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## Engineering of structural variants of the major peanut allergens Ara h 2 and Ara h 6 for allergen-specific immunotherapy



### To the Editor:

Emerging data from a phase 2 multicenter study confirm the utility of allergen-specific immunotherapy (AIT) with natural proteins to induce desensitization and tolerance to peanut.<sup>1</sup> Alternatively, hypoallergenic variants of major peanut allergens have been designed to increase the safety of AIT by reducing IgE-mediated side effects.<sup>2-5</sup> Hypoallergens have a reduced capacity to bind IgE while retaining their T-cell stimulatory ability and could be administered at higher doses and consequently increase the safety and efficacy of AIT. Two basic approaches to produce hypoallergens for peanut AIT have been reported: the chemical modification of the major peanut allergens Ara h 2 and 6<sup>2,3</sup> and the modification of Ara h 1-3 by site-directed mutagenesis.<sup>4,5</sup>

This study reports a novel concept for genetic engineering of hypoallergenic variants of Ara h 2 and Ara h 6, the 2 most frequently recognized and most potent peanut allergens. We generated modified variants of these allergens with preserved

T-cell stimulatory capacities and reduced IgE binding to maintain the desensitizing potential. Hypoallergenic variants were designed on the basis of *in silico* analyses of the known 3-dimensional structures and amino acid sequences of Ara h 2 (PBD: 3OB4) and Ara h 6 (PBD: 1W2Q) and taking into account linear IgE<sup>4,6</sup> and T-cell epitopes<sup>7</sup> (Fig 1, A; see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). For detailed material and methods, see this article's Methods section in the Online Repository at [www.jacionline.org](http://www.jacionline.org). Our analyses revealed that most of the known linear IgE epitopes were located in the unstructured loops of Ara h 2, whereas T-cell epitopes were mostly identified in the  $\alpha$ -helical core. Thus, the loops that form major parts of the antibody-accessible surface of these proteins were modified by changing the order of neighboring segments of the protein sequences. In each loop, the sequence was divided into short stretches of 5 to 10 amino acid residues. This caused modifications of sequential and conformational IgE epitopes. In contrast, cysteines involved in the formation of disulfide bonds,  $\alpha$ -helices, and short turns were left unchanged to preserve the protein core, which is essential for protein stability and contains most of the known T-cell epitopes.<sup>7</sup> The modified and the wild-type allergens were expressed as soluble proteins in *Escherichia coli* with structures typical for proteins dominated by  $\alpha$ -helices (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Sera from 34 well-characterized individuals with peanut allergy sensitized to Ara h 2 and Ara h 6 (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) were analyzed for IgE binding to the mutated variants by IgE ELISA (Fig 1, B; see Fig E3 and Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Mutated molecules showed significantly less IgE-binding capacities than did rAra h 2 wt ( $55\% \pm 22\%$  SD reduction;  $P < .001$ ), rAra h 6 wt ( $39\% \pm 23\%$  SD reduction;  $P < .001$ ), and purified nAra h 2 ( $61\% \pm 18\%$  SD reduction;  $P < .001$ ) and nAra h 6 ( $62\% \pm 19\%$  SD reduction;  $P < .001$ ). For more than 60% of the sera, IgE binding to the mutated allergens was reduced by 50% to 94% as compared with their natural counterparts. However, some patient's sera retained a high level of IgE reactivity to the modified allergens (IgE-binding reduction  $<50\%$ ; Fig 1, B; Table E2). The reduction in IgE binding was not associated with allergen-specific IgE levels ( $P = .8061$  for Ara h 2;  $P = .2672$  for Ara h 6). In addition, immunoblotting demonstrated a strong specific IgE reactivity to the purified natural and recombinant wild-type allergens, whereas IgE binding to the mutated proteins dropped by more than 50% (Fig E2, A and B). Notably, the IgE binding of the hypoallergens was also significantly reduced compared with the recombinant wild-type allergens, which clearly illustrated the effects of the introduced modifications. In line with reduced IgE binding, the modified variants of Ara h 2 and Ara h 6 displayed reduced basophil activation, suggesting their markedly reduced allergenic potency (Fig 1, C; see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). This was reflected by significantly lower percentages of activated basophils at Ara h 2 concentrations ranging from 1 pg/mL to 1 ng/mL ( $P < .01$ ), illustrating an average reduction of 3 logs. A similar trend, a 5- to 10-fold reduction, was observed for Ara h 6 mut using basophils from 3 donors with peanut allergy (Fig 1, C; Fig E4). This reduction in IgE binding and effector cell activation, achieved as a result of the modification of unstructured regions, was similar or higher to the ones achieved for Ara h 2 and Ara h 6 linearized

