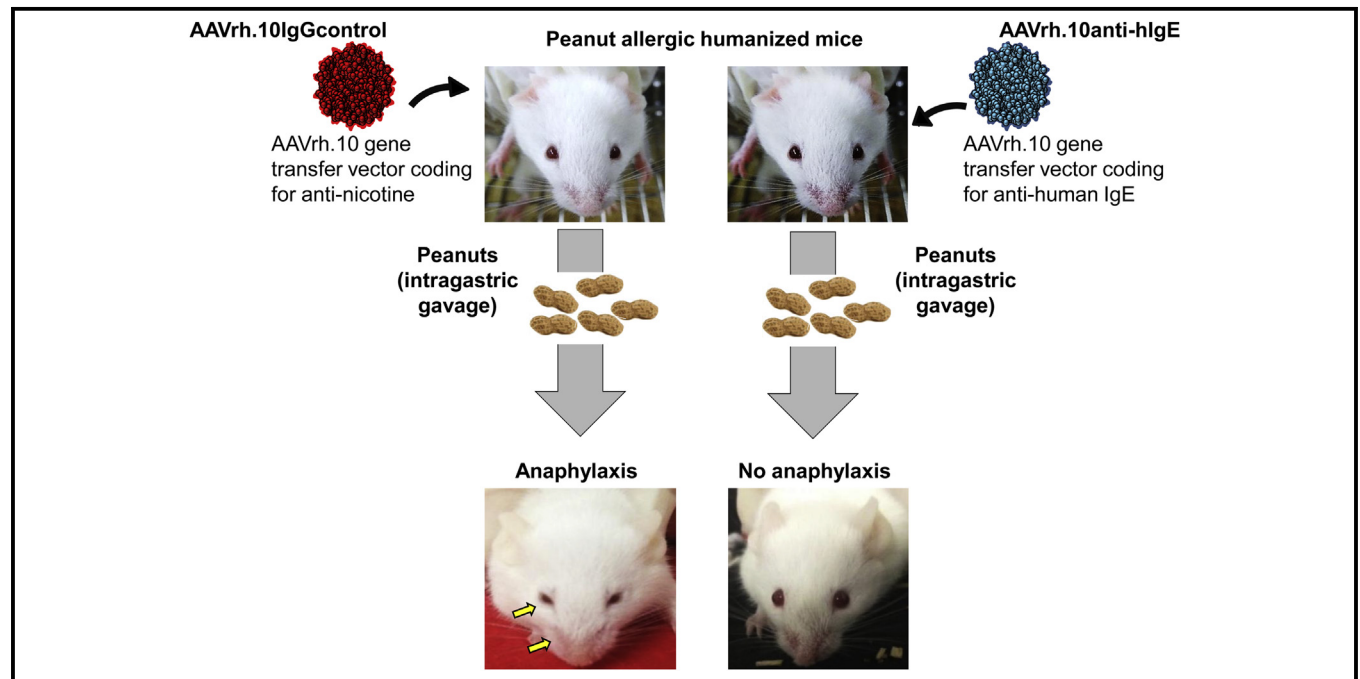


Anti-hIgE gene therapy of peanut-induced anaphylaxis in a humanized murine model of peanut allergy

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GRAPHICAL ABSTRACT



Background: Peanuts are the most common food to provoke fatal or near-fatal anaphylactic reactions. Treatment with an anti-hIgE mAb is efficacious but requires frequent parenteral administration.

Objective: Based on the knowledge that peanut allergy is mediated by peanut-specific IgE, we hypothesized that a single administration of an adeno-associated virus (AAV) gene transfer vector encoding for anti-hIgE would protect against repeated peanut exposure in the host with peanut allergy.

Methods: We developed a novel humanized murine model of peanut allergy that recapitulates the human anaphylactic response to peanuts in NOD-*scid* IL2Rgamma^{null} mice

transferred with blood mononuclear cells from donors with peanut allergy and then sensitized with peanut extract. As therapy, we constructed an adeno-associated rh.10 serotype vector coding for a full-length, high-affinity, anti-hIgE antibody derived from the Fab fragment of the anti-hIgE mAb omalizumab (AAVrh.10anti-hIgE). In the reconstituted mice peanut-specific IgE was induced by peanut sensitization and hypersensitivity, and reactions were provoked by feeding peanuts to mice with symptoms similar to those of human subjects with peanut allergy.

Results: A single administration of AAVrh.10anti-hIgE vector expressed persistent levels of anti-hIgE. The anti-hIgE vector,

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administered either before sensitization or after peanut sensitization and manifestation of the peanut-induced phenotype, blocked IgE-mediated alterations in peanut-induced histamine release, anaphylaxis scores, locomotor activity, and free IgE levels and protected animals from death caused by anaphylaxis.

Conclusion: If this degree of persistent efficacy translates to human subjects, AAVrh.10anti-hIgE could be an effective 1-time preventative therapy for peanut allergy and possibly other severe, IgE-mediated allergies. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Peanut allergy, food allergy, omalizumab, IgE, gene therapy, mouse model

Peanuts account for the majority of fatal and near-fatal food-induced, anaphylactic reactions in the United States.^{1,2} Peanut-induced anaphylaxis typically manifests as vomiting, diarrhea, abdominal pain, angioedema, bronchospasm, hypotension, loss of consciousness, and sometimes death after allergen ingestion.³ Laryngeal edema, lower-airway obstruction, and hypotension are the most serious manifestations.⁴ Unlike other food allergies, peanut allergy is rarely outgrown,^{2,5} and it is essential for affected subjects to strictly avoid peanuts and have quick access to an epinephrine autoinjector at all times.³ Although strides have been made in food allergy awareness and prevention of peanut sensitization, there is no satisfactory therapy to prevent or reverse this disease.⁶⁻⁹

Like other food allergies, peanut allergy is mediated by type I hypersensitivity responses linked to peanut antigen-specific IgE.¹⁰⁻¹² The IgE-allergen complex activates mast cells and basophils, with release of mediators responsible for the clinical anaphylaxis response to peanuts (see Fig E1 in this article's Online Repository at www.jacionline.org).^{10,12} Based on this knowledge, there has been considerable interest in treating patients with peanut allergy with therapies that interrupt peanut-specific IgE from eliciting anaphylaxis. One approach is treatment with omalizumab (Xolair, Genentech Inc, South San Francisco, Calif, and Novartis Pharmaceuticals Corporation, East Hanover, NJ), a recombinant DNA-derived humanized IgG_{1κ} mAb that binds to human IgE.¹³ Omalizumab inhibits binding of IgE to the IgE receptor (FcεRI) on the surfaces of mast cells and basophils, thus limiting the degree of release of mediators of the allergic response from the FcεRI-bearing cells. Once circulating IgE antibodies are bound by anti-hIgE, they cannot bind to specific high-affinity Fc receptors on mast cells and basophils, and the complexes of anti-hIgE bound to IgE are then cleared from the circulation.¹³ Studies with humanized recombinant anti-hIgE therapy for food allergy have shown promise, at least in the short term.^{2,8,13,14} However, using an anti-hIgE mAb as prophylaxis against peanut-induced anaphylaxis in peanut-sensitive subjects has challenges because the protection provided by a single administration of omalizumab is short, estimated at 2 to 4 weeks, requiring at least monthly parenteral administration of the mAb to maintain persistent effective therapy.¹⁵

As a strategy to circumvent the requirement for repeated anti-hIgE mAb administration, we hypothesized that a single administration of an adeno-associated virus (AAV) coding for omalizumab would provide long-term expression of anti-hIgE, protecting against peanut-induced allergic reactions on a persistent basis. To evaluate this hypothesis, we created a unique humanized

Abbreviations used

AAV: Adeno-associated virus
GVHD: Graft-versus-host disease
hIgE: Human IgE
NSG: NOD-*scid* IL2Rgamma^{null}
PCA: Passive cutaneous anaphylaxis

mouse model of human IgE-mediated anaphylaxis in response to peanuts. Murine models (not humanized) of peanut allergy are complicated in that both IgE and IgG₁ elicit allergic reactions in mice, whereas IgG antibodies appear to play a smaller role in human anaphylaxis.^{16,17} To circumvent this, we created a humanized IgE anti-peanut-induced anaphylaxis NOD-*scid* IL2Rgamma^{null} (NSG) murine model using immunodeficient mice reconstituted with peanut-allergic human blood mononuclear cells. In these mice total and peanut-specific IgE levels are present after engraftment of mononuclear cells from patients with peanut allergy, and peanut-specific anaphylaxis is induced by the release of mediators after challenge with peanut extract. When treated with a single administration of a serotype rh.10 AAV vector coding for omalizumab, there is persistent prevention of peanut-induced severe allergy, either when administered as prophylaxis before peanut sensitization or as therapy after the mice exhibit the peanut-induced anaphylaxis-related symptomatology.

