

Evaluating the potential allergenicity of dietary proteins using model strong to non-allergenic proteins in germ-free mice



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ABSTRACT

Currently no validated animal model is predictive of human responses in ranking purified dietary proteins in the prevalence or potency of food allergy in humans. Since the gastrointestinal microbiota is thought to influence oral tolerance, we hypothesize that a germ-free mouse model will more accurately predict atopic human responses than conventional mice. Germ-free C3H/HeN mice were immunized with 60 µg Ara h 2, BLG, or LOX by three weekly intraperitoneal (IP) injections with alum adjuvant. One week following the final immunization an IP challenge of 500 µg of Ara h 2, BLG, or LOX was administered. Thirty minutes post-challenge clinical scores were graded and body temperatures recorded. The presence of protein-specific IgE and mast cell protease concentrations in mouse sera were determined using ELISA. Upon challenge germ-free mice sensitized with Ara h 2 and BLG exhibited significantly more severe clinical scores compared to germ-free mice immunized with LOX. Hypothermic responses in challenged mice differed between the three proteins post-challenge. Results indicate that this model can differentiate between potent and non-allergens based on temperature drop, clinical scores, and biomarkers. Additional proteins with known human exposure and allergenicity are needed to confirm the predictive accuracy.

1. Introduction

The prevalence of food allergies are increasing at a faster rate than can be explained by genetic factors (Berin and Sampson, 2013), which suggests a role for environmental factors that have yet to be identified. Although there are many new theories discussed as having a role in the apparent increases in food allergy prevalence, such as vitamin D deficiency (Bozzetto et al., 2012), the over-use of antibiotics (Metsala et al., 2013), and even the avoidance of consumption of allergenic foods in early childhood/life (Palmer and Prescott, 2012). The potential causes are complex and there is no single model that can be used to test the theories in an unbiased way.

While it is clear that a few major allergenic foods are responsible for the majority of life-threatening reactions, the general public perception of an epidemic of food allergy to peanut and other foods is leading to shifts in public policy that impact the food industry, regulators and ultimately consumers (Waggoner, 2013). Food processing methods and

genetic engineering are both being used to introduce novel proteins in foods for specific functional properties. Therefore regulatory agencies are requiring safety assessments of such products including evaluation of the potential allergenicity of the new proteins. The Codex Alimentarius Commission of the Food and Agricultural Organization and World Health Organization of the United Nations developed international guidelines for the evaluation of the potential allergenicity of genetically engineered (GE) crops (Codex, 2003, 2009). Earlier FAO/WHO guidelines (2001) called for the use of animal models to evaluate potential sensitization of new proteins in GE plants. However, there has been a lack of rigorous testing to demonstrate the predictive power of various animal models to consider human food safety (Goodman et al., 2008). Codex guidelines were established to frame a multistep assessment strategy that results in a conclusion of the likelihood that the newly introduced protein is an allergen. In GE crops the identity of the gene donor must be known so that literature regarding human exposure can be reviewed to establish if there is a “history of safe use”, if the

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protein is a known allergen, or if the protein was isolated from an important source of allergy (Goodman and Tetteh, 2011). In addition, a comparison of the amino acid sequence of the new protein with those of known allergens is conducted to evaluate whether the new protein is sufficiently similar to a known allergen to suspect that it would pose a modest risk of allergic cross-reactivity due to shared IgE binding. If the sequence of the protein expressed by the transferred gene has a high identity match to a known allergenic protein using FASTA or BLASTP comparison, protein-specific IgE testing would be required using sera from individuals allergic to the source of the gene (Goodman, 2008). There is some uncertainty and differences of opinion regarding the degree of sequence identity that would indicate likely risk. Sequences sharing greater than 70% identity across their full-length indicates a high likelihood of IgE cross-reactivity, while less than 50% identity generally does not (Aalberse, 2000). Goodman (2006) noted that Pearson, the developer of the FASTA program, suggested that identity matches of 50% over contiguous stretches of 20–40 amino acids occur rather commonly by random chance. There are very few studies that have compared serum IgE binding using competitive inhibition assays for structural protein families that are known to share cross-reactivity. One study by Scheurer et al. (1999) showed reductions in IgE binding of 100 to 1000 fold for different homologues of Bet v 1 that were only 40% identical compared to the specific protein that were thought to be the primary allergen for the serum donors (Goodman, 2006). The suggested high-risk total sequence identity matches would be for those of greater than 50% identity over essentially the full-length of the protein, with an *E* score match smaller than $1e-7$ as an indication that serum testing is needed (Goodman, 2006). However, the Codex recommendation that short identity matches to an allergen of greater than 35% identity in any span of 80 amino acids be used as a trigger for human serum testing, is likely overly conservative, frequently giving false-positive identification (Goodman, 2008; Mirsky et al., 2013). The utility of the bioinformatics strategy is dependent on a well-curated database of known allergens, such as www.Allergenonline.org (Goodman, 2006). Regarding potential allergenicity, the greatest risk in GE crops is the introduction of a protein that is a major allergen from a different food that the allergic subjects are already sensitized and allergic to as demonstrated by Nordlee et al. (1996). The allergic individuals would not know to avoid the GE food containing the allergen, eat it, and experience a potentially severe allergic reaction.

