashew immunotherapy mice (data not shown).

Serum immunoglobulin levels with reactivity to cashew and walnut were measured in the multisensitized mouse model after

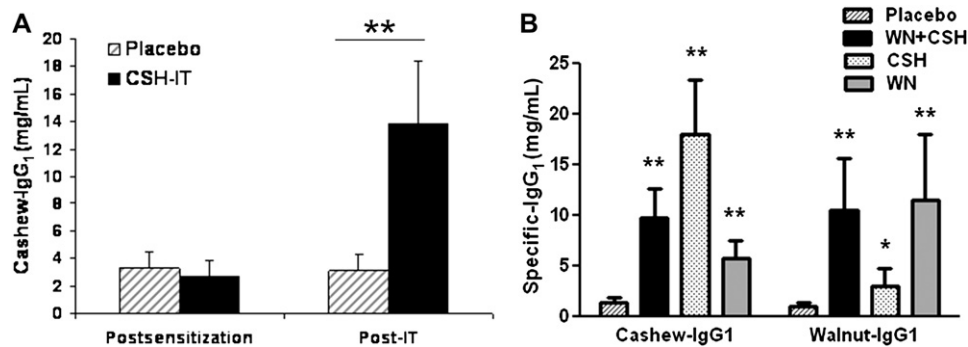


FIG 4. Specific IgG₁ levels after immunotherapy (IT) are shown as averages with SDs. **A**, Cross-reactivity model: cashew-specific IgG₁ levels for placebo and cashew immunotherapy (CSH-IT) groups. **B**, Multisensitization model: cashew-specific and walnut-specific IgG₁ levels for placebo, walnut plus cashew immunotherapy (WN+CSH), cashew immunotherapy (CSH), and walnut immunotherapy (WN). Significant differences (* $P < .05$ or ** $P < .01$).

immunotherapy. As with the cross-reactivity model, IgE and IgG_{2a} levels were not significantly different between groups, whereas significant increases in IgG₁ levels were found (Fig 4, B). Cashew-specific and walnut-specific IgG₁ had highly significant increases ($P < .01$) in the walnut plus cashew immunotherapy and the walnut immunotherapy groups relative to placebo. The cashew immunotherapy group had a highly significant ($P < .01$) increase in cashew-specific IgG₁, whereas the walnut-specific IgG₁ also increased ($P < .05$), albeit with lower concentrations.

Cellular responses were assessed postimmunotherapy for responses to cashew proteins in the cross-reactivity model. Two important T_H2-type cytokines, IL-4 and IL-5, were significantly lower in the immunotherapy group than the placebo group ($P < .05$; Fig 5, A and B). The T_H1-type cytokine, IL-12, was significantly higher ($P < .05$) from cashew immunotherapy splenocytes with a mean of 193.3 pg/mL compared with placebo mice at a mean of 116.5 pg/mL (Fig 5, C). Another T_H1 cytokine, IFN- γ , was not statistically different between immunotherapy and placebo mice; neither were the cellular proliferation responses (data not shown). Importantly, cashew immunotherapy had no effect on egg-sensitized mouse cell proliferation (data not shown) or IL-5 secretion (Fig 5, B).

DISCUSSION

Cross-reactivity of tree nut allergens has been demonstrated by using human IgE, and it is well established that multi-tree nut sensitizations often occur. Recently, we have shown that cross-reactivity between cashew and walnut exists in mice and can cause allergic reactions on first exposure, primarily because of vicilin cross-reactivity.¹⁵ Cross-reactivity at the T-cell level was also shown to occur in sensitized mice, leading to the hypothesis that a single-tree nut immunotherapy approach may provide broad protection from multiple tree nut allergies, because it is well established that allergen immunotherapy works at the T-cell level.²⁰ Because it is unknown whether human beings with multiple tree nut allergies have experienced several sensitizing events or whether there is only 1 sensitizing event followed by a large degree of cross-reactivity among the homologous seed storage allergens, we aimed to address 2 hypotheses that should be useful for future clinical studies aimed at treatment of multi-tree nut allergy: (1) to determine whether treatment of the underlying tree nut

sensitization will prevent allergic reactions to cross-reacting tree nuts, and (2) to determine the effects of single-tree nut immunotherapy on true multi-tree nut sensitization.