METHODS

Humanized murine model

NSG mice (6-8 weeks old) were housed in microisolator cages and maintained on peanut-free chow, according to standard guidelines. All food and water was autoclaved. Mice were bred as pairs (1 female with 1 male) or trios (2 females with 1 male); only female mice were used in the allergy experiments because female mice have been found to be more susceptible to anaphylaxis than male mice.¹⁸

All experiments using human cells and sera were approved by the institutional review boards at Weill Cornell Medical College and Yale New Haven Hospital, and written informed consent was obtained from each subject enrolled in the study. Heparinized blood was obtained from donors with allergy to peanut or nonallergic healthy control subjects (see Table E1 in this article's Online Repository at www.jacionline.org). Specific sensitization was documented by detection of allergen-specific IgE in the sera of donors (ImmunoCAP specific IgE blood test; Phadia AB, Uppsala, Sweden). Blood mononuclear cells were isolated from heparinized blood by using Ficoll-Paque density centrifugation (Ficoll-Paque Plus; Sigma-Aldrich, St Louis, Mo). The absence of detectable human IgG in mouse sera was assessed by means of ELISA before reconstitution with human cells.

Cells isolated by using the Ficoll-Paque method were administered to 6- to 8-week-old NSG mice. Each animal received intraperitoneally 3×10^7 blood mononuclear cells in RPMI (Sigma-Aldrich) mixed with 100 μg of crude peanut extract in a total volume of 200 μL of 0.9% sodium chloride split in 2 separate injection sites (100 μL each).

Protein extracts from roasted unsalted peanuts (*Arachis hypogaea*; Hampton Farms, Severn, NC) were made on the same day of administration to mice by mixing 25 g of ground peanut with 250 mL of 20 mmol/L Tris buffer (pH 7.2). After 2 hours at 23°C, the aqueous fraction was collected and subsequently centrifuged to remove residual traces of fat and insoluble particles.¹⁹ Protein concentrations were determined by using Bradford analysis with BSA as a standard. The presence of expected peanut allergens in the peanut extract preparation was confirmed by using SDS-PAGE (see Fig E2 in this article's Online Repository at www.jacionline.org). Mice were sensitized once weekly at weeks 0 to 4 with 100 μg of crude peanut extract through intraperitoneal injections. Mice were then challenged at weeks

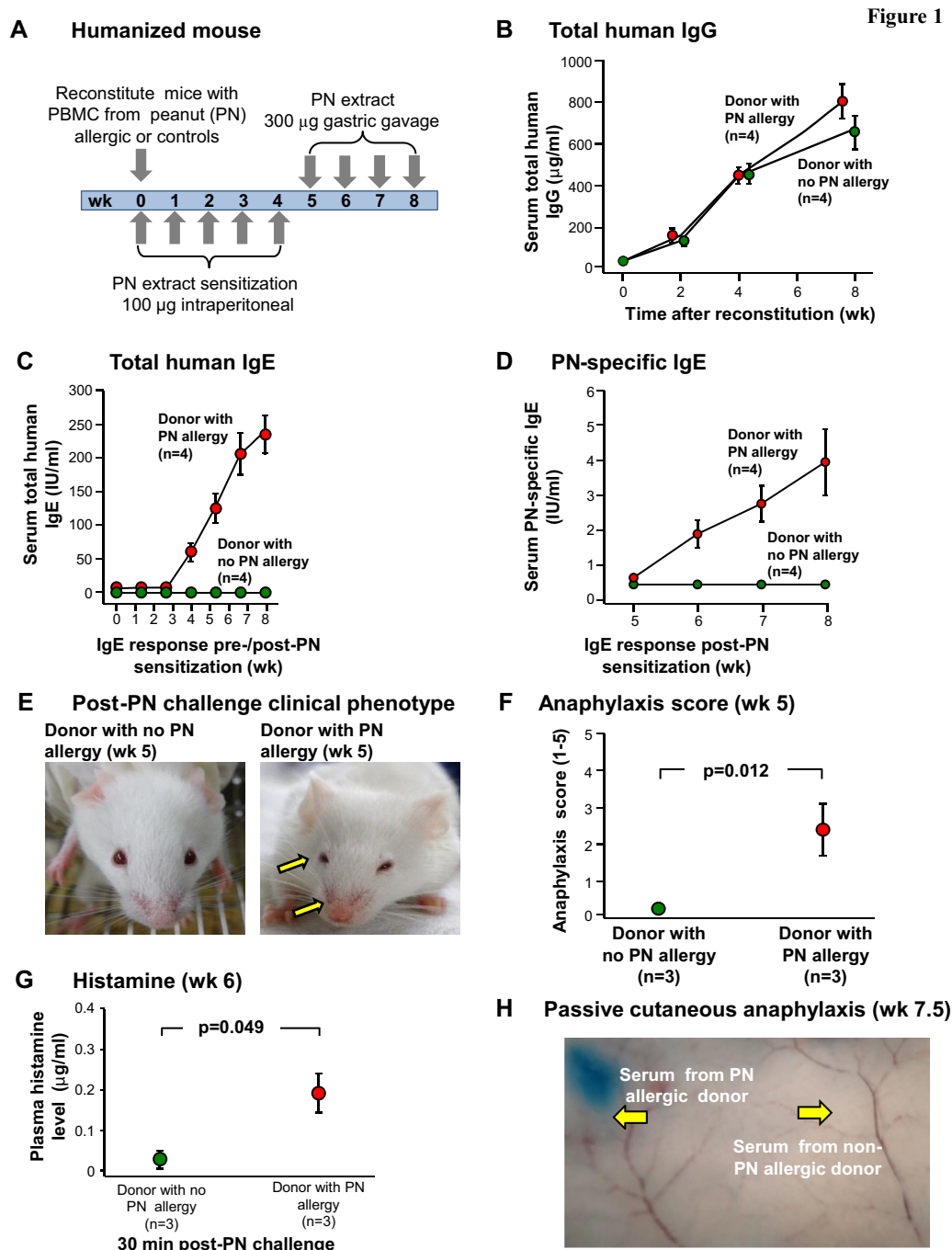


FIG 1. Characterization of NSG mice reconstituted with human blood mononuclear cells from a donor with peanut (PN) allergy and a control donor. The example shown is with reconstitution from a donor with peanut allergy (PN01) compared with the control donor (C01; see Table E1). **A**, Timeline of reconstitution, peanut sensitization, and challenge, with interventions indicated by arrows. **B**, Human IgG levels (ELISA). **C**, Total human IgE levels (ELISA). **D**, Peanut-specific IgE (ELISA). **E**, Examples of appearance of mice after peanut extract challenge at week 5. *Left*, Mouse reconstituted with mononuclear cells from a nonallergic donor appears normal after peanut challenge. *Right*, Mouse reconstituted with mononuclear cells from a donor with peanut allergy displaying puffiness around the eyes/snout, ears erect, and itching/ruffling of fur 1 minute after peanut challenge. **F**, Anaphylaxis score (1-5) 30 minutes after challenge at week 5: mouse donor with no peanut allergy and mouse donor with peanut allergy. **G**, Plasma histamine levels 30 minutes after peanut challenge at week 6: mouse donor with no peanut allergy and mouse donor with peanut allergy. In Fig 1, **B-D**, **F**, **G**, all data are expressed as means $\pm$ SEMs. **H**, PCA. Shown is extravasation of blue dye using human serum from a patient with peanut allergy and serum from a subject without peanut allergy.

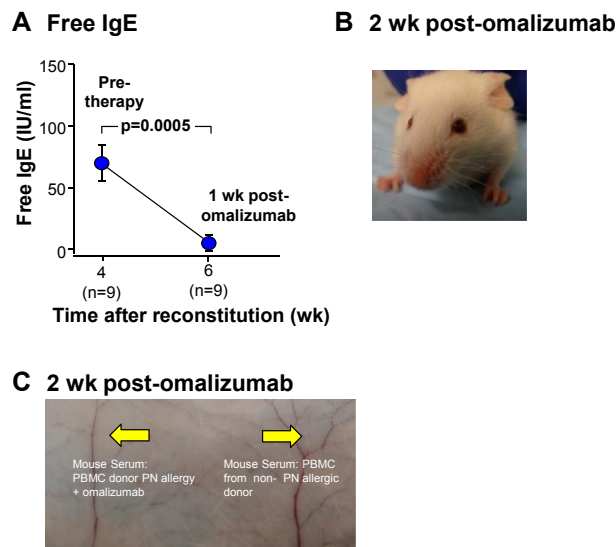


FIG 2. Treatment of peanut (PN) antigen-induced anaphylaxis with omalizumab after sensitization and peanut challenge. The examples shown are from donors PN01 and PN02. **A**, Free IgE levels quantified by means of ELISA 1 week before and 1 week after therapy with omalizumab (250 μ g). Data are expressed as means $\pm$ SEMs. **B**, Mice after peanut extract challenge. Shown are data 2 weeks after therapy. The mouse treated with omalizumab appeared normal after peanut challenge (compare with Fig 1, E). **C**, Omalizumab-mediated suppression of PCA. Omalizumab blocked peanut-induced, peanut-specific IgE-mediated PCA (compare with Fig 1, H).

5 to 10 through intragastric gavage with 300 μ g of crude peanut extract using a curved 20-gauge needle (Kent Scientific, Torrington, Conn) and observed for up to 4 hours after the procedure for signs of an allergic reaction (see Table E2 in this article's Online Repository at www.jacionline.org). All mice were fasted for 8 hours before peanut challenge to maximize absorption of peanut antigen across the gastric mucosa. The mice were assessed for human IgG, total human IgE, total mouse IgE, mouse IgG, peanut-specific human IgE, free human IgE, plasma histamine, anaphylactic symptoms, passive cutaneous anaphylaxis (PCA), mouse locomotor activity, and histology (to assess human graft vs mouse host reactions; see the Methods section in this article's Online Repository at www.jacionline.org).