The previously described methods including serum IgE tests, are not useful for allergenicity assessment of proteins that are not from known allergenic sources or sequence similar to allergens. However, some proteins may not be similar to any known allergen, but may present an important risk of sensitization and then elicitation of new allergic responses. Potential risk factors may include inherent properties of the protein such as resistance to proteolysis (Ladics et al., 2014), stability, immunogenicity, hydrophobicity, or enzymatic activity. Other risk factors may be associated with the interaction of the protein and the whole food such as high abundance (Taylor et al., 2010), or adjuvant activity of other components of the food matrix in the GE crop. In these experiments, we aimed to test a germ-free C3H/HeN mouse model's ability to rank known proteins of varying allergenicity and to establish point of reference or "standard" allergic responses for proteins that are potent, moderate, or non/weak allergens (Smit et al., 2016). This would establish a model for evaluation of sensitizing and eliciting capacity of novel proteins, and allow for comparison to the standard proteins as an indicator of potential allergenicity. An animal model for predicting whether a novel protein has the ability to sensitize consumers *de novo* would be useful for assessing future GE products that are highly abundant or stable in the pepsin-resistance tests.

While a number of researchers have published studies using animal models that show some promise in differentiating strongly allergenic from weakly allergenic proteins in the context of whole food sources, clear inconsistencies have been reported from other labs (Birmingham et al., 2003; Bogh et al., 2016; Gonipeta et al., 2009; Ahrens et al.,

2014). However, other studies have reported that allergic responses measured in mice, whether based on specific IgE (sIgE) or clinical symptoms do not correlate well with reported human responses to potent and weak food allergens (Selgrade et al., 2009; Ladics et al., 2010). Many animal models produce clear sensitization with ovalbumin in mice (Ganeshan et al., 2009). However, major allergens from peanut such as Ara h 1 and Ara h 2, and the major milk allergen, β -lactoglobulin (BLG) often appear less able to induce sensitization than weak or non-allergens such as spinach RUBISCO and soybean lipoxygenase (LOX), in a variety of mouse strains utilizing similar sensitization protocols (Ladics et al., 2010; Bowman and Selgrade, 2008). It should be noted that Smit et al. developed a two model system that seems to be able to differentiate allergens from non-allergens using DC-T cell assay *in vivo* mouse model in conventional mice (Smit et al., 2016).

Results from the current studies using three proteins (Ara h 2, BLG and Soybean LOX), suggest that our germ-free murine model may be predictive of human allergic responses to dietary proteins. Studies by others suggest that the GI microbiota may increase oral tolerance (Rodríguez et al., 2011). Thus, we predict that a mouse with no intestinal microbes (germ-free) may be better suited for avoiding tolerance and eliciting an allergenic response of varying intensities that are determined by factors intrinsic to the sensitizing protein.

1.1. Hypothesis

We hypothesize that a germ-free mouse model will more accurately predict human responses than a model using mice with a conventional microbiota. This study was undertaken to test the sensitizing and eliciting responses comparing a potent dietary peanut allergen, Ara h 2; a moderately potent bovine milk allergen, BLG; and a non-allergenic protein, soybean LOX.