To study immunotherapy efficacy on cross-reactivity, we sensitized mice to cashew and measured allergic responses to pistachio after treatment with cashew immunotherapy. Pistachio was chosen for cross-reactivity studies in cashew-sensitized mice because it belongs to the same botanical family, Anacardiaceae, as cashew and is clinically relevant.^{8,11,21} In this report, we demonstrated that pistachio has high levels of cross-reactive IgE with legumin and vicilin proteins representing allergens in mice (Fig 1). Mice treated with cashew immunotherapy experienced median symptom scores of 0 (no reaction) on challenge with either cashew or pistachio, whereas placebo-treated mice had median symptom scores of 3. Body temperatures remained unchanged in cashew immunotherapy mice, whereas large decreases in temperature were measured in placebo mice on challenge with either cashew or pistachio. These data clearly demonstrate that cashew immunotherapy prevented allergic reactions to cashew as well as the highly cross-reactive pistachio. The results lead us to believe that allergic reactions to tree nuts because of cross-reactivity will be abrogated by treating the underlying sensitization.

To demonstrate that immunotherapy efficacy was a result of the protein components in the cashew extract, 2 groups of mice were sensitized to egg and treated with placebo or cashew immunotherapy. Allergic reactions after egg challenge were not different between the groups because both experienced severe symptoms and large decreases in body temperature. The egg-sensitized groups thus rule out nonspecific immune modulators, such as T_H2-modifying agents, immunosuppressing compounds, or Toll-like receptor agonists that could be present in the cashew extract. Demonstrating specificity and attributing the protein allergens as the active compounds in the immunotherapy is critical because current clinical trials on immunotherapy for food allergy rely on proteins for efficacy.²²⁻²⁴

Immunologic changes prompted by cashew immunotherapy were investigated at the humoral and T-cell levels in the cross-reactivity model. Serum analyses showed a highly significant increase in cashew-specific IgG₁, whereas no changes were measured for IgE or IgG_{2a}. Conflicting reports on the role of IgG₁ in the mouse have emerged, with convincing evidence that IgG₁ plays a direct role in mouse anaphylaxis,^{25,26} whereas others