As further evidence that peanut-induced anaphylaxis in the reconstituted NSG mice was mediated by human IgE and to compare single omalizumab with single AAVrh.10anti-hIgE administration (see below), a subset of reconstituted mice were treated a single time with 250 μ g of omalizumab (Xolair), a dose based on a per-weight basis as used in other murine studies.²⁰ The mice were evaluated before therapy and 1 week after therapy for IgE, and 2 weeks after therapy for physical assessment of the mice for signs of anaphylaxis and PCA.

Therapy with AAVrh.10anti-hIgE

AAVrh.10anti-hIgE and control AAVrh.10antiNic vector (referred to as AAVrh.10IgGcontrol)²¹ are based on the nonhuman primate-derived rh.10 capsid pseudotyped with AAV2-inverted terminal repeats surrounding the anti-hIgE antibody expression cassette (see the Methods section in this article's Online Repository). The ability of the AAVrh.10anti-hIgE vector to direct expression of anti-hIgE *in vitro* and *in vivo* is detailed in the Methods section in this article's Online Repository.

To test whether pretreatment (prophylactic therapy) with AAVrh.10anti-hIgE would protect mice with peanut allergy, NSG mice (6–8 weeks old) were treated with AAVrh.10anti-hIgE (10^{11} genome copies) or AAVrh.10IgGcontrol (10^{11} genome copies) at week -3 and then reconstituted with human donor blood mononuclear cells at week 0. The

mice were subsequently challenged with peanut extract. From 2 weeks after vector injection, anti-hIgE antibody levels were evaluated every 2 weeks. Mice were sensitized (weeks 0–4) and challenged with crude peanut extract (weeks 5–8). Human IgG levels were evaluated at weeks 2, 4, and 8. Human IgE, peanut-specific IgE, and free IgE levels were evaluated at week 4. An anaphylaxis score was evaluated 30 minutes after each peanut challenge. In addition, locomotor activity was evaluated at week 6, histamine levels at week 7, and PCA at week 7.5.

To determine whether treatment with AAVrh.10anti-hIgE could protect mice with peanut allergy after the mice had been sensitized with peanut extract and exhibited peanut extract-induced allergic reactions, NSG mice were reconstituted with human donor blood mononuclear cells at week 0 and then sensitized and challenged with crude peanut extract at weeks 0 to 4 and 5 to 10, respectively. After signs of anaphylaxis associated with peanut challenge (week 5), mice were administered AAVrh.10anti-hIgE (10^{11} genome copies), AAVrh.10IgGcontrol (10^{11} genome copies), or the humanized anti-IgE mAb omalizumab (250 μ g) in 200 μ L of 0.9% NaCl. Anti-hIgE antibody levels were evaluated at fixed intervals (every 2 weeks). Human IgG levels were evaluated at weeks 2, 4, and 8. Human IgE levels were evaluated at weeks 4 and 6, free IgE levels at weeks 4 and 6, and peanut-specific IgE levels at week 4. An anaphylaxis score was evaluated 30 minutes after each peanut challenge at weeks 7 and 10 (see Table E2). Locomotor activity was evaluated at weeks 7 and 10, histamine levels at weeks 6 and 9, and PCA at weeks 7 and 10.

Survival

Mice exhibiting clinical signs of allergy after peanut sensitization were observed for 24 hours after peanut challenge and daily thereafter for signs of deterioration in health. If deemed moribund (severe shaking, labored breathing, wheezing, cyanosis, and no activity after prodding; all without recovery after 1–2 hours after peanut challenge), mice were killed, and the date of death was recorded.

Statistics

See the Methods section in this article's Online Repository for details regarding statistics.

RESULTS

Humanized murine model of peanut allergy

Immunodeficient NSG mice humanized by means of intraperitoneal administration of blood mononuclear cells from patients with peanut allergy with a clinical history of anaphylaxis (Fig 1, A) had immune and clinical phenotypes that mimic human peanut allergy (Fig 1, B–G). As expected, mice reconstituted with mononuclear cells from either a donor with peanut allergy or a donor without peanut allergy had increasing levels of human IgG after reconstitution (Fig 1, B). In contrast, after peanut sensitization, only the mice reconstituted with mononuclear cells from patients with peanut allergy expressed total human IgE and peanut-specific IgE (Fig 1, C and D). Importantly, mice reconstituted with blood mononuclear cells from a donor with peanut allergy showed a clinical phenotype associated with an allergic response. Mice displayed puffiness around the eyes and snout, pilar erecti, itching/ruffling of fur, and decreased ambulation and respiratory rate (Fig 1, E), with an anaphylaxis score of 2 ± 1 (Fig 1, F). These clinical characteristics were not observed in the animals that received blood mononuclear cells from a nonallergic subject. Histamine levels in mice reconstituted with blood mononuclear cells from a donor with peanut allergy and then challenged with crude peanut extract displayed increased levels of histamine after peanut challenge when

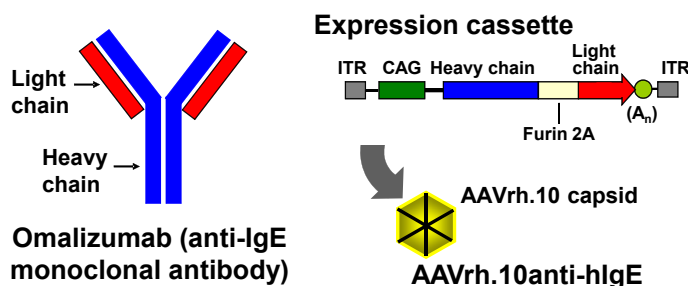
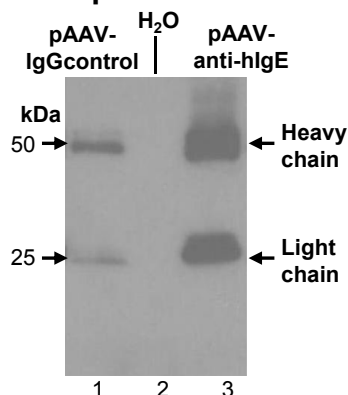
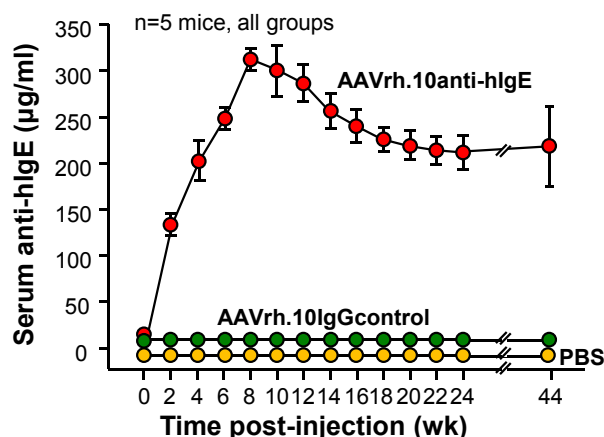
A AAVrh.10anti-hlgE**B *In vitro* directed expression****C NOD-scid IL2Rgamma^{null}**

FIG 3. Characterization of AAVrh.10anti-hlgE. **A**, Schematic with CMV enhancer/chicken β -actin (CAG) promoter, heavy and light chains of the anti-hlgE mAb omalizumab, furin 2A cleavage site, and polyadenylation signal. **B**, *In vitro* pAAV-anti-hlgE directed expression of omalizumab in 293HEK cells. **C**, Persistence of expression of the anti-hlgE antibody over time after a single intravenous administration of AAVrh.10anti-hlgE (10^{11} genome copies) to NSG mice. AAVrh.10IgGcontrol (10^{11} genome copies) and PBS were controls. Values are presented as means $\pm$ SEMs.

compared with those in mice reconstituted with blood mononuclear cells from a nonatopic donor without peanut allergy (Fig 1, G).

Two approaches were used to confirm that IgE was responsible for the induction of peanut hypersensitivity: PCA testing and response to activation of omalizumab. For PCA, human serum from a patient with peanut allergy induced a peanut-specific IgE-mediated PCA reaction in the mice with peanut allergy with extravasation of blue dye of greater than 3 mm, whereas PBS and serum from a donor without peanut allergy induced no visible dye extravasation. As an additional test of the model, the reconstituted mice were sensitized and then challenged with peanut extract (300 μ g once weekly by means of oral gavage). Mice were treated with omalizumab after the first sign of anaphylactic symptoms 1 week after peanut challenge. Free IgE levels were significantly lower ($P = .0005$) 1 week after omalizumab administration compared with levels 1 week before therapy (Fig 2, A). The omalizumab-treated mice appeared normal after peanut challenge 2 weeks after omalizumab therapy (compare Fig 2, B, to Fig 1, E). Finally, omalizumab blocked peanut-induced, peanut-specific IgE-mediated PCA similar to what was observed with serum from a donor without peanut allergy, which induced no dye extravasation (Fig 2, C).