1.2. Assessing potency

Sensitization is usually induced when a susceptible individual is exposed to an allergen, if the amount and quality of the allergen are sufficient the individual may be sensitized. What makes a patient susceptible involves a myriad of processes including various innate immune cells interacting to elicit a Th2 response which leads to the induction of an IgE response. Factors that have been shown to impact this process include the gut microbiota, genetics, chemical properties of the protein, food matrix, the dose and route of exposure, and the digestibility of the allergen. Once sensitized, elicitation thresholds will vary based on the route and dose of exposure and the extent to which the individual was sensitized. Potency of an allergen's sensitization and elicitation potential should be considered (Kimber et al., 2001). Sera from severely allergic patients may be used to assess the relative sensitization potency of allergens from the same food source (i.e. ranking relative potency of various peanut allergens), for the subjects included in the test. Serum donors should be clinically diagnosed with allergy to the relevant source. Antigen specific serum IgE should be evaluated by immunoblots or ELISA or ImmunoCAP® tests with well characterized antigens (Palmer et al., 2005). However, *in vitro* IgE binding by direct assays may not be specific or biologically active. Specificity may be verified by inhibition assays and biological reactivity may be evaluated by either basophil activation or histamine release in culture or by *in vivo* challenge of allergic subjects (Palmer et al., 2005). Previously a humanized rat basophilic leukemia (hRBL) cell assay was used with peanut allergic human sera and purified peanut proteins to demonstrate that the 2S albumin (Ara h 2) is more potent than vicilin (Ara h 1) by Palmer et al. (2005). However, it is important to include a sufficient number of clinical subjects with diverse genetic, environmental exposure and clinical symptoms in the tests to be able to rank allergens for the broad population of allergic subjects. It is also important to test biological activity of the IgE binding by basophil assays or by skin prick tests in subjects with direct IgE binding.

Ranking the potency of proteins as allergens between different food sources is a more challenging task. Although there are atopic individuals with allergies to multiple sources or foods, allergy is very much an individual disease with some having severe symptoms to peanuts, others to specific tree nuts, milk, eggs, fish or crustacean shellfish. Each of these allergenic sources has multiple proteins that may be responsible for all or some of the reactivity for a given individual. Thus judging potency of proteins between food sources adds complexity far beyond evaluating allergenicity of proteins within a single source. The true test of elicitation potency requires clinical threshold studies to determine the eliciting dose (Taylor et al., 2010).

Aside from potency, other risk factors that are considered are epidemiological data on prevalence and incidence, evidence of causality with consistency of association, and the ranked severity of symptoms (subjective and objective). When reviewing epidemiological data special consideration is necessary if self-reports were utilized as they often over estimate allergic population (Sicherer et al., 2010; Nwaru et al., 2014; Rona et al., 2007).

1.3. Representative allergenic foods

There are eight food sources that contribute to the majority of allergic reactions in the U.S. We selected proteins from three of these foods: peanut, milk, and soybean. The proteins used in this study were carefully selected to test our model's range of measurable allergic responses. A spectrum of responses ranging from severe anaphylaxis to little or no response depending on the protein used was our goal. This model has the potential to become a primary test to assess the allergenic potential of novel foods or proteins, produced through genetic modification or new food processing techniques.

1.3.1. Ara h 2

Ara h 2 is a major peanut food allergen that belongs to the prolamin superfamily of proteins, which includes other known allergens in the 2S albumin, alpha-amylase and trypsin inhibitor protein families. Ara h 2 consists of two isoforms, Ara h 2.01 and Ara h 2.02, that migrate as a doublet in reducing SDS-PAGE with molecular weights of approximately 17 and 18 kDa. The proteins are almost identical except that Ara h 2.02 contains an additional 12 amino acid repeat of a major IgE binding epitope compared to Ara h 2.01 (Chatel et al., 2003). *In vitro* digestion with trypsin, pepsin, and chymotrypsin indicated that Ara h 2 is highly resistant to GI digestion (Sen et al., 2002), a common trait in food allergens (Nowak-Węgrzyn, 2007). Ten sequential IgE-binding epitopes were identified through tests with pooled peanut allergic sera and 15 amino acid overlapping peptides in SPOTS analysis (Stanley et al., 1997).