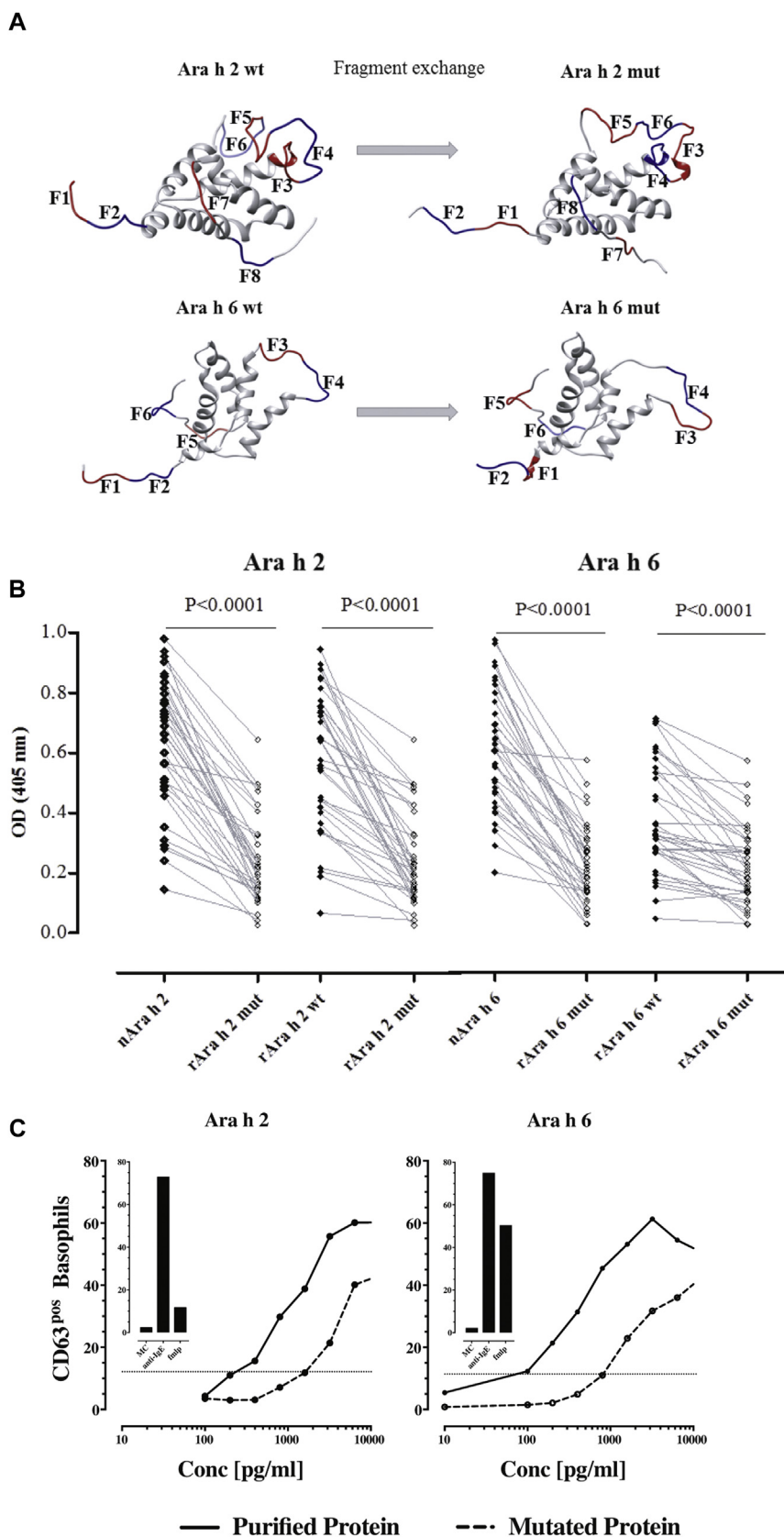


**FIG 1.** **A**, The order of fragments (in red and blue) in the loops of Ara h 2 (F1-F8) and Ara h 6 (F1-F6). **B**, Individual binding characteristics of IgE from 34 sera of patients with peanut allergy sensitized to Ara h 2 and Ara h 6 to the respective natural, recombinant wild-type, and the mutated proteins are shown. Data from individual patients are connected with lines (Wilcoxon signed rank test). **C**, Dose-dependent activation of basophils from donors with peanut allergy. One representative experiment of 9 for Ara h 2 and 1 of 3 for Ara h 6.

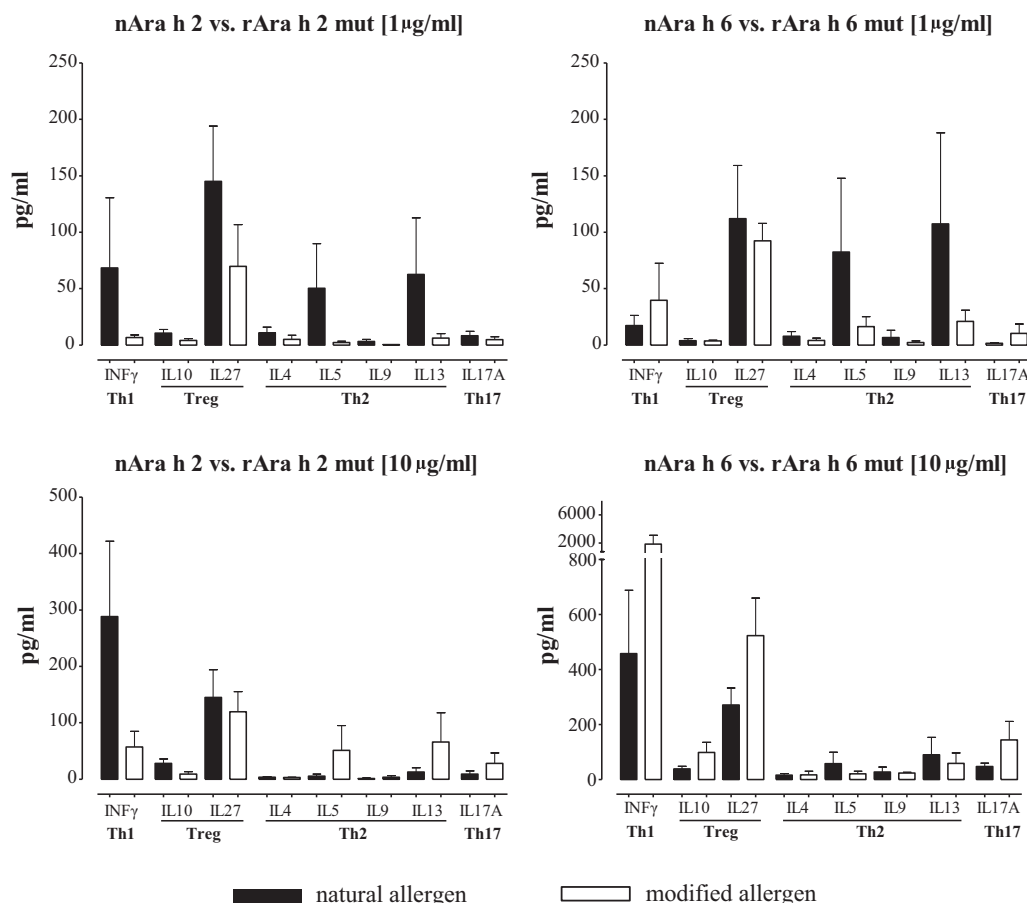


**FIG 2.** Allergen-specific cytokine production to wild-type and modified Ara h 2 and Ara h 6. Peripheral blood-derived mononuclear cells from subjects with peanut allergy ( $n = 5$ ) were stimulated with hypoallergenic variants of Ara h 2 and Ara h 6 or the corresponding wild-type controls (1 and 10 µg/mL). Cytokine production was measured via bead-based assay, and statistical analysis was performed using 2-way ANOVA with  $P$  value of less than .05 as the level of significance. *Treg*, Regulatory T.

by irreversible reduction of disulfide bonds by alkylation.<sup>2,3</sup> Notably, the ability of our modified proteins to activate basophils was not completely lost, which may be important for the early phase of oral immunotherapy when desensitization plays a central role.<sup>8</sup>

Indeed, modified Ara h 2 and 6 were fully capable to induce allergen-specific T-cell proliferation (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). At a concentration of 1 µg/mL, both mutated allergens showed T-cell stimulatory capacities comparable to those of their natural counterparts when using T cells from individuals with or without peanut allergy. The modified allergens induced a substantially lower type 2 cytokine response (especially for IL-5 and IL-13) than did the natural allergens (Fig 2). No evidence for the induction of a type 2 response in PBMCs from healthy controls was observed (see Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). At 10 µg/mL, the proliferation induced by Ara h 2 mut was significantly higher ( $P < .05$ ) than for the natural allergen (Fig E5). Low-stimulation indices for higher concentrations of natural Ara h 2 have been observed before.<sup>9</sup> The type 2 cytokine response induced by Ara h 2 mut at 10 µg/mL corresponded to those induced by 1 µg/mL natural Ara h 2 (Fig 2; eg, IL-5:  $50.32 \pm 88.1$  vs

$51.32 \pm 98.3$  pg/mL; IL-13:  $66.12 \pm 115.8$  vs  $62.66 \pm 112.1$  pg/mL). Mutated Ara h 6 did not induce an elevated type 2 response at both concentrations compared with the wild-type (Fig 2). IL-10 and IL-27, characteristic for regulatory T cells, were induced by both hypoallergens. These were desirable effects in concert with the observed T-cell stimulatory properties. In conclusion, these designer hypoallergens that display significantly reduced but not completely absent IgE binding and preserved T-cell activation capacity may provide valuable options for a safer immunotherapy of peanut allergy. The heterogeneity of individual responses to the modified allergens warrants caution and indicates that careful selection and characterization of patients must be performed before immunotherapy.

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## Novel locus for atopic dermatitis in African Americans and replication in European Americans



To the Editor:

Atopic dermatitis (AD) is a common chronic inflammatory skin disease that affects up to 30% of children and is highly heritable. To date, 31 genetic loci have been identified through genome-wide association studies (GWASs), mapping to genes involved in immune regulation and skin barrier deficiencies.<sup>1</sup> However,

despite the higher prevalence of AD in African Americans (AAs; 15.9% vs 9.7% in European ancestry),<sup>2</sup> this population has been underrepresented in GWASs and specific genetic risk factors remain elusive.