Graft-versus-host defense

As has been previously described in humanized models of immunodeficient mice reconstituted with human mononuclear cells,^{22,23} inflammatory and immune infiltrates of human cells were present in both the lungs and small intestines in humanized mice 6 to 7 weeks after reconstitution with mononuclear cells from both allergic and nonallergic donors. The infiltrates included diagnostic features of graft-versus-host disease (GVHD) in the lung, including lymphohistiocytic, plasmacytic, and neutrophilic perivascularitis and peribronchiolitis; diffuse interstitial neutrophilia; and multifocal bronchiolar intraepithelial eosinophilic inclusions. The small intestine also showed histologic features consistent with GVHD, including eosinophilic, neutrophilic, and lymphocytic infiltrates (see Table E3 in this article's Online Repository at www.jacionline.org). As a consequence of GVHD, the model was limited to approximately 10 weeks after reconstitution with the human mononuclear cells.

AAVrh.10anti-hlgE

The expression cassette of the AAVrh.10anti-hlgE construct (Fig 3, A) was evaluated for vector expression *in vitro* by means of transfection of the expression plasmid into HEK293T cells.

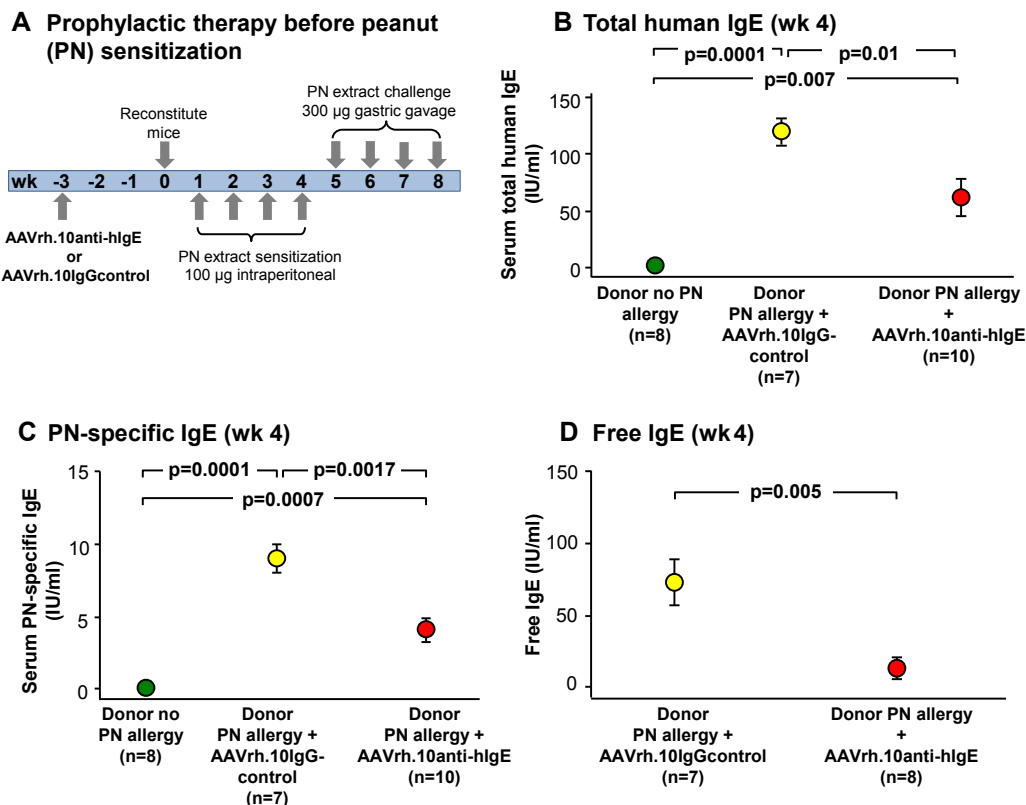


FIG 4. Prophylaxis of peanut (PN) antigen-induced anaphylaxis by treatment with AAVrh.10anti-hlgE before sensitization. **A**, Prophylaxis timeline of reconstitution, peanut sensitization, and challenge, with interventions indicated by arrows. The examples shown are from donors PN01 and PN03 with peanut allergy and control donor C01 (see Table E1). **B**, Total human IgE evaluated after completion of peanut sensitization protocol (week 4). **C**, Total peanut specific IgE after completion of peanut sensitization protocol (week 4). **D**, Free IgE levels evaluated by using ELISA at week 4. Data in Fig 4, B-D, are expressed as means $\pm$ SEMs.

Seventy-two hours after transfection, both heavy and light chains of the anti-hlgE antibody were detected in cell-culture supernatants (Fig 3, B). *In vivo* expression of human anti-IgE after AAVrh.10anti-hlgE gene transfer was demonstrated in NSG mice after a single intravenous administration of the vector; levels of greater than 200 µg/mL were achieved in NSG female mice for at least 44 weeks, the duration of the experiment. No anti-human IgE was detected in serum from mice that received intravenous AAVrh.10IgGcontrol or PBS (Fig 3, C).

Prophylactic therapy

NSG mice were treated with AAVrh.10anti-hlgE or the control vector before reconstitution with human cells and before peanut sensitization and challenge to assess the effects that a single prophylactic administration of AAVrh.10anti-hlgE would have on the allergic response to peanuts (Fig 4, A). All mice were successfully reconstituted with mononuclear cells, as measured by persistent levels of human IgG after reconstitution, and mouse IgE and IgG levels were confirmed to be absent (not shown). After blood mononuclear cell transfer and sensitization with peanut extract, total human IgE, peanut-specific IgE, and free IgE levels were induced (Fig 4, B-D). An IgE response developed only when the specific allergen was administered to NSG mice reconstituted with blood mononuclear cells from a donor with peanut allergy.

Mice treated with AAVrh.10anti-hlgE had significantly lower levels of total and peanut-specific IgE from week 4 after reconstitution onward when compared with mice treated with AAVrh.10IgGcontrol ($P = .01$ and $P = .0017$, respectively; Fig 4, B and C). Importantly, free IgE levels were significantly lower in AAVrh.10anti-hlgE-treated mice compared with the AAVrh.10IgGcontrol-treated mice ($P = .005$; Fig 4, D). Anaphylactic responses were evaluated 30 minutes after intragastric challenge with peanut extract. Strikingly, AAVrh.10anti-hlgE-treated mice 6 to 7.5 weeks after reconstitution displayed a far less severe allergic phenotype compared with those that received the control vector, as defined by clinical phenotype, improved locomotor activity, a lower anaphylaxis score, decreased histamine release, and decreased PCA (Fig 5, A-D).

Therapy after sensitization

Although AAVrh.10anti-hlgE prophylaxis before sensitization suppressed the peanut-induced allergic phenotype, a more realistic scenario relevant to human subjects would be treatment after peanut sensitization and peanut-induced symptoms have been observed. NSG mice were treated with an AAVrh.10anti-hlgE or a control vector after reconstitution, peanut challenge, and peanut allergy symptomatology to assess the effects that a