1.3.2. β -lactoglobulin

Bovine β -lactoglobulin (BLG, Bos d 5) is a major allergen from milk. BLG is common in most mammalian milk, although it is not found in mice or human milk. It is a globular 18.4 kDa protein. In physiological conditions it is generally found as a dimer comprised of two identical subunits that have β -barrel structure forming a lipid-binding site (Bossios et al., 2011). Two intra-molecular disulfide bonds stabilize the structure, and likely make the protein stable to GI proteolysis (Breiteneder and Mills, 2005). Sequential IgE binding epitopes have been characterized using microarray analysis with 20 amino acid peptides with serum samples from 31 milk allergic subjects (Cerecedo et al., 2008).

1.3.3. Soybean lipoxygenase

Soybean, *Glycine max*, contains three lipoxygenase isozymes that together account for nearly 1% of the protein in the seed. They are referred to as LOX-1, LOX-2 and LOX-3 (Mandal et al., 2014). The natural proteins were purified by TNO in the Netherlands and characterized. The SDS-PAGE bands identified using a sample of the purified

protein showed a band as expected of 94 kDa (Tranbarger et al., 1991). Mass spectrometry of the purified protein demonstrated that it includes sequences of all three isoforms. The LOX proteins likely serve both as a dioxygenase, and as a source of nitrogen for the embryonic soybean. It is a non-heme iron-containing enzyme important in the biosynthesis of plant signaling compounds (Prescott and John, 1996). A thorough literature review indicates this protein has not been reported to be a human allergen. There are not positive markers to identify a "non-allergen" so care needs to be taken when defining a protein as a non-allergen. For the purposes of this study LOX is referred to as a non-allergen.

2. Materials and methods

2.1. Germ-free C3H/HeN mice

Germ-free C3H/HeN mice were purchased from Anaxem (INRA, Jouy-en-Josas, France). Mice were bred in the GGg Germ-free Mouse Facility at the University of Nebraska-Lincoln, and weaned at four weeks of age. Germ-free mice were housed in flexible sterile isolators with controlled temperature and 12-h light/dark cycle. All mice were allowed *ad libitum* intake of autoclaved water and pellet chow (Harlan Teklad, product 2019S) that was free of test protein sources to avoid induction of tolerance or dietary sensitization including peanut, milk and soy according to manufacturer listed ingredients. The germ-free status was monitored using standard culture and PCR based methods. All procedures were performed in accordance with The University of Nebraska-Lincoln's Institutional Animal Care and Use Committee (IACUC) approved protocol (project identification number 732).

2.2. Test proteins

Peanut allergen Ara h 2 (> 95% pure) was purified from lightly roasted natural peanut flour produced by Golden Peanut Co. LLC, (Alpharetta, GA, Prod # 521271) by Fulei Luan at the University of Nebraska, Lincoln. The BLG (Brix et al., 2003) (> 95% pure, a mixture of variant A + B, prepared by Arla Foods Ingredients, Videbæk, Denmark). Soybean lipoxygenase (LOX), purified (~90%) by TNO Nutrition and Food Research in the Netherlands and a gift of DuPont) were used for sensitization and elicitation.

2.2.1. Allergen characterization

Ara h 2, BLG (β -lactoglobulin) (Arla Foods Ingredients), and soybean lipoxygenase (LOX) (donated to FARRP by DuPont) were characterized under reduced and non-reduced conditions using SDS-PAGE and immunoblot using standard procedures. Briefly protein samples (5 μ g/well) were separated under reducing conditions (2-mercaptoethanol, heated for 5 min at 100 °C), or under non-reducing conditions (without 2-mercaptoethanol and without heating) in 10–20% tris-glycine SDS-PAGE, then stained with colloidal blue G250 (Sigma, product B2025). Following destaining the gel was then scanned with the Kodak Gel Logic 440 imaging system and the density of pixels of the primary band at the appropriate molecular weight in reducing conditions was compared to the total density of all protein bands in the sample lane. The purity estimated by this method was > 90%. In additional gels, protein samples separated under similar conditions were transferred to PVDF membranes (Invitrogen, product LC2005), blocked with 1% Bovine Serum Albumin (BSA) in PBS and incubated with primary antibody (rabbit anti-BLG, LOX, or Ara h 2 IgG diluted to 1:10,000). HRP-conjugated goat anti-rabbit antibodies were used as secondary antibody. Super Signal West Dura substrate (Pierce, product 34075) was used and images were captured (Kodak Gel Logic 440 imaging system). BLG samples were also separated in SDS-PAGE and stained with EZ Blue stain (Sigma, product G1041). Bands of interest were digested with trypsin (2%) and excised for LC-MS/MS analysis.