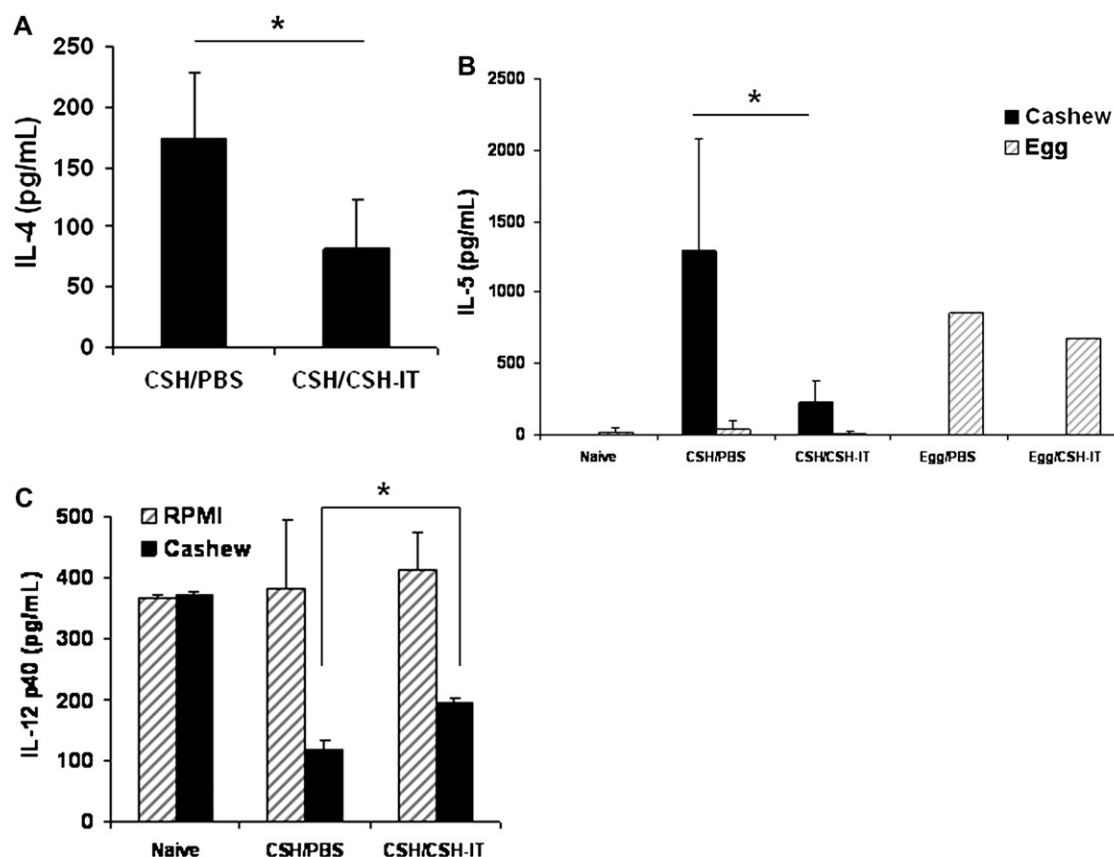


FIG 5. Cytokines secreted from splenocytes after immunotherapy. IL-4 (A), IL-5 (B), and IL-12 (C) were measured after 96 hours of stimulation with the indicated antigen; *RPMI*, medium alone. *CSH/PBS* or *CSH/CSH-IT*, cashew-sensitized mice treated with placebo or cashew immunotherapy, respectively; *Egg/PBS* or *Egg/CSH-IT*, egg-sensitized mice treated with placebo or cashew immunotherapy, respectively. *Significant differences ($P < .05$).

have elegantly demonstrated a nonanaphylactic type of IgG₁.^{27,28} IgG₁ has also been considered the mouse analog of human IgG₄,²⁹ which is known to increase in successful clinical applications of allergen immunotherapy.²⁰ In our model, it seems evident that the IgG₁ generated by immunotherapy is nonanaphylactic; otherwise, the cashew immunotherapy mice would be more reactive than placebo. The highly increased IgG₁ coupled with the unchanged IgE leads us to believe that IgG₁ may play a protective role *in vivo*. Cellular studies after immunotherapy showed decreased IL-4 and IL-5 and increased IL-12 for cashew immunotherapy mice compared with placebo. These results indicate a shift away from a T_H2 phenotype toward a T_H1 profile, which is a typical outcome seen in successful immunotherapy.²⁰ Cashew immunotherapy did not affect proliferations or IL-5 secretion by egg stimulation of egg-sensitized mouse splenocytes, further demonstrating that no immunomodulating compounds are present in the cashew extract.

In the multisensitization model, walnut immunotherapy effectively protected mice with cashew plus walnut allergy from both cashew and walnut challenges. The allergic responses, including symptom scores, decreased body temperature, and MMCP-1 release, were significantly lower ($P < .01$) for the walnut immunotherapy group compared with the placebo-treated mice for both cashew and walnut challenges. Cashew immunotherapy fully protected mice from reactions to cashew challenge ($P < .01$ for all

challenge parameters) and had a favorable impact on the walnut challenge, leading to significantly decreased allergic symptoms ($P < .05$). However, cashew immunotherapy did not cause significant changes in body temperature or MMCP-1 release in the walnut challenge. Interestingly, the walnut-specific IgG₁ increase after cashew immunotherapy was lower than the cashew-specific IgG₁ increase from walnut immunotherapy, suggesting that an IgG₁ response above an as yet undetermined critical threshold may be necessary to offer maximal therapeutic benefit.