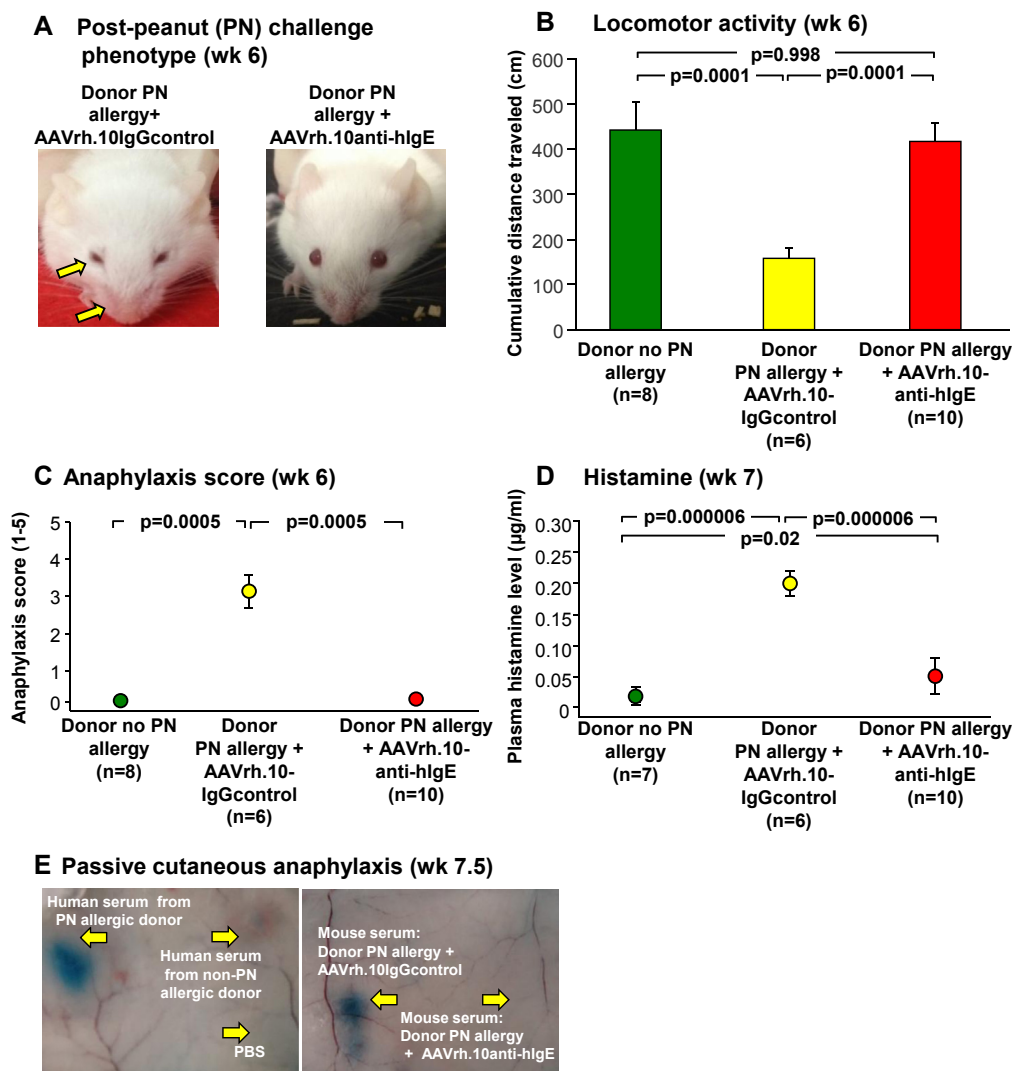


FIG 5. Prophylaxis of peanut (PN) antigen-induced anaphylaxis by prior treatment with AAVrh.10anti-hlgE. **A**, Mice after peanut extract challenge at week 6. *Left*, Mouse treated with a control vector at week -3 displayed puffiness around the eyes/snout and pilar erecti, itching/ruffling of fur, and decreased ambulation and respiratory rate after peanut challenge. *Right*, Mouse treated with AAVrh.10anti-hlgE at week -3 appeared normal after peanut challenge. **B**, Locomotor activity. Shown are data of the distance traversed over 30 minutes after peanut challenge in vector- and control-treated mice assessed at week 6. **C**, Anaphylaxis score 30 minutes after peanut challenge at week 6. **D**, Plasma histamine levels 30 minutes after peanut challenge at week 7. Data in Fig 5, *B-D*, are expressed as means $\pm$ SEMs. **E**, AAVrh.10anti-hlgE-mediated suppression of PCA. *Left*, Peanut extract-induced PCA mediated by peanut-specific IgE from the serum of a donor with peanut allergy. *Right*, Peanut extract induced PCA-mediated by peanut-specific IgE from the pooled serum of the humanized NSG mice with peanut allergy reconstituted by using the same donor as in the *left panel* but treated prophylactically with AAVrh.10anti-hlgE at week -3 before sensitization. The sera from the AAVrh.10anti-hlgE-treated mice blocked peanut-induced, peanut-specific IgE-mediated PCA compared with the AAVrh.10IgGcontrol.

single therapeutic administration of AAVrh.10anti-hlgE would have on the allergic response to peanuts after the sensitivity and allergic phenotype has been established. NSG mice were successfully reconstituted with mononuclear cells, as measured based on persistent levels of human IgG after reconstitution. Sensitized NSG mice were then treated with an AAVrh.10anti-hlgE or a control vector after the first sign of peanut-induced anaphylaxis (week 5; Fig 6, *A*). Mice that received blood mononuclear cells from a donor with normal IgE levels that did not have a clinical history of atopy and were administered peanut extract served as control animals. After blood mononuclear cell

transfer and sensitization with peanut extract, an IgE response developed only when the specific allergen was injected in the group of NSG mice reconstituted with blood mononuclear cells from a donor with peanut allergy and was sustained after peanut challenge (Fig 6, *B*). All mice had peanut-specific IgE after completing peanut sensitization at week 4, 1 week before therapy. There was no difference in peanut-specific serum IgE levels between groups ($P = .17$; Fig 6, *C*). Free IgE levels in the AAVrh.10anti-hlgE-treated mice were significantly reduced 1 week after therapy (week 6) compared with the AAVrh.10IgG-control mice ($P = .0006$ and $P = .16$, respectively; Fig 6, *D*).

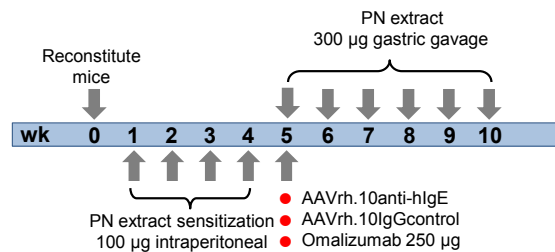
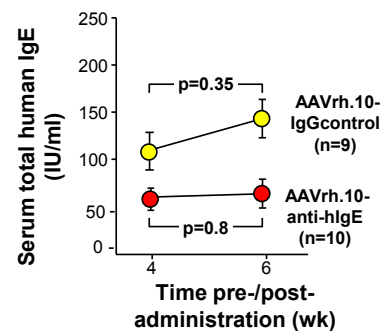
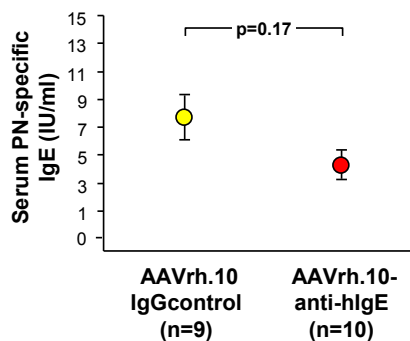
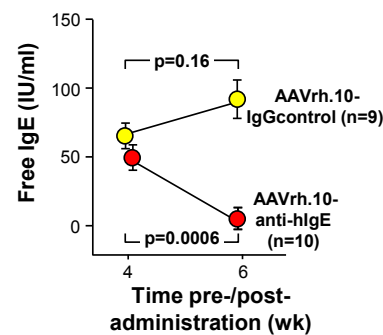
A Treatment after peanut (PN) sensitization**B Total human IgE****C Peanut-specific IgE (wk 4)****D Free IgE**

FIG 6. Treatment of peanut (PN) antigen-induced anaphylaxis with AAVrh.10-anti-hIgE or omalizumab after mice exhibited peanut-specific allergic symptoms after sensitization and peanut challenge. **A**, Therapeutic timeline of reconstitution, peanut sensitization, and challenge, with interventions indicated by arrows. Examples shown in Fig 6, **B-D**, are from donors PN01 and PN02. **B**, Total human IgE evaluated by means of ELISA (week 4, 1 week before therapy; week 6, 1 week after therapy). **C**, Total peanut-specific IgE evaluated by means of ELISA at week 4, 1 week before therapy. **D**, Free IgE evaluated by means of ELISA at week 4, 1 week before therapy, and week 6, 1 week after therapy. Data in Fig 6, **B-D**, are expressed as means $\pm$ SEMs.

At week 10, 5 weeks after therapy with AAVrh.10anti-hIgE, the mice with peanut allergy had no clinical signs of peanut-induced allergy, whereas mice with peanut allergy treated with a single administration of omalizumab 5 weeks previously had puffiness around the eyes and snout, pilar erecti, itching, and ruffling of fur (Fig 7, A). At week 7, 2 weeks after therapy, both AAVrh.10anti-hIgE- and omalizumab-treated mice were significantly more ambulatory than the control mice (Fig 7, B, left), but at week 10, 5 weeks after therapy, the AAVrh.10anti-hIgE mice were significantly more ambulatory than the single administration of omalizumab-treated mice ($P = .0001$; Fig 7, B, right; by week 10, 89% of the control mice had died). Consistent with the clinical phenotype and ambulatory data, the anaphylaxis score at 7 weeks (2 weeks after therapy) was significantly lower for both the AAVrh.10anti-hIgE- and omalizumab-treated mice compared with the control mice, but at 10 weeks (5 weeks after therapy), only the AAVrh.10anti-hIgE therapy continued to be efficacious, with the omalizumab-treated mice similar to the control mice at week 7 (by week 10, 89% of the control mice had died; Fig 7, C). The same observations were made for plasma histamine levels (Fig 7, D) and PCA (Fig 7, E).

The most striking observation was analysis of survival. Forty days after therapy, only mice that received AAVrh.10anti-hIgE were protected against death (Fig 8). Ninety percent of

AAVrh.10anti-hIgE-treated mice (9/10) survived until 40 days after therapy (the last time point assessed), whereas 70% (7/10) of single administration of omalizumab-treated mice died and 89% (8/9) of AAVrh.10IgGcontrol mice died over the same time period.