LPS contamination was quantified using the Limulus Amoebocyte

Lysate (LAL) Assay (Association of Cape Cod, Boston, MA, product C0031) according to the manufacturer's instructions. LPS contamination in Ara h 2, BLG, and LOX were found to be 1, 3, and 9 EU/mg, respectively. The analysis is based on initial protein concentration 1 mg/ml for each protein with samples diluted and tested with the least diluted protein sample that fit the standard curve of LPS used as the basis for the calculated LPS level.

2.3. Preliminary sensitization dose response

Dose response experiments were conducted with all three allergens to determine the optimal sensitization dose, 12, 60, or 300 µg, by intraperitoneal immunization with alum three times on a weekly intervals. Ten mice were used per treatment. One week after the third immunization, each mouse was challenged with 500 µg of test protein by i.p. injection. The dose response was performed as we recognize that varying potency might be due to differences in protein size, and number of epitopes. Doses were weight/volume of each protein. The final design was to use one dose for each protein in replicate experiments. These results (data not shown) prompted the use of 60 µg test protein dose as the standard for the sensitization protocol for this study. All lyophilized proteins were weighed and then solubilized prior to injection. These proteins are not recombinant, they were purified from native sources and were not reduced or modified before sensitization or challenge. The same batch of protein was used in all treatments.

2.4. Intraperitoneal sensitization and immune challenge

Each of the three proteins were used in two replicate experiments. The sum of the replicates equaled approximately 20 protein and adjuvant immunized mice, and 10 alum injected control mice for each of the three proteins (Ara h 2, BLG, or LOX), for a total of six experiments. Experimental mice were age matched and received the first immunization dose between four and five weeks of age. Male and female mice were utilized in a 1:1 ratio. Mice were immunized with 60 µg of protein three times on weekly intervals (Fig. 1). Protein solutions were prepared with 60 µg of protein in 100 µl of buffer and filtered through cellulose acetate membrane (0.45 µm pore size syringe filter). The BLG and LOX solutions were prepared in 0.9% sodium chloride for injection, (Hospira, product 0409-7983-61) and Ara h 2 solution was prepared using phosphate buffered saline (PBS) solution 1x (Fisher Scientific, product BP399). The filtered solutions were then mixed with an equal volume of autoclaved Thermo Scientific Inject Alum Adjuvant by adding the alum dropwise while mixing. The suspensions were injected within an hour of mixing. The protein and adjuvant suspensions were injected intraperitoneally (IP) at a volume of 200 µL per mouse. Control mice were injected with an alum adjuvant and buffer solution at 200 µL/mouse. One week after the last immunization dose all mice were challenged with 500 µg of the appropriate test protein in 200 µL of buffer, by IP injection.

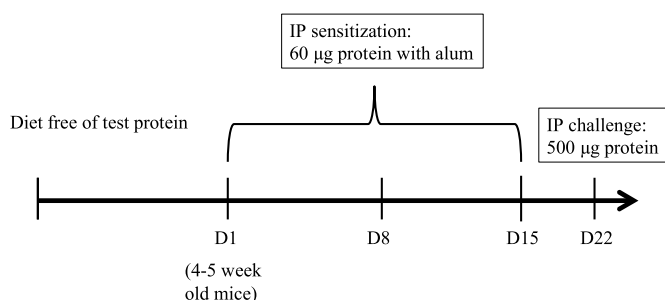


Fig. 1. Mouse immunization protocol. Four to five-week old germ-free C3H/HeN mice were intraperitoneally immunized three times on weekly intervals with protein and adjuvant. Control mice were immunized with buffer solution and alum alone. All mice were challenged with test protein on day 22.

Table 1

Clinical score assigned 30 min post-challenge.

Score	Observed Symptoms
0	no symptoms
1	scratching and rubbing around nose and head
2	puffiness around eyes and mouth, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate
3	wheezing, labored breathing, and cyanosis around the mouth and tail
4	no activity after prodding, or tremor and convulsion observed
5	death

2.5. Evaluation of allergic response

Prior to challenge a baseline temperature was measured using a Microtherma 2t with Ret-3 rectal probe (AD Instruments, product MLT1404). Mice were monitored and 30 min post-challenge a final temperature was measured, and a clinical score assigned based on a six-point clinical observation scale (Table 1). Blood was collected from anesthetized mice via cardiac puncture and transferred to serum separator tubes (BD microtainer with clot activator gel additive, product 02-675-188) to isolate sera. Sera were stored at -80°C until thawing for measurement of protein-specific IgE ELISA or mast cell protease.