In this study, we report an AD GWAS in the largest AA sample studied to date. For case and control selection, we developed, validated, and implemented an AD phenotyping algorithm based on electronic health record data mining, in collaboration with the electronic Medical Records and Genomics (eMERGE) network. Cases were defined as subjects 2 months or older with either (1) 2 or more AD diagnoses (International Classification of Diseases, Ninth Revision [ICD-9] code 691.8), on separate calendar days, and 1 or more prescriptions of AD-related medications (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)); or (2) 3 or more AD diagnoses, on separate calendar days. Subjects with scabies, Wiscott-Aldrich syndrome, allergic purpura, ichthyosis congenita, and chromosomal anomalies were excluded. Controls were subjects with no ICD-9 code for AD, no ICD-9 codes for any skin condition, allergy, asthma, nutritional deficiencies, or skin cancer (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), and no relevant medications for AD (see Table E1).

The algorithm was validated internally at the Center for Applied Genomics (CAG) and also externally at the Marshfield Clinic, with positive predictive values over 92% for cases and controls at both sites (for details, see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Implementation of the algorithm at CAG accrued 12,345 AD cases and controls with imputed genotyping data (see Table E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), including 5,843 of AA ancestry (2,420 cases and 3,423 controls; see Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), which were selected for the analysis. GWASs on 11,743,754 variants in the AA sample did not result in any variants surpassing genome-wide significance; however, 150 variants at 49 loci showed nominal *P* values of less than  $10^{-5}$  that were followed-up in an independent AA sample from the SAGE II study. Meta-analysis of the 2 AA samples resulted in a genome-wide significant association at rs3811419 (C>T) (Allele C:  $\beta$  (SE) = 0.573 (0.098);  $P_{\text{META}} = 5.64 \times 10^{-9}$ ; MAF(T) = 3.7%;  $P_{\text{CAG}} = 1.48 \times 10^{-7}$ ;  $P_{\text{SAGEII}} = .011$ ) 1.5 kb upstream of *RORC* (Fig 1; see Table E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). This association also replicated in the 6502 AD cases (N = 510) and controls (N = 5998) of European ancestry that were identified using the same phenotyping algorithm ( $\beta$  (SE) = 0.405 (0.149);  $P = 6.61 \times 10^{-3}$ ; MAF = 11.6%).

We explored the potential cis-expression quantitative trait locus (eQTL) effects of rs3811419 (C) and 7 variants in linkage disequilibrium (LD) ( $r^2 > 0.8$ ) with it, in skin and blood, using HaploReg v4,<sup>3</sup> the National Center for Biotechnology Information (NCBI) Genotype-Tissue Expression version 6 data,<sup>4</sup> and studies reporting eQTL effects in those tissues (see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Seven of the 8 single nucleotide polymorphisms (SNPs) investigated showed eQTL effects on the expression of *THEM4* in blood (top effect for rs3811418 (G);  $P$  value =  $6.22 \times 10^{-14}$ ) and 5 showed effects in skin (top effect for rs11582525 (G);  $P$  value =  $1.50 \times 10^{-5}$ ) (see Table E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Ferreira et al<sup>5</sup> recently reported association at this locus in a large meta-analysis of allergic disease in individuals of European ancestry. Their top variant, rs11204896, was independent of rs3811419 and did not

## METHODS

### Design of hypoallergenic Ara h 2 and Ara h 6

Hypoallergenic variants of Ara h 2.0201 and Ara h 6.0101 were designed on the basis of an *in silico* analysis of the known 3-dimensional structures and amino acid sequences of Ara h 2 (PBD: 3OB4; UniProt: Q6PSU2) and Ara h 6 (PBD: 1W2Q; UniProt: Q647G9) as well as taking into account the knowledge of linear IgE-cell<sup>E1,E2</sup> and T-cell epitopes<sup>E3,E4</sup> of these 2 allergens (Fig 1, A; Fig E1). Helices, short turns (<10 amino acid residues), and cysteine residues remained unchanged. Long loops (with at least 10 amino acid residues) between the unchanged parts were mutated. In each loop, the sequence was split into short stretches of 5 to 10 amino acid residues in length. In loops that comprised 2 segments, the order of these segments was swapped. Hence, the N-terminus of the second segment was connected to the C-terminus of the preceding helix, the N-terminus of the first segment was connected to the C-terminus of the second segment, and the C-terminus of the first segment was connected to the N-terminus of the succeeding helix. In loops comprising more than 2 segments, the same procedure as outlined above was used but the order of the segments was altered (Fig 2). The changes were made to the sequences of the N-terminus (aa 1-10 of Ara h 2 and aa 2-12 of Ara h 6), the C-terminus (aa 131-145 of Ara h 2 and aa 111-123 of Ara h 6), and in the large loops (aa 35-74 of Ara h 2 and aa 35-48 of Ara h 6). Localization of the changes in the structures of Ara h 2 and Ara h 6 are shown in Fig 1. Nonstructured loops missing in the crystal structures were added using Modeller 9v3.<sup>E5</sup> Graphics were generated using UCSF Chimera.<sup>E6</sup>

### Production and purification of natural and recombinant allergens

Previously developed purification protocols were used for the preparation of natural (n) Ara h 2<sup>E7</sup> and Ara h 6.<sup>E8</sup> Recombinant (r) wild-type proteins Ara h 2 wt and Ara h 6 wt as well as mutated proteins rAra h 2 mut and rAra h 6 mut were expressed from *E coli* Rosetta-gami (DE3)pLysS. Expression constructs were generated by inserting synthetic codon-optimized DNA sequences into the *E coli* vectors pMW172HisN for rAra h 2 wt and rAra h 2 mut and pET32a(+) for rAra h 6 wt and rAra h 6 mut (Eurofins MWG Operon, Ebersberg, Germany). rAra h 6 wt and rAra h 6 mut expressed in the latter vector yielded fusion proteins with an N-terminal 6x His-tagged thioredoxin. For removal of the His-tagged thioredoxin, an oligonucleotide encoding a PreScission Protease (GE Healthcare, Little Chalfont, UK) cleavage site (5'-LeuGluValLeuPheGlnGlyPro-3') was inserted directly upstream of the Ara h 6 wt and Ara h 6 mut coding sequence between the NcoI and BglII restriction sites. For rAra h 2 wt and rAra h 2 mut expression, bacterial cells were grown in 1 L of 2XTy media containing 100 µg/mL ampicillin at 30°C, overnight. For rAra h 6 wt and rAra h 6 mut, optimal expression conditions were at 18°C for 72 hours. Soluble recombinant proteins were purified through a HiTrap HP nickel column (GE Healthcare Life Sciences, Freiburg, Germany). Final purification of rAra h 6 wt and rAra h 6 mut was obtained after cleavage of the thioredoxin by PreScission Protease, removal of thioredoxin using a HisTrap HP nickel column, and removal of PreScission Protease using a GSTrap 4B column (GE Healthcare Life Sciences).

Purity of the recombinant proteins was analyzed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining. The identity was confirmed by N-terminal sequencing, and the secondary structure was analyzed as described in the Online Repository. Bacterial endotoxins were removed from rAra h 2 mut using Phenyl Sepharose chromatography and from nAra h 2, nAra h 6, and rAra h 6 mut by treating proteins with Endotoxin Removal Beads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The content of endotoxins was determined by the Limulus Amebocyte Lysate assay (Lonza, Walkersville, Md).