DISCUSSION

Peanut allergy, one of the most common food allergies, can generate fatal or near-fatal episodes if not immediately treated.^{3,24} The number of children in the United States given a diagnosis of peanut allergy more than doubled between 1997 and 2007,⁹ and it is estimated that more than 50% of patients with peanut allergy will experience an accidental exposure to peanut over a 2-year period.^{4,8} At present, strict avoidance of peanuts and ready access to self-injectable epinephrine is the cornerstone of management for peanut allergy.^{3,25,26}

IgE plays an important role in mediating type 1 hypersensitivity in response to antigens in human subjects,^{10,12} and studies involving patients with high-risk peanut allergy treated with a humanized IgG₁ mAb directed against IgE facilitated rapid oral desensitization and qualitatively improved the desensitization process.^{2,8,14,15} The main limiting factors of current anti-IgE

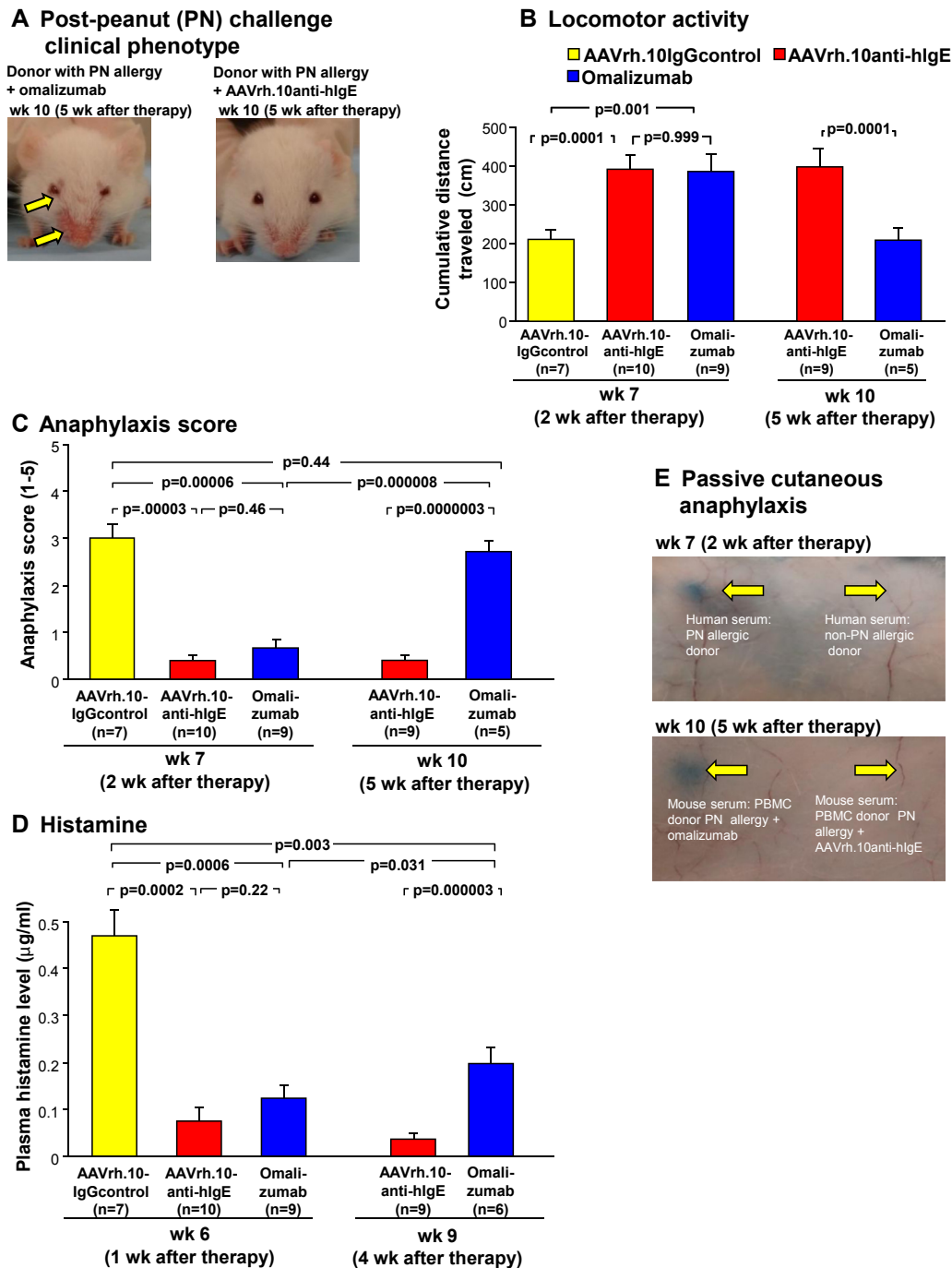


FIG 7. Treatment of peanut (PN) antigen-induced anaphylaxis with AAVrh.10-anti-hlgE compared with omalizumab after sensitization and peanut challenge. **A**, Mice after peanut extract challenge. Shown are data for week 10, 5 weeks after therapy. *Left*, Mouse treated with omalizumab (week 5) displayed puffiness around the eyes/snout and pilar erect, itching/ruffling of fur, and decreased ambulation and respiratory rate after peanut challenge (compare with Fig 2, B: mouse 2 weeks after treatment with omalizumab). *Right*, Mouse treated 5 weeks previously with AAVrh.10anti-hlgE appeared normal after peanut challenge. **B**, Locomotor activity. Shown are data of the distance traversed over 30 minutes after peanut challenge for week 7, 2 weeks after therapy, and week 10, 5 weeks after therapy. **C**, Anaphylaxis score (1-5) 30 minutes after peanut challenge. Shown are data for week 7, 2 weeks after therapy, and week 10, 5 weeks after therapy. **D**, Plasma histamine levels 30 minutes after peanut challenge. Shown are data for week 6, 1 week after therapy, and week 9, 4 weeks after therapy. Only 1 AAVrh.10IgG control-treated mouse was alive at weeks 9 and 10, and therefore statistical analysis was not included. Data for Fig 7, B-D, expressed as means $\pm$ SEMs. **E**, AAVrh.10anti-hlgE-mediated suppression of PCA. *Upper panel*, Peanut extract-induced PCA mediated by peanut-specific IgE from serum of a donor with peanut allergy but not from a nonallergic control donor. *Lower panel*, Week 10, 5 weeks after therapy. Persistent expression of AAVrh.10anti-hlgE blocks extravasation of dye, whereas a 1-time injection of omalizumab 5 weeks previously was no longer protective. Omalizumab blocked peanut-induced, peanut-specific IgE-mediated PCA 2 weeks after omalizumab therapy (Fig 2, C).

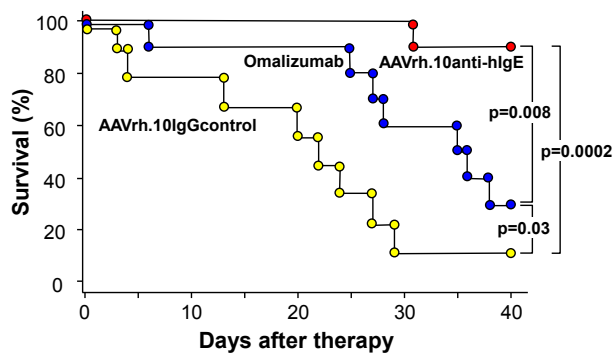


FIG 8. Mouse survival after treatment with a single administration of AAVrh.10anti-hIgE, single administration of omalizumab alone, or control vector. Survival duration and treatment type are shown days after therapy.

therapy are the inconvenience and high cost of frequent subcutaneous administration of an mAb.^{13,15,27}

To circumvent the requirement of receiving monthly administration of the anti-hIgE monoclonal antibody, the economic burden of this repeated treatment, and the associated complications with repeated administration, we developed a novel AAV-mediated gene therapy strategy to deliver persistent therapeutic serum levels of omalizumab using a single administration of a gene transfer vector coding for anti-hIgE. By using a humanized immunodeficient murine model of peanut allergy that, once sensitized with peanut extract, mimics human peanut-induced anaphylaxis, the data demonstrate that prophylactic AAV-mediated anti-hIgE therapy before sensitization, as well as treatment after the peanut-induced anaphylaxis has manifested, markedly reduces the peanut-induced phenotype. Importantly, the data demonstrate that a single administration protects a humanized murine model of peanut allergy from peanut-induced anaphylaxis after the severe allergy has manifested. In contrast, after a single administration of omalizumab, survival 5 weeks after therapy is markedly less.

To our knowledge, our study is the first report to demonstrate a gene therapy platform for severe human-related allergy. This study proposes a novel approach to treat peanut allergy that could change the clinical approach for patients with clinically significant peanut allergy. Because the anti-hIgE therapy is not targeted against a specific allergen, this therapy could be applied as treatment for a spectrum of severe allergies and other atopic disorders, including asthma.

AAV vectors

AAV vectors are widely used for gene transfer, with a high safety record in clinical trials. There are several features that make AAV vectors attractive for persistent anti-hIgE therapy, including their relatively low immunogenicity, ability to efficiently transduce nondividing cells, and capability to direct sustained long-term transgene expression after *in vivo* delivery.^{28,29} With delivery of the AAV vector through the intravenous route, transduction of the vector occurs mainly in the liver.²⁹⁻³¹ Intravenous delivery of the AAVrh.10anti-hIgE vector mediated long-term expression of omalizumab after a single administration. Thus, in a clinical setting AAVrh.10anti-hIgE therapy could offer long-term protection to allergic patients after a single administration of the vector.

Humanized models of peanut allergy

Significant challenges to the development of food allergy models are the lack of a relevant animal model for the high risk of anaphylaxis in human subjects, the tendency of animals to have immunologic tolerance to ingested antigens, and antigen-induced anaphylaxis in the mouse being mediated primarily through IgG.¹⁶ In the present study we established and characterized a humanized peanut-sensitive NSG mouse model that reproduces a robust allergic response that mimics the human allergic cascade at both the molecular and phenotypic levels. Peanut exposure was necessary to trigger IgE production, even when mononuclear cells from subjects with high IgE levels were transferred. Induction of allergen-specific IgE was always paralleled by high total IgE titers, and enhancement of total IgE was required with both allergen sensitization and challenge. The phenotype of the murine model was defined with an anaphylaxis score, histamine levels, ambulatory activity, clinical phenotype, and PCA.