2.6. Clinical scoring

The clinical scoring method was adapted from published methods (Sun et al., 2007; Smit et al., 2011). Briefly, the clinical score was assessed 30 min post-challenge with scores assigned as a number with 0, indicating no symptoms, and 5, indicating death (Table 1).

2.7. Protein-specific IgE direct ELISA

Direct ELISA assays were used for detection of specific IgE for each protein. Samples of Ara h 2, BLG, or LOX were coated on flat-bottomed 96-well plates (NUNC Immuno MaxiSorp 96-MicroWell plates, product 439454) using 2 µg protein per ml in sodium bicarbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate [pH 9.6]), applied at 100 µL/well. Plates were sealed and incubated overnight at 4°C . Unbound BLG was removed using three cycles of washing with 250 µL/well wash buffer (PBS, 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl [pH 7.4], with Tween-20 at 0.05%) using an automated plate washer. Assay diluent (PBS, 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl [pH 7.4], and bovine serum albumin 1%) was used as a blocking agent. Plates were blocked for 1 h at room temperature using 200 µL/well. Sera were diluted 1:10 using assay diluent and incubated for 1 h at room temperature, in non-stick 96 well polypropylene plates, before being pipetted into predetermined wells in the washed ELISA plate at 100 µL/well. Mouse serum was tested in duplicate wells. Plates were then sealed and incubated overnight at 4°C . Plates were washed and soaked five times with 60 s soak-times with 250 µL/well washing buffer and bound IgE was detected using LO-ME-3 rat anti-mouse IgE-HRP (LifeSpan BioSciences, Inc. #C58447), diluted 1:2000 (v:v) in assay diluent with diluted detection antibody added at 100 µL/well. Plates were sealed and incubated for 1 h at room temperature. Plates were washed five times with 250 µL/well followed by addition of detection substrate, ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] (1 mM; Roche, product 11204530001) in citrate buffer (100 mM citric acid, 50 mM sodium phosphate [pH 4.2]) containing 0.03% hydrogen peroxide, which was prepared as a stock and was added at 100 µL/well. Plates were developed for 30 min at room temperature and then ODs were read at 405 nm (BioTek Synergy HT Microplate Reader, Gen5 software), no stop solution was used. Control mice were immunized with adjuvant only and challenged with one of the three test proteins; separate control mice were used for each test protein, on the day of challenge. The mean 405 nm OD observed from control mice sera

plus two standard deviations were considered the cutoff for a positive, protein-specific IgE, response in treatment mice. Cutoff values were calculated for each ELISA plate to account for background variation.

2.8. Measurement of plasma mMCP-1

Mast cell protease was measured using an ELISA kit (eBioscience Mouse MCPT-1 Ready-SET-Go) as described by the manufacturer. Sera were diluted between 1:50 and 1:1600 in assay diluent depending on treatment. Serum from each mouse was tested in duplicate wells (BioTek Synergy HT Microplate Reader, Gen5 software). Reactions were stopped by addition of 2N sulfuric acid was used at 50 μ L/well at a fixed incubation time. The absorbance at 450 and 570 nm was recorded and used to calculate mMCP-1 concentrations against a standard curve.

2.9. Statistical analyses

All data were tested for normality using three tests: D'Agostino and Pearson omnibus, Shapiro-Wilk, and KS normality tests with $\alpha = 0.05$. In order to treat all experiments the same way a non-parametric test was utilized, as some of the treatment and control data were not normally distributed. Kruskal-Wallis test was used to analyze temperature drops, mMCP-1, clinical scores, and protein-specific IgE. In order to correct for multiple comparisons Dunn's multiple comparisons test was used, therefore all p-values reported are adjusted p-values. The test results were considered statistically significant when the adjusted p-value was < 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, or ns for not significant). All analyses were completed using GraphPad Prism 8.