### Patients' sera

Sera from 34 patients with clinically relevant, moderate-to-severe allergic reactions following the ingestion of peanuts and containing Ara h 2- and Ara h 6-specific IgE antibodies were included in this study (Table E1). Symptoms suggestive of allergy were classified into groups according to Sicherer and

Sampson.<sup>E9</sup> Sensitization patterns of the patients' sera were identified against peanut extract and the individual allergen components Ara h 1-3, 6, 8, and 9 by ImmunoCAP and ImmunoCAP ISAC as previously described.<sup>E10</sup> A negative control group consisted of 5 nonatopic subjects. The use of clinical data and serum samples for this study was approved by the respective institutional review boards (the Ethics Committee of the Medical University of Vienna, approval no. 848/2011; the Ethics Committee of the Medical University of Graz, approval no. 24-006 ex 11/12; and the Ethics Committee of Lower Austria, approval no. GSK-EK-4/143-2011), and signed informed consent was obtained from the patients or their legal representatives.

### SDS-PAGE and IgE immunoblotting

For immunoblot experiments, 50 µg of peanut extract or 2 µg of each purified protein per lane was subjected to denaturing 15% SDS-polyacrylamide gel electrophoresis. Proteins were then either stained with Coomassie Brilliant Blue or blotted onto nitrocellulose membranes. Membranes were blocked by incubation with 50 mM sodium phosphate buffer, pH 7.5, containing 3% (w/v) BSA, 0.5% (v/v) Tween 20. For visualization of IgE-binding proteins, blots were incubated overnight with a pool of 3 sera containing Ara h 2- and Ara h 6-specific IgE. Bound IgE antibodies were detected by incubating the strips with 1:20 diluted <sup>125</sup>I-labeled antihuman IgE (MALT Allergy System Isotope Reagent, IBL Hamburg, Germany).

### Circular dichroism spectroscopy

Circular dichroism spectra of natural and recombinant Ara h 2 and Ara h 6 molecules were recorded (from 260 to 190 nm) with a J-710 spectrometer (Jasco Corporation, Tokyo, Japan) using a 0.2-cm path length quartz cell. Samples (0.1 mg/mL) were prepared in 50 mM Na-phosphate buffer, pH 7, and measured at room temperature. To obtain mean spectra, the average of 4 accumulations was collected at 100 nm/min with a 2-second time constant, 0.5 nm resolution, and sensitivity of ±100 mdeg. Spectra are represented as mean molar circular dichroism (with respect to moles of amide bonds).

### IgE ELISA

Sera from 34 patients with peanut allergy and 5 donors without allergy were tested for the presence of IgE antibodies specific to purified natural Ara h 2 and Ara h 6, recombinant wild-type proteins rAra h 2 wt and Ara h 6 wt, as well as recombinant mutated proteins Ara h 2 mut and Ara h 6 mut. For ELISA, microtiter plates (Maxisorp; Nalge Nunc International, Roskilde, Denmark) were coated with 100 µL per well of individual allergens (1 µg/mL) in 50 mmol/L sodium carbonate buffer, pH 9.6, overnight at 4°C. After blocking with 200 µL of TBS containing 3% of BSA, appropriately diluted sera (100 µL per well) from patients with peanut allergy or donors without allergy were incubated in duplicates overnight at 4°C. Specific IgE was detected according to manufacturer's instructions by using an alkaline phosphatase-conjugated mouse antihuman IgE mAb (BD Pharmingen, San Jose, Calif), followed by color development with Sigma FAST p-nitrophenyl phosphate tablets (Sigma-Aldrich, St Louis, Mo) and measurement of the absorbance at 405 nm. OD values exceeding the mean negative control value by more than 3 SDs were considered positive. The Friedman test ( $\alpha = 0.05$ ) was performed to test whether the amount of IgE binding to each of the recombinants molecules differed significantly from that binding to their natural counterparts.

### Basophil activation assay

To assess the biological activity of the modified molecules on effector cell populations that contribute to acute allergic responses, basophil activation tests were performed. In brief, whole blood from patients with peanut allergy was exposed to stimulation buffer alone (MC), the positive controls (anti-FcεRI antibody, formyl-methionyl-leucyl-phenylalanine), and Ara h 2 (n = 9), Ara h 6 (n = 3), or the respective mutants thereof, and activation/degranulation was defined as CD63 expression on the surface of basophils gated as SSC<sup>low</sup>/CCR3<sup>+</sup> population. The assay was performed according to the manufacturer's instructions (FlowCAST, Buehlman, Schoenenbuch, Switzerland).

## T-cell proliferation and cytokine responses

PBMCs from 5 individuals with peanut allergy and 6 age- and sex-matched individuals without peanut allergy were cultured with 0.01, 0.1, 1, and 10  $\mu\text{g}/\text{mL}$  of the respective allergens or their mutated versions. Allergen-specific proliferation was assessed by the [ $^3\text{H}$ ]-thymidine incorporation assay as described previously.<sup>E11,E12</sup> On day 7, supernatants were collected and allergen-specific cytokine production was assessed for allergen and hypoallergen concentrations of 1 and 10  $\mu\text{g}/\text{mL}$  with a cytokine multiplex cytometric bead array (Millipore, Darmstadt, Germany). Statistical analysis was performed using 2-way ANOVA or Wilcoxon matched pairs test where appropriate with *P* value of less than .05 as the level of significance.

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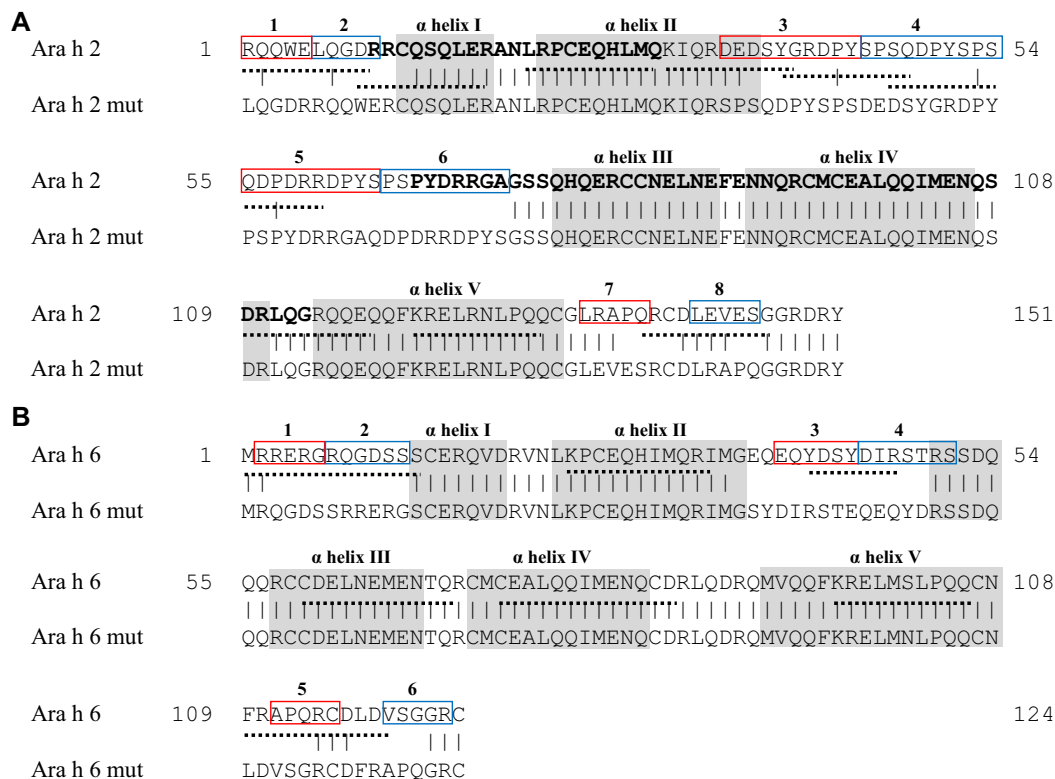


**FIG E1.** Amino acid sequence alignments. Amino acid sequence alignments of (A) Ara h 2 and Ara h 2 mut and (B) Ara h 6 and Ara h 6 mut. Sequences were colored according to secondary structure elements of Ara h 2 and Ara h 6:  $\alpha$  helices in gray, fragments of Ara h 2 and Ara h 6 that were swapped in red and blue, Ara h 2 and Ara h 6 IgE epitopes underlined with dotted lines, Ara h 2 T-cell epitopes in bold.