Therapy with AAVrh.10anti-hIgE

This study demonstrates that a 1-time therapy with AAVrh.10anti-hIgE reduced serum IgE levels, free IgE levels, and plasma histamine levels and abrogated the clinical response associated with peanut-induced anaphylaxis. Therapy with AAVrh.10anti-hIgE was effective in both a prophylactic mouse model with treatment when the immune system is still naive with respect to peanut antigen and in a therapeutic model in which the immune system has already committed to an anaphylactic pathway. The data demonstrate the potent and consistent efficacy of AAVrh.10anti-hIgE therapy on peanut allergy, as shown by locomotor activity, anaphylaxis scores, histamine release, and PCA data. Peanut-specific IgE levels demonstrated therapeutic benefits and clinical reactivity to peanut in the prophylactic model, suggesting that the total amount of serum peanut-specific IgE is a sensitive biomarker for therapeutic effect in this model. The protection against peanut-induced anaphylaxis in both models coupled to the long-term gene expression studies suggests that AAVrh.10anti-hIgE has a long-lasting protective effect and indicates that protection can be achieved against allergen challenge by a single dose, an advantage of the delivery system.

There are limitations to our study that will require further assessment as AAV-mediated gene therapy is translated to the clinic. First, the humanized mouse model has a maximum of a 10-week window of analysis because of the development of xenographic GVHD.^{22,23} Therefore long-term effectiveness of the vector, as well as long-term study of the host immune response, particularly the development of neutralizing anti-AAV antibodies, cannot be assessed. However, by week 5, although single-dose therapy with AAVrh.10anti-hIgE was effective in anaphylaxis protection, single-dose therapy with omalizumab was not.

Second, although AAVrh.10anti-hIgE therapy is effective in the humanized mouse model, translation of this therapy to the clinic has challenges. Adverse side effects have been reported with omalizumab therapy, including anaphylaxis, cardiovascular and cerebrovascular events, and malignancy, and there are reports of associated risks of low IgE levels and autoimmunity or increased risk for parasitic disease.³²⁻³⁷ The overall incidence of adverse events reported has been similar across studies between subjects receiving omalizumab versus placebo, and the majority of treatment-related adverse events, mostly

cardiovascular and cerebrovascular, and risk of parasitic infections were mild or moderate in severity in treated subjects. To date, there is no evidence that omalizumab enhances the risk of anaphylactic reactions, immune complex diseases, or malignancy,^{35,38-40} and studies support that omalizumab has demonstrated efficacy, with a well-established favorable safety and clinical profile.

We thank N. Mohamed for editorial assistance and Sebastien Monette for assistance with the analysis of histologic specimens.

Key messages

- A novel humanized murine model of peanut allergy was created by reconstituting NSG mice with mononuclear cells derived from patients with peanut allergy, which reproduces the allergic phenotype at both the molecular and phenotypic levels.
- A single administration of a nonhuman primate serotype rh.10 AAV expressing omalizumab (AAVrh.10anti-hlgE) provides long-term protection from peanut allergen challenge in the mouse with peanut allergy.
- If this strategy can be translated to human subjects, it would represent a paradigm shift for the treatment of patients with clinically significant peanut allergy.

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METHODS

Assessment of mice

Human IgG, total human IgE, total mouse IgE, and mouse IgG levels were evaluated in mouse sera by means of ELISA (Abcam, Cambridge, Mass). Peanut allergen-specific human IgE levels were measured by using an ELISA kit (Biopanda, Dundonald, United Kingdom) and a free human IgE kit (Eaglebio, Nashua, NH), according to the manufacturer's instructions. Plasma histamine levels were assessed 30 minutes after challenge with crude peanut extract and analyzed with an enzyme immunoassay kit (Abnova, Walnut, Calif), as described by the manufacturer (detection limit, 0.2 ng/mL). Symptoms of anaphylaxis were evaluated 30 minutes after challenge with a mouse defined anaphylaxis score (see Table E2).^{E1-E3} Scoring was performed in a blind manner by 3 independent investigators, and the results were averaged.

To assess PCA, abdomens of naive wild-type mice were shaved, and 24 hour later, animals received a 50- μ L intradermal injection of pooled sera from NSG mice that were reconstituted either with blood mononuclear cells from a patient with peanut allergy or from a donor with no history of peanut allergy and had been sensitized with peanut extract, as described above. Sera from donors with peanut allergy were used as a positive control, and PBS was used as a negative control. Twenty-four hours after intradermal injection, mice were injected intravenously with a mixture of 100 μ L of 0.5% Evans blue dye and 100 μ g of crude peanut extract. Thirty minutes after injection, mice were killed, the skin of the abdomen was inverted, and reactions were examined based on a visible blue color. A reaction was scored as positive if bluing of the skin at the injection sites was greater than 3 mm in diameter in any direction.

To evaluate post-peanut challenge behavior, we recorded mouse locomotor behavior with infrared beam-equipped activity open-field chambers (20 $\times$ 20 cm chamber; AccuScan Instruments, Columbus, Ohio) to track motions in both horizontal (X and Y) and vertical (Z) directions. Mice were allowed to habituate to the box before each test. Mice were placed in the box 30 minutes after challenge with crude peanut extract by means of oral gavage. Locomotor activity was collected as the cumulative distance travelled (in centimeters) in 30 minutes.

One limitation inherent in the model is that reconstitution of NSG mice with human mononuclear cells leads to human graft versus mouse host reaction, resulting in organ infiltration with human immune cells.^{E4,E5} At 6 to 7 weeks after therapy, mice were killed by means of CO₂ inhalation to assess this. The lungs and small intestines were removed and placed in formalin for paraffin sections. Staining was performed with hematoxylin and eosin (Sigma-Aldrich, St Louis, Mo). Slides were analyzed with an Olympus BX40 microscope (Olympus, Center Valley, Pa) using the ColorView Soft Imaging system camera and software. Evaluation of the lung and small intestine was done in a blind fashion by using semiquantitative assessment (0 to 4+) of inflammatory infiltrates.

AAVrh.10 vectors

The expression cassette consists of the cytomegalovirus enhancer chicken β -actin promoter, the anti-hIgE monoclonal heavy and light chain cDNA sequence, and the rabbit β -globin polyadenylation signal. The full-length heavy and light chain amino acid sequences from the humanized anti-IgE antibody were back-translated by using human preferred codons, and sequences were optimized for improved mRNA stability and protein expression. Immunoglobulin heavy and Ig κ secretion signals were added to the heavy and light chains, respectively. Heavy and light chains were cloned in the same open reading frame by using a *Thosea asigna* virus 2A cleavable sequence downstream of a furin cleavage recognition site. Both antibody chains were expressed from the same open reading frame in an equimolar ratio.

The optimized full-length anti-hIgE cDNA sequence was synthesized and cloned into the pAAV plasmid under control of the cytomegalovirus enhancer chicken β -actin promoter. The AAVrh.10anti-hIgE vector was produced by cotransfection into human embryonic kidney 293T cells (HEK 293T; American Type Culture Collection, Manassas, Va) of the pAAV plasmid together with a plasmid carrying the AAV Rep proteins derived from AAV2 needed for vector replication; the AAVrh.10 viral structural proteins VP1,

VP2, and VVP3, which define the serotype of the produced AAV vector; and the adenovirus helper functions of E2, E4, and VA RNA. The AAVrh.10anti-hIgE vector was purified by means of iodixanol gradient and QHP anion exchange chromatography, as previously described.^{E6} Vector genome titers were determined by using quantitative TaqMan real-time PCR analysis. A vector coding for an irrelevant antibody directed against nicotine, AAVantiNic (referred to as AAVrh.10IgGcontrol) was used as a control for the *in vivo* studies.^{E7}

Assessment of AAVrh.10anti-hIgE expression

To assess AAVrh.10anti-hIgE-directed expression of the mAb *in vitro*, we transfected HEK 293T cells with the pAAVanti-hIgE plasmid or a pAAV control plasmid coding for an unrelated human antibody control,^{E7} and the supernatant was harvested 72 hours later. Anti-hIgE antibody expression in supernatants was evaluated by means of Coomassie blue stain SDS-PAGE and Western blot analysis with peroxidase-conjugated goat anti-human kappa light chain antibody and peroxidase-conjugated goat anti-human IgG antibody and enhanced chemiluminescence substrate (Bio-Rad Laboratories, Hercules, Calif).

For *in vivo* studies, all animal studies were conducted under protocols reviewed and approved by the Weill Cornell Institutional Animal Care and Use Committee. Female NSG mice, 4 to 6 weeks old (Jackson Laboratories, Bar Harbor, Me), were housed under specific pathogen-free conditions. At 6 to 8 weeks of age, the mice received AAVrh.10anti-hIgE or AAVrh.10IgGcontrol at 10¹¹ genome copies by means of intravenous injection in a 100- μ L volume.