3. Results and discussion

To investigate this model's potential to be predictive for estimating a novel protein's potential to sensitize and elicit allergy (immediate type I hypersensitivity) three proteins were used to evaluate the range of responses. Following further validation of this model using additional proteins with known histories of allergenicity, proteins of unknown allergenicity could be tested using this protocol to judge their allergenic potential. While intragastric sensitization is an appealing route, early pilot studies had high variability and required large amounts of purified protein, which is why we used IP sensitization and challenge in this study. Similarly we used germ-free mice over conventional mice with a full microbiota as an effort to create an exaggerated Th2 response from the lack of LPS signaling through TLR4 which in turn creates a more robust allergic response to the sensitization proteins thereby allowing us to assess inherent allergenicity of the proteins used by analyzing the spectrum of responses from various protein treatments in the germ-free mice.

3.1. Protein characterization

SDS-PAGE and IgG immunoblot confirmed the identity of our protein sources based on migration patterns. Then an EZ blue gel was ran and the bands of interest were excised and the partial amino acid sequence of the protein of interest was confirmed using LC-MS/MS. For example, with BLG three main bands were seen after the trypsin digestion when ran on SDS-PAGE. The gel was 10–20% Tris-Glycine and was ran at 125 V for 90 min. The gel was stained using EZ Blue G. After excising the bands and using LC-MS/MS bands were identified to be BLG, soy lipoxigenase, and Ara h 2.

3.2. Preliminary sensitization dose response

Recognizing that varying potency, protein size, and number of epitopes could potentially prevent maintaining a consistent sensitization dose amount (weight/volume), dose response experiments were

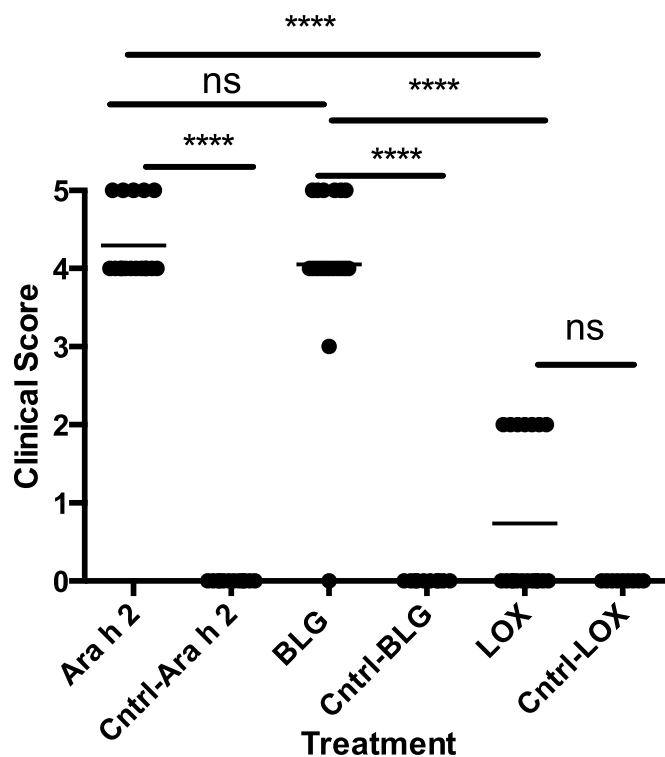


Fig. 2. Clinical scores post challenge with potent or non-allergen. Germ-free C3H/HeN mice approximately 20 treatment mice and 10 control mice per protein (combined results of two separate experiments for each protein). Controls were sensitized with alum alone and challenged with the indicated allergen. Male and female mice were approximately represented equally in all treatments. Clinical scores (Y-axis) assessed 30 min post-challenge. Results analyzed using Kruskal-Wallis test and Dunn's test for multiple comparisons, **** $p < 0.0001$, and ns = not significant.

completed for each protein using 12, 60, or 300 μ g test protein with alum. All treatment and control animals received the same challenge dose of 500 μ g test protein. A BLG dose response was completed first indicating a non-significant trend that 60 μ g/sensitization elicited a stronger response, based on hypothermia and clinical symptoms post-challenge, to a higher and lower dose. In an effort to limit variables it was decided that for the following protein dose response experiments 60 μ g of allergen per sensitization would be selected as the optimal sensitizing dose if post-challenge 60 μ g sensitized treatment mice experienced the same or greater allergic response (based on hypothermia and clinical symptoms) than the 12 or 300 μ g sensitized treatment mice. For LOX all doses resulted in equally low responses post-challenge. In the Ara h 2 dose response experiment 12 and 60 μ g doses had similar outcomes, however 300 μ g resulted in 50% mortality within an hour following the third sensitization. Based on high mortality the 300 μ g-dose was unacceptable for potent allergens.