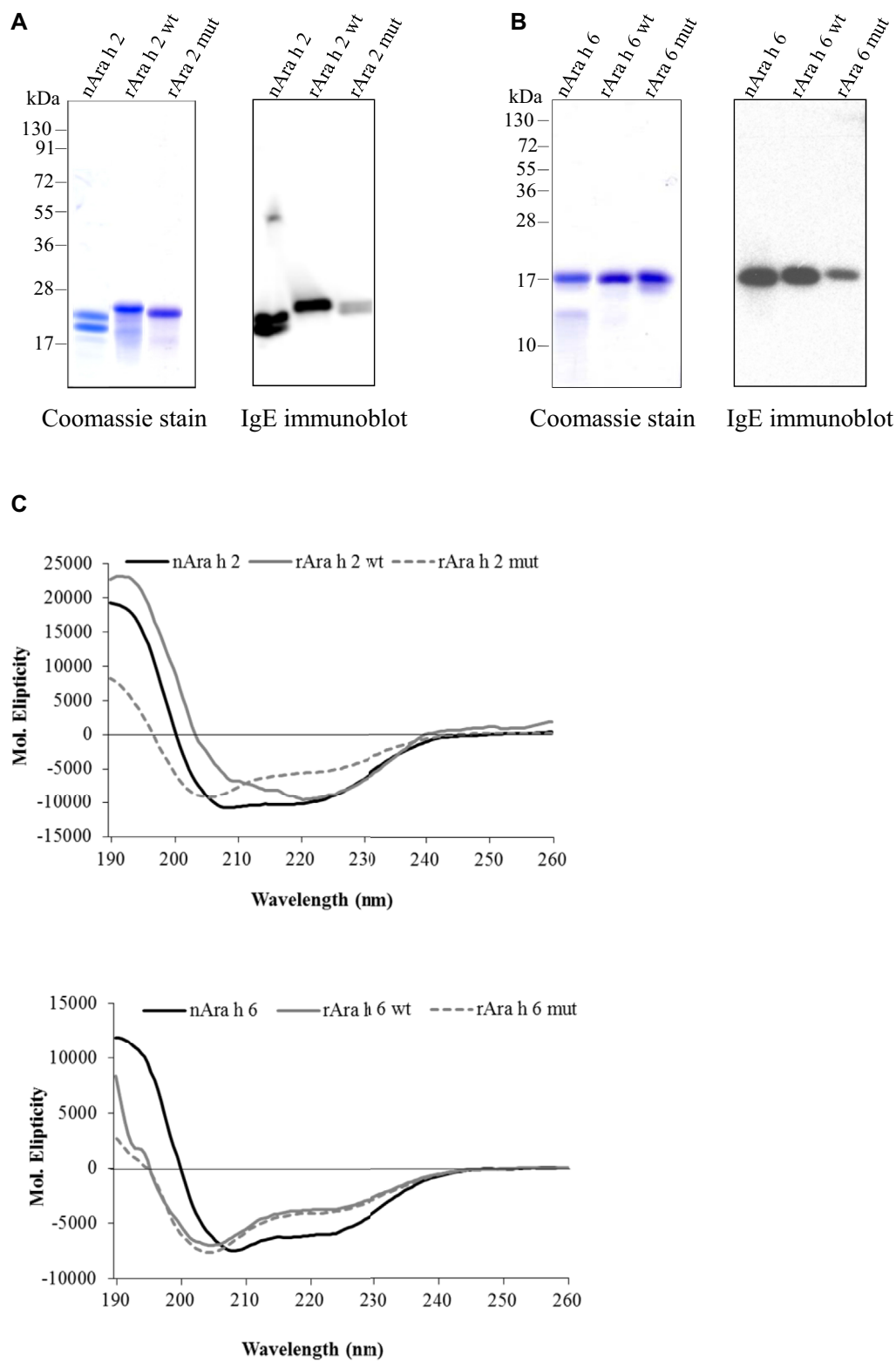


**FIG E2.** Coomassie stain and IgE immunoblot analysis of purified natural Ara h 2 (**A**) and Ara h 6 (**B**) and their recombinant wild-type and modified counterparts. Far ultraviolet circular dichroism spectra of natural, recombinant wild-type, and modified ([Fig E2, A](#)) Ara h 2 and ([Fig E2, B](#)) Ara h 6 molecules (**C**).

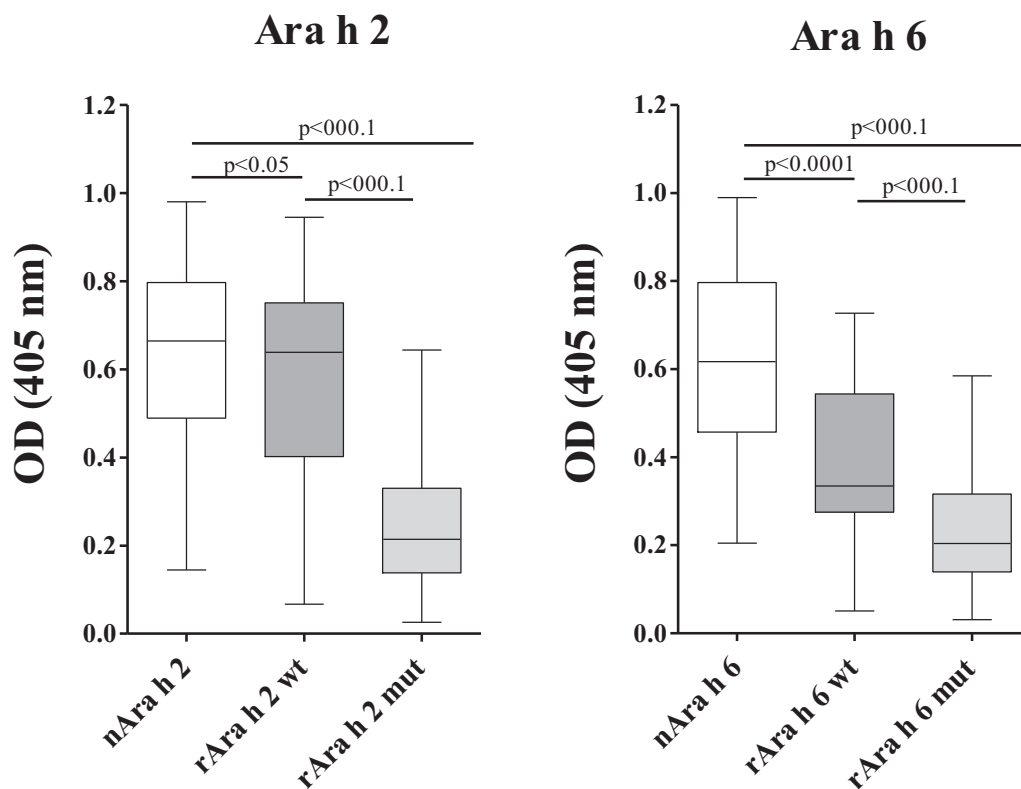


**FIG E3.** IgE binding of the hypoallergenic wild-type mutants of Ara h 2 and Ara h 6 is significantly reduced compared with their natural and recombinant wild-type counterparts. IgE binding from 34 sera of patients with peanut allergy sensitized to Ara h 2 and Ara h 6 to the natural and recombinant wild-type and mutated proteins assessed with ELISA.