The results from the multisensitization model provide important evidence that the single-nut immunotherapy approach may not be fully effective for all combinations of tree nuts. A possible explanation for walnut immunotherapy fully protecting against cashew challenge, but cashew immunotherapy not protecting as well against walnut challenge (improved symptoms, but not body temperature or MMCP-1), arises from the differing allergen profile of these nuts in the mice. In this model, cashew vicilin and legumin proteins are allergens, whereas walnut vicilin, legumin, and 2S albumin are allergens.¹⁵ It seems the 2S albumins in these nuts are not homologous at the T-cell epitope level in mice; otherwise, mice would be expected to generate immune responses to the cashew 2S albumin, because they are fully capable of an antibody response against walnut 2S albumin.¹⁵ Therefore, using cashew immunotherapy to downregulate walnut hypersensitivity would not be fully effective because of low-level homology of

T-cell epitopes of the 2S albumins. Putative T-cell epitope homology between the vicilins and legumins explains why some degree of protection is achieved on walnut sensitization via cashew immunotherapy and fits well with walnut immunotherapy downregulating cashew allergy in this model. These findings indicate that it may be critical to characterize which protein families are allergenic for a specific patient, and even which allergens between nuts will share enough T-cell epitope homology, if a single tree nut immunotherapy approach can be successful for multi-tree nut allergy.

In conclusion, cross-reactions from homologous tree nut proteins are prevented by treating the underlying sensitization. In a true multisensitization model, single-tree nut immunotherapy can have a significant positive impact on multiallergic status, which appears to be dependent on the selection of tree nut used as the immunotherapy.

Clinical implications: Immunotherapy with a single tree nut can decrease allergic responses in mice reacting to multiple tree nuts. This finding may be applicable to individuals with clinical reactivity to several tree nuts.