Blood (100 μ L) from the tail vein was assessed at time 0 and at various time points until 24 weeks. The blood samples were allowed to clot for 1 hour at 23°C, followed by 30 minutes at 4°C, and then spun at 1800g for 20 minutes to collect serum. The concentration of anti-hIgE antibody was then determined by means of ELISA. Wells of flat-bottomed 96-well EIA/RIA plates (Corning, Corning, NY) were coated with 0.2 μ g of human IgE in 100 μ L of carbonate buffer (pH 9.6) overnight at 4°C and then washed with 0.05% Tween 20 in PBS (PBS-Tween) and blocked with 5% dry milk in PBS for 60 minutes at 23°C. Serial dilutions of sera were added to wells and incubated for 60 minutes at 23°C. The plates were washed 3 times with PBS-Tween and 100 μ L of 1:2000 diluted mouse anti-human IgG conjugated to horseradish peroxidase (Abcam) in 1% dry milk in PBS incubated for 60 minutes at 23°C. After 4 wash steps, peroxidase substrate (100 μ L/well, Bio-Rad Laboratories) was added to each well and incubated for 15 minutes at 23°C, and the reaction was stopped with addition of 2% oxalic acid (100 μ L/well). Absorbance was measured at 415 nm.

Anti-hIgE antibody titers were calculated by means of interpolation of the log (OD)–log (dilution) with a cutoff value equal to twice the absorbance of background and converted to micrograms per milliliter based on a standard curve with omalizumab antibody (Xolair, Genentech Inc, and Novartis Pharmaceuticals Corporation) quantified by using the Pierce BCA Protein Assay Kit (Life Technologies, Grand Island, NY).

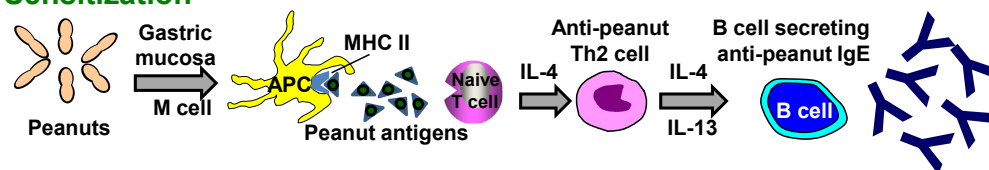
Statistics

All data are presented as means $\pm$ SEMs, unless otherwise stated; the *n* value for each group is stated in the figures. Differences between groups were analyzed by using the 2-tailed Student *t* test. In experiments comparing the different prophylactic and therapeutic treatment groups, each group contained mice reconstituted with blood mononuclear cells from 2 different donors. For analyses of human IgE, peanut-specific IgE, free IgE, and histamine levels, these levels were adjusted for the effects of donor. This was done by using a regression procedure in which human IgE, peanut-specific IgE, free IgE, or histamine levels were regressed on the donor by using dummy variables, residuals from these regressions were added to the grand mean, and the data were presented as donor-adjusted values. Differences of locomotor activity between groups were analyzed by using the Kolmogorov-Smirnov test. For symptom score data, differences between groups were analyzed by using the Kruskal-Wallis test. Survival curves were estimated by using the Kaplan-Meier method, and differences between survival curves were assessed with the Peto and Peto modification of the Gehan-Wilcoxon test (using the R package “survival”)^{E8} because we did not assume proportional hazards. *P* values of less than .05 were considered significant for all comparisons.

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Sensitization



Exposure to peanuts



Therapy with anti-IgE

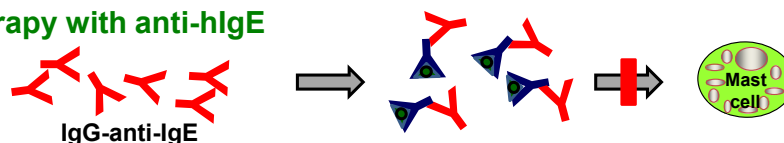


FIG E1. Major cellular interactions during the mucosal allergic immune response and therapy with anti-IgE. *Sensitization:* In the gastric mucosa peanut allergens are taken up by M cells and transferred to antigen-presenting cells (APC), where they are processed into peptide fragments. Complexes of peanut-allergen peptide and major histocompatibility complex (MHC II) are presented to naive T cells, which differentiate into T_H2 cells. Activated T_H2 cells recognize peanut allergen peptide–MHC complexes on the surfaces of B cells, releasing the cytokines IL-4 and IL-13, which promote IgE antibody production by B cells. Secreted IgE antibodies bind to FcεRI receptors on effector cells, such as mast cells, which become sensitized. *Exposure to peanuts:* On secondary encounter with the same peanut allergen, the allergen crosslinks cell-bound IgE, activating mast cells to release inflammatory mediators. Additional production of IL-4 and IL-13 by mast cells and basophils results in further T_H2 cell differentiation and IgE synthesis. This cascade of events ultimately leads to induction of symptoms commonly associated with peanut allergy. *Therapy with anti-IgE:* Anti-IgE binds to the third constant domain of the IgE heavy chain (Cε3), the same site at which IgE normally binds to both high- and low-affinity IgE receptors on mast cells, basophils, and other cell types. Anti-IgE forms complexes with free IgE and prevents its interaction with these receptors.

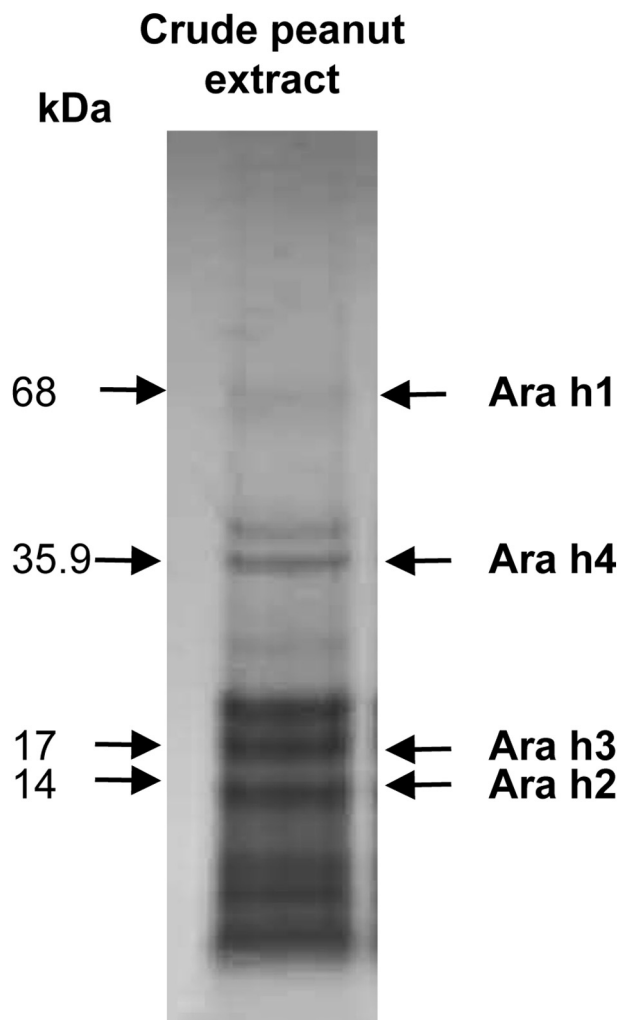


FIG E2. SDS-PAGE of peanut allergens. Arrows indicate the known peanut extract proteins Ara h1, Ara h2, Ara h3, and Ara h4.^{E9}

TABLE E1. Characteristics of human donors for NSG mouse reconstitution*

Donor phenotype	Study ID	History of peanut-induced anaphylaxis	Total IgE (kU/L)	Antigen-specific IgE (kU/L)
Patient with peanut allergy	PN01	Yes	616	35.0
	PN02	Yes	31	2.5
	PN03	Yes	10	18.5
Nonallergic control subject	C01	No	≤7	<0.1

*Allergy status and clinical history were assessed in all donors.

TABLE E2. Mouse anaphylaxis score*

Score	Symptom
0	No signs of shock
1	Itching/ruffling fur
2	Puffiness around eyes/mouth Diarrhea, pilar erecti, decreased activity Decreased respiratory rate
3	Wheezing/labored breathing Cyanosis
4	No activity (after prodding)
5	Death

*Severity of the anaphylactic response was scored 30 minutes after peanut challenge by using a scoring system from 0 (no signs of shock) to 5 (death) in all mice.^{E1-E3}

TABLE E3. Histologic assessment of the lungs and small intestines in NSG immunodeficient mice 6 to 7 weeks after reconstitution

NSG mice*	Lung				Small intestine			
	Lymphocytic	Plasmacytic	Neutrophilic	Eosinophilic	Lymphocytic	Plasmacytic	Neutrophilic	Eosinophilic
Control†	0	0	0	0	0	0	0	0
Nonallergic	+++	+++	+++	++	++	0	0	0
With peanut allergy	+++	+++	+++	++	++	0	++	++

+, Minimal; ++, mild; +++, moderate; +++++, marked.

*n = 1, control; n = 2, nonallergic; n = 2 peanut allergy.

†NSG immunodeficient mouse not reconstituted with mononuclear cells and not treated with vector.