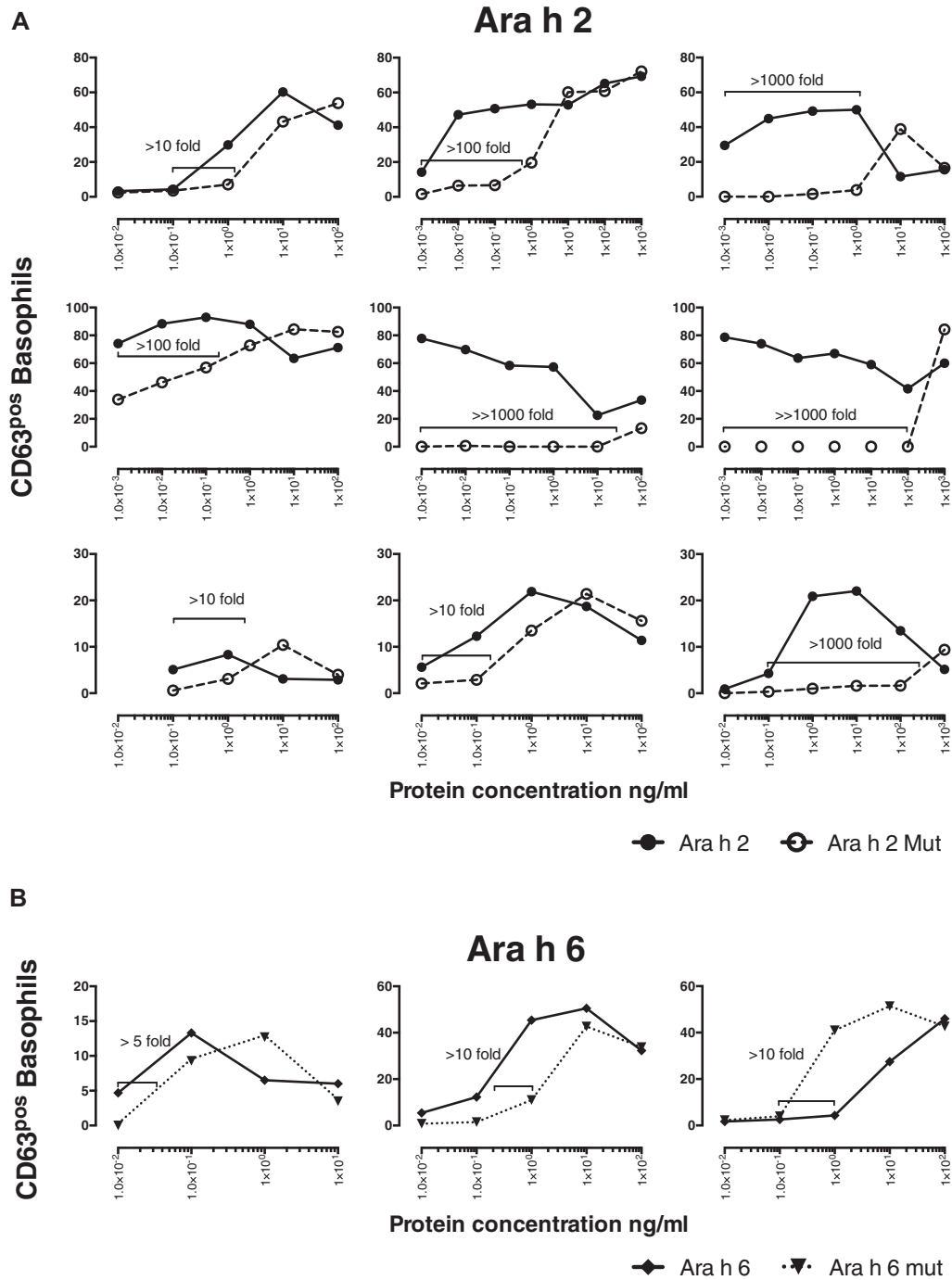


**FIG E4.** Dose-dependent basophil activation (percentage of CD63<sup>+</sup>) from individual patients with peanut allergy by the modified Ara h 2 and Ara h 6 compared with their natural counterparts.

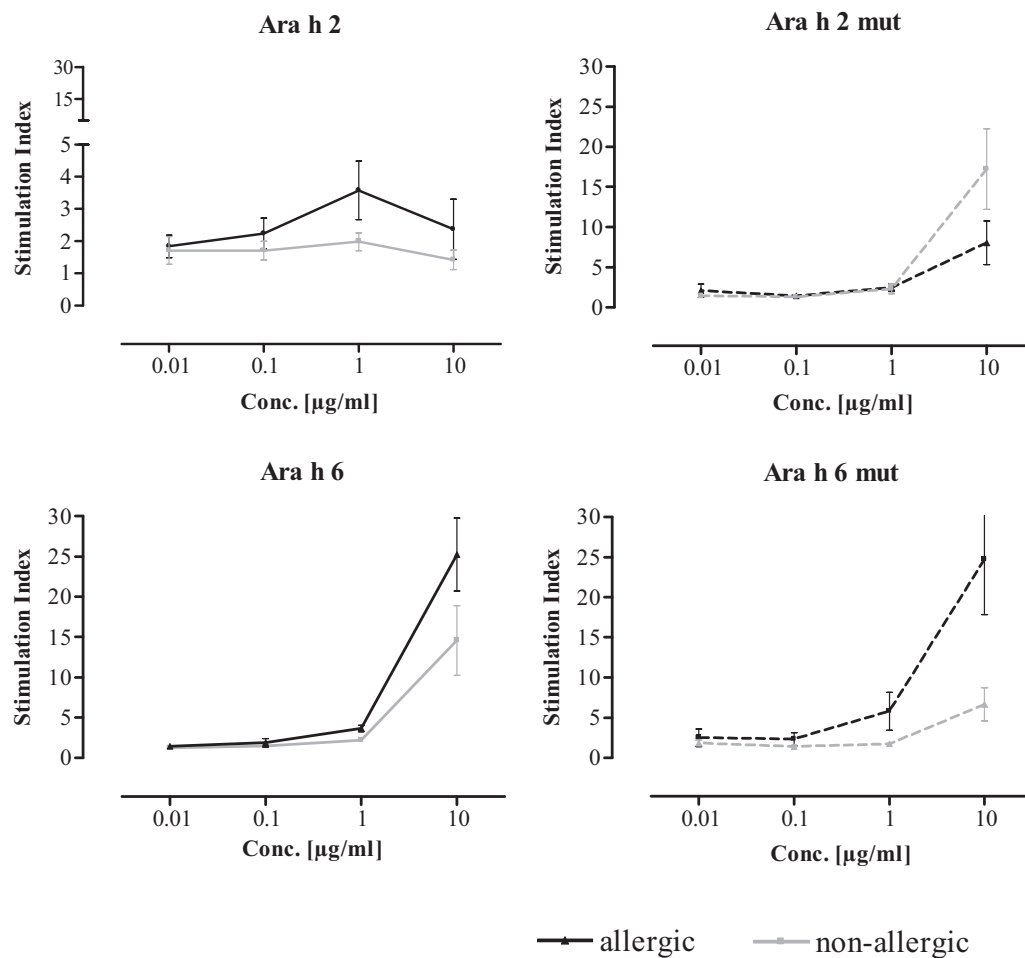


**FIG E5.** Hypoallergenic Ara h 2 and Ara h 6 retain T-cell stimulatory capacities. Peripheral blood-derived mononuclear cells from subjects with peanut allergy ( $n = 5$ ) and subjects without peanut allergy ( $n = 6$ ) were stimulated with the wild-type controls or the corresponding hypoallergenic variants of Ara h 2 and Ara h 6.