REFERENCES

1. Sicherer SH, Munoz-Furlong A, Sampson HA. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J Allergy Clin Immunol* 2003;112:1203-7.
2. Fleischer DM, Conover-Walker MK, Matsui EC, Wood RA. The natural history of tree nut allergy. *J Allergy Clin Immunol* 2005;116:1087-93.
3. Roux KH, Teuber SS, Sathe SK. Tree nut allergens. *Int Arch Allergy Immunol* 2003;131:234-44.
4. Sicherer SH, Furlong TJ, Munoz-Furlong A, Burks AW, Sampson HA. A voluntary registry for peanut and tree nut allergy: characteristics of the first 5149 registrants. *J Allergy Clin Immunol* 2001;108:128-32.
5. Clark AT, Ewan PW. The development and progression of allergy to multiple nuts at different ages. *Pediatr Allergy Immunol* 2005;16:507-11.
6. Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 2001;107:191-3.
7. Clark AT, Anagnostou K, Ewan PW. Cashew nut causes more severe reactions than peanut: case-matched comparison in 141 children. *Allergy* 2007;62:913-6.
8. Ahn K, Bardina L, Grishina G, Beyer K, Sampson HA. Identification of two pistachio allergens, Pis v 1 and Pis v 2, belonging to the 2S albumin and 11S globulin family. *Clin Exp Allergy* 2009;39:926-34.
9. Willison LN, Tawde P, Robotham JM, Penney RM 4th, Teuber SS, Sathe SK, et al. Pistachio vicilin, Pis v 3, is immunoglobulin E-reactive and cross-reacts with the homologous cashew allergen, Ana o 1. *Clin Exp Allergy* 2008;38:1229-38.
10. Teuber SS, Sathe SK, Peterson WR, Roux KH. Characterization of the soluble allergenic proteins of cashew nut (*Anacardium occidentale* L.). *J Agric Food Chem* 2002;50:6543-9.
11. Goetz DW, Whisman BA, Goetz AD. Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis, and human specific IgE serologic surveys. *Ann Allergy Asthma Immunol* 2005;95:45-52.
12. de Leon MP, Gaspoli IN, Drew AC, Rolland JM, O'Hehir RE, Suphioglu C. Immunological analysis of allergenic cross-reactivity between peanut and tree nuts. *Clin Exp Allergy* 2003;33:1273-80.
13. Fernandez C, Fianador A, Martinez-Garate A, Martinez Quesada J. Allergy to pistachio: crossreactivity between pistachio nut and other Anacardiaceae. *Clin Exp Allergy* 1995;25:1254-9.
14. Maloney JM, Rudengren M, Ahlstedt S, Bock SA, Sampson HA. The use of serum-specific IgE measurements for the diagnosis of peanut, tree nut, and seed allergy. *J Allergy Clin Immunol* 2008;122:145-51.
15. Kulis M, Pons L, Burks AW. In vivo and T-cell cross-reactivity between walnut, cashew, and peanut. *Int Arch Allergy Immunol* 2009;148:109-17.
16. Sathe SK, Venkatachalam M, Sharma GM, Kshirsagar HH, Teuber SS, Roux KH. Solubilization and electrophoretic characterization of select edible nut seed proteins. *J Agric Food Chem* 2009;57:7846-56.
17. Pons L, Ponnappan U, Hall RA, Simpson P, Cockrell G, West CM, et al. Soy immunotherapy for peanut-allergic mice: modulation of the peanut-allergic response. *J Allergy Clin Immunol* 2004;114:915-21.
18. 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.
19. Sathe SK, Clara Sze-Tao KW, Wolf WJ, Hamaker BR. Biochemical characterization and in vitro digestibility of the major globulin in cashew nut (*Anacardium occidentale*). *J Agric Food Chem* 1997;45:2854-60.
20. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol* 2007;119:780-91.
21. Hasegawa M, Inomata N, Yamazaki H, Morita A, Kirino M, Ikezawa Z. Clinical features of four cases with cashew nut allergy and cross-reactivity between cashew nut and pistachio. *Allergol Int* 2009;58:209-15.
22. Buchanan AD, Green TD, Jones SM, Scurlock AM, Christie L, Althage KA, et al. Egg oral immunotherapy in nonanaphylactic children with egg allergy. *J Allergy Clin Immunol* 2007;119:199-205.
23. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, et al. Clinical efficacy and immune regulation with peanut oral immunotherapy. *J Allergy Clin Immunol* 2009;124:292-300, e1-97.
24. Skripak JM, Nash SD, Rowley H, Bereton NH, Oh S, Hamilton RG, et al. A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. *J Allergy Clin Immunol* 2008;122:1154-60.
25. Finkelman FD, Rothenberg ME, Brandt EB, Morris SC, Strait RT. Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *J Allergy Clin Immunol* 2005;115:449-57, quiz 58.
26. Strait RT, Morris SC, Yang M, Qu XW, Finkelman FD. Pathways of anaphylaxis in the mouse. *J Allergy Clin Immunol* 2002;109:658-68.
27. Faquim-Mauro EL, Jacysyn JF, Macedo MS. Anaphylactic and non-anaphylactic murine IgG1 differ in their ability to bind to mast cells: relevance of proper glycosylation of the molecule. *Immunobiology* 2003;207:169-77.
28. Silva SR, Casabuono A, Jacysyn JF, Favoretto BC, Fernandes I, Macedo MS, et al. Sialic acid residues are essential for the anaphylactic activity of murine IgG1 antibodies. *J Immunol* 2008;181:8308-14.
29. Musio S, Pedotti P, Mantegazza R, Ohtsu H, Boon L, Steinman L, et al. Anaphylaxis to a self-peptide in the absence of mast cells or histamine. *Lab Invest* 2009;89:398-405.