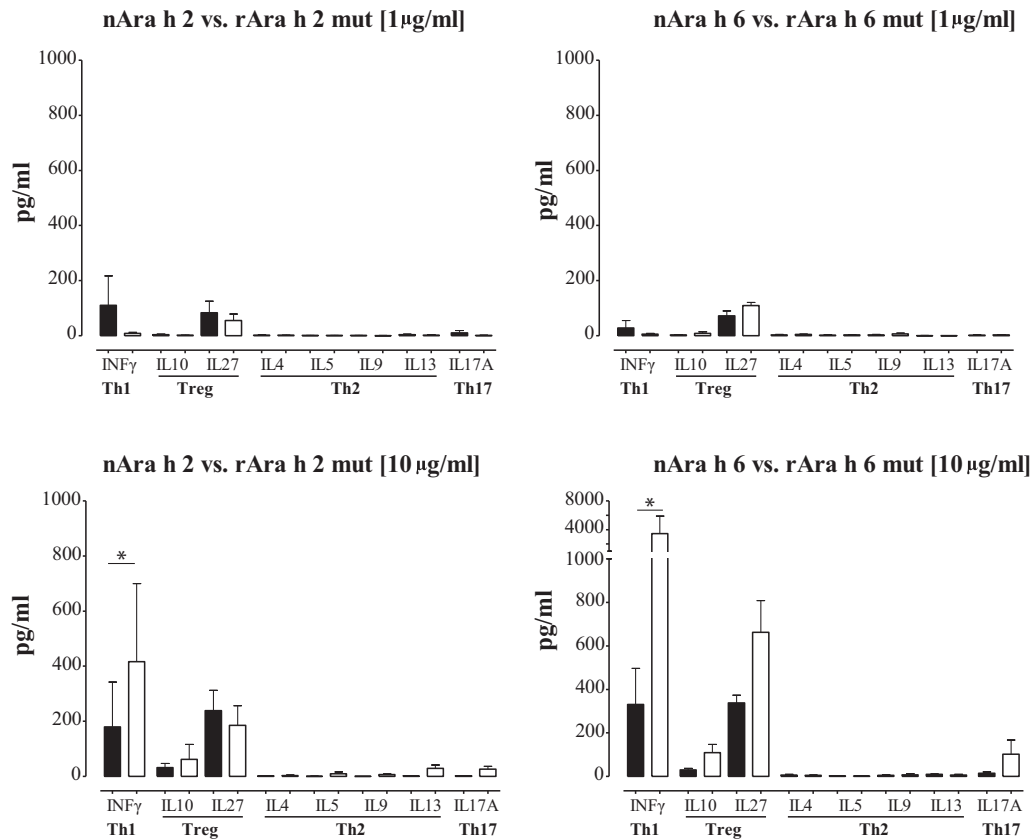


**FIG E6.** Allergen-specific cytokine production to wild-type and modified Ara h 2 and Ara h 6. Peripheral blood-derived mononuclear cells from subjects without allergy ( $n = 6$ ) were stimulated with the natural or the corresponding hypoallergenic variants of Ara h 2 and Ara h 6 (1 and 10  $\mu\text{g/mL}$ ). Cytokine production was measured via bead-based assay, and statistical analysis was performed using 2-way ANOVA with  $P$  value of  $<.05$  as the level of significance. *Treg*, Regulatory T.

**TABLE E1.** Clinical data of 34 patients with peanut allergy

| Patients | Age (y) | Sex | Symptoms to peanut                 | Total IgE (kU/L) | Peanut-specific IgE (kU/L) | Ara h 2-specific IgE (kU/L) | ISAC Values (ISU-E) |         |         |         |         |         |
|----------|---------|-----|------------------------------------|------------------|----------------------------|-----------------------------|---------------------|---------|---------|---------|---------|---------|
|          |         |     |                                    |                  |                            |                             | Ara h 1             | Ara h 2 | Ara h 3 | Ara h 6 | Ara h 8 | Ara h 9 |
| 1        | 31      | M   | GI symptoms, bronchial obstruction | 112              | 10.8                       | 4.06                        | 5.64                | 6.06    | 4.52    | 10.49   | 6.36    | 0       |
| 2        | 40      | F   | Anaphylaxis                        | 21.3             | 0.69                       | 0.31                        | 0.26                | 0.6     | 0       | 1.53    | 0.36    | 0       |
| 3        | 33      | M   | OAS                                | 74.9             | 3.37                       | 2.02                        | 5.66                | 1.74    | 0       | 6.83    | 0       | 0       |
| 4        | 36      | F   | Abdominal pain, vomiting           | 121              | 10.4                       | 6.12                        | 8.05                | 4.41    | 0.29    | 9.84    | 3.44    | 0       |
| 5        | 28      | F   | Eczema, GI symptoms                | 67.9             | 1.7                        | 1.29                        | 0.16                | 1.07    | 0       | 2.83    | 0       | 0       |
| 6        | 25      | F   | Anaphylaxis                        | 771              | 19.4                       | 17.69                       | 8.38                | 11.43   | 0.97    | 21.09   | 2.37    | 0       |
| 7        | 17      | M   | Anaphylaxis                        | 2236             | >100                       | >100                        | 133.27              | 108.26  | 133.86  | 173.1   | 2.36    | 0.44    |
| 8        | 25      | F   | Anaphylaxis                        | 212              | 12.8                       | 8.48                        | 1.26                | 3.95    | 1.44    | 13.35   | 0.2     | 0.18    |
| 9        | 10      | M   | OAS                                | 345              | 15.9                       | 17.36                       | 20.03               | 13.29   | 0       | 16.56   | 0       | 0       |
| 10       | 15      | F   | Anaphylaxis                        | 361              | >100                       | 15.35                       | 43.11               | 49.4    | 32.32   | 57.02   | 1.47    | 0       |
| 11       | 5       | F   | NK                                 | 157              | 5.57                       | 84.5                        | 16.47               | 49.6    | 12.31   | 92.09   | 0       | 0       |
| 12       | 20      | M   | Anaphylaxis                        | 208              | 66.4                       | 98.4                        | 14.68               | 56.71   | 2.74    | 106.54  | 0       | 0       |
| 13       | 24      | M   | Anaphylaxis                        | 717              | >100                       | >100                        | 48.32               | 39.1    | 57.32   | 98.98   | 2.09    | 0       |
| 14       | 7       | M   | Anaphylaxis                        | 732              | >100                       | 87.7                        | 6.89                | 9.73    | 7.41    | 31.84   | 0       | 0       |
| 15       | 7       | F   | Urticaria, erythema                | 95.1             | 40.9                       | 17.00                       | 1.84                | 8.04    | 0.3     | 24.64   | 0       | 0       |
| 16       | 6       | M   | Anaphylaxis by inhalation          | Not available    | 10.1                       | 6.21                        | 0.67                | 5.16    | 0       | 12.34   | 3.46    | 0       |
| 17       | 6       | F   | Deterioration of eczema            | 135              | 22.8                       | 10.07                       | 0.06                | 13.59   | 0       | 48.92   | 2.56    | 0       |
| 18       | 24      | F   | Anaphylaxis                        | 708              | >100                       | 47.3                        | 51.08               | 20      | 21.05   | 62.5    | 0.2     | 0       |
| 19       | 5       | F   | Anaphylaxis                        | 1268             | 56.1                       | 38.9                        | 5.75                | 7.94    | 1.09    | 28.85   | 3.05    | 0.27    |
| 20       | 10      | M   | Anaphylaxis                        | 421              | >100                       | >100                        | 53.86               | 59.53   | 21.83   | 160.23  | 0.12    | 0       |
| 21       | 10      | F   | Deterioration of eczema            | 1010             | >100                       | 90.30                       | 20.35               | 20.53   | 3.48    | 102.23  | 0       | 0       |
| 22       | 5       | M   | NK                                 | 342              | 61.7                       | 9.0                         | 1.63                | 4.94    | 0       | 16.61   | 0       | 0       |
| 23       | 19      | M   | Anaphylaxis                        | 564              | 12.6                       | 3.2                         | 1.08                | 6.45    | 0       | 13.29   | 13.09   | 0       |
| 24       | 7       | F   | Anaphylaxis                        | 186              | 14.5                       | 8.96                        | 0.49                | 4.77    | 0       | 24.62   | 0       | 0       |
| 25       | 4       | M   | Urticaria                          | 218              | 6.91                       | 8.50                        | 0.18                | 5.32    | 0.23    | 2.12    | 0       | 0       |
| 26       | 9       | M   | Anaphylaxis                        | 62.3             | 28.2                       | 26.80                       | 3.31                | 13.65   | 0.42    | 26.81   | 0       | 0       |
| 27       | 6       | M   | Anaphylaxis                        | 80.7             | 30                         | 26.90                       | 18.95               | 11.21   | 6.3     | 18.13   | 0       | 0       |
| 28       | 23      | M   | Anaphylaxis                        | 677              | >100                       | >100                        | 53.1                | 19.09   | 23.79   | 36.7    | 0.29    | 1.47    |
| 29       | 55      | F   | Urticaria, dyspnea                 | 136              | 8.1                        | 5.77                        | 6.63                | 3.32    | 1.07    | 10.53   | 1.18    | 0       |
| 30       | 23      | F   | Anaphylaxis                        | 311              | 80                         | 40.2                        | 66.7                | 40.5    | 8.6     | 1.67    |         | 32      |
| 31       | 38      | F   | Abdominal pain                     | 914              | 4.78                       | 58.8                        | 36.12               | 27.44   | 12.14   | 67.88   | 0.11    | 0       |
| 32       | 39      | F   | Anaphylaxis                        | 29.8             | 7.98                       | 7.1                         | 7.5                 | 14.9    | 0.17    | 27.62   | 0.42    | 0       |
| 33       | 9       | M   | Anaphylaxis                        | >5000            | >100                       | >100                        | 167.46              | 103.51  | 165.14  | 178.32  | 35.68   | 0       |
| 34       | 14      | F   | Anaphylaxis                        | 136              | 340                        | 81.20                       | 18.4                | 50.78   | 24.03   | 36.82   | 1.29    | 0       |

F, Female; GI, gastrointestinal; ISAC, Immuno-solid-phase allergen chip; ISU-E, Standardized Units for specific IgE; M, male; NK, not known; OAS, oral allergy syndrome.

**TABLE E2.** Percentage of IgE-binding reduction to hypoallergenic molecules of Ara h 2 and Ara h 6 compared with their natural and wild-type counterparts

| Patients | rAra h 2 mut1<br>compared with<br>nAra h 2 | rAra h 2 mut1<br>compared with<br>rAra h 2 wt | rAra h 2 wt<br>compared with<br>nAra h 2 | rAra h6 mut1<br>compared with<br>nAra h 6 | rAra h 6 mut1<br>compared with<br>rAra h 6 wt | rAra h 6 wt<br>compared with<br>nAra h 6 |
|----------|--------------------------------------------|-----------------------------------------------|------------------------------------------|-------------------------------------------|-----------------------------------------------|------------------------------------------|
| 1        | 76                                         | 70                                            | 22                                       | 57                                        | 30                                            | 38                                       |
| 2        | 48                                         | 27                                            | 29                                       | 83                                        | 72                                            | 39                                       |
| 3        | 69                                         | 35                                            | 52                                       | 33                                        | 29                                            | 6                                        |
| 4        | 50                                         | 23                                            | 35                                       | 51                                        | 45                                            | 11                                       |
| 5        | 53                                         | 32                                            | 30                                       | 61                                        | 49                                            | 22                                       |
| 6        | 55                                         | 50                                            | 10                                       | 63                                        | 29                                            | 47                                       |
| 7        | 71                                         | 69                                            | 7                                        | 67                                        | 20                                            | 59                                       |
| 8        | 65                                         | 76                                            | 0                                        | 73                                        | 66                                            | 20                                       |
| 9        | 49                                         | 33                                            | 23                                       | 67                                        | 31                                            | 53                                       |
| 10       | 58                                         | 68                                            | 0                                        | 91                                        | 36                                            | 85                                       |
| 11       | 63                                         | 55                                            | 19                                       | 44                                        | 12                                            | 37                                       |
| 12       | 82                                         | 86                                            | 0                                        | 84                                        | 60                                            | 59                                       |
| 13       | 48                                         | 53                                            | 0                                        | 77                                        | 62                                            | 39                                       |
| 14       | 81                                         | 76                                            | 20                                       | 88                                        | 80                                            | 39                                       |
| 15       | 88                                         | 82                                            | 32                                       | 55                                        | 21                                            | 44                                       |
| 16       | 36                                         | 27                                            | 12                                       | 53                                        | 25                                            | 63                                       |
| 17       | 66                                         | 62                                            | 11                                       | 55                                        | 31                                            | 34                                       |
| 18       | 76                                         | 79                                            | 0                                        | 67                                        | 4                                             | 66                                       |
| 19       | 46                                         | 42                                            | 7                                        | 05                                        | 20                                            | 0                                        |
| 20       | 72                                         | 53                                            | 42                                       | 64                                        | 44                                            | 36                                       |
| 21       | 77                                         | 78                                            | 0                                        | 70                                        | 49                                            | 41                                       |
| 22       | 82                                         | 35                                            | 72                                       | 50                                        | 30                                            | 29                                       |
| 23       | 94                                         | 93                                            | 20                                       | 31                                        | 22                                            | 12                                       |
| 24       | 66                                         | 53                                            | 26                                       | 44                                        | 42                                            | 3                                        |
| 25       | 41                                         | 34                                            | 11                                       | 74                                        | 35                                            | 61                                       |
| 26       | 52                                         | 76                                            | 0                                        | 90                                        | 75                                            | 59                                       |
| 27       | 37                                         | 40                                            | 0                                        | 82                                        | 35                                            | 72                                       |
| 28       | 34                                         | 24                                            | 14                                       | 49                                        | 7                                             | 45                                       |
| 29       | 81                                         | 84                                            | 0                                        | 57                                        | 43                                            | 25                                       |
| 30       | 69                                         | 49                                            | 41                                       | 47                                        | 36                                            | 17                                       |
| 31       | 43                                         | 46                                            | 0                                        | 44                                        | 2                                             | 43                                       |
| 32       | 13                                         | 14                                            | 0                                        | 66                                        | 3                                             | 65                                       |
| 33       | 78                                         | 77                                            | 0                                        | 57                                        | 46                                            | 20                                       |
| 34       | 41                                         | 53                                            | 0                                        | 94                                        | 90                                            | 35                                       |