3.3. Stronger response with potent allergens than with non-allergen

Overall, there was a marked increase in severity of symptoms (Fig. 2), specific IgE positivity (Fig. 3 and Table 2), and mast cell protease in mice sensitized with potent allergens (Ara h 2 and BLG) compared to the non-allergen (LOX) when immunized IP with 60 μ g protein and alum adjuvant (Fig. 4) post IP challenge. While specific IgE can be misleading when it is the only marker utilized, in this case the trends of specific IgE mirrors MCPT-1 and the clinical scores of the mice in the different treatment groups. There were no statistically significant differences based on the sex of the animal.

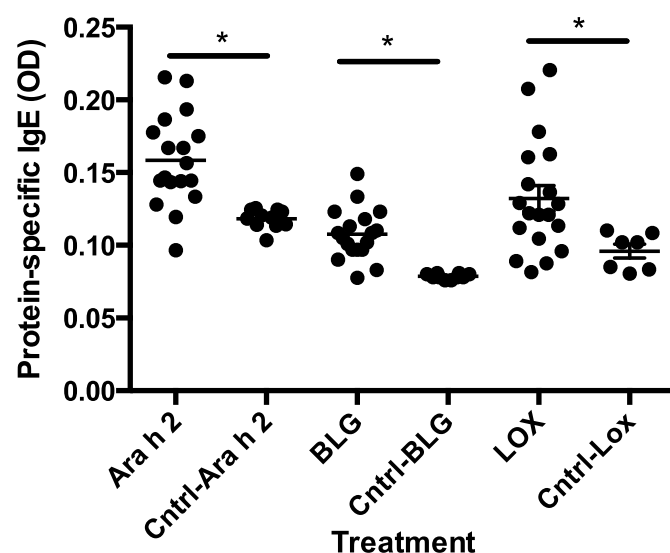


Fig. 3. Protein-specific IgE. Germ-free C3H/HeN mice approximately 20 treatment mice and 10 control mice per protein (combined results of two separate experiments for each protein). Controls were immunized with alum alone and challenged with the indicated allergen. Direct ELISA was performed coating treatment allergen on NUNC plate at 2 μ g/ml. Germ-free mice sera diluted 1:10 in PBS-1% (w/v) BSA and OD was read at 405 nm. Statistical analysis was only done to compare treatment and control due to the fact that the assays are different for each of the three proteins used for the direct ELISAs. * $P < 0.05$.

Table 2

Ratio of allergen-specific IgE mice per treatment. Cutoff (405 nm OD) for specific IgE positive mice is equal to the mean OD of control mice sera plus two standard deviations. Each row represents two separate experiments that were combined after testing that results did not differ. Allergen-specific IgE treatment mice were classified as positive if the ELISA OD value was higher than the mean OD value plus two standard deviations of control mice sera. Each cutoff value was determined using sera from control mice (mock-sensitized with alum and buffer, then challenged with the appropriate protein), from the same experiment and tested in the same ELISA plate to control for background variation.

Allergen	Cutoff (OD 405 nm)	Allergen-specific IgE positive mice % (# of pos/n)
Ara h 2	0.131	89% (16/18)
BLG	0.082	94% (17/18)
LOX	0.121	57% (11/19)

3.4. Mortality in GF C3H/HeN mice

Five out of 18 mice sensitized and challenged with Ara h 2 died post challenge (Table 3). The remaining Ara h 2 treatment mice showed clear signs of anaphylaxis, as they did not respond to prodding (clinical score of 4). The mortality of germ-free C3H/HeN mice sensitized and challenged with BLG (Arla Foods) was six out of 19 mice. Although the mortality was similar for Ara h 2 and BLG treatments (28 and 31%) respectively, the remaining mice challenged with Ara h 2 all had a clinical score of 4, they did not show statistically significant differences (Fig. 2).

3.5. Hypothermia measurements differentiates strong and non-allergenic proteins

A significant drop in body temperature, measured rectally, was observed 30 min post-challenge in mice sensitized with either Ara h 2 or BLG. Germ-free mice sensitized and challenged with Ara h 2, experienced a statistically greater drop in body temperature than did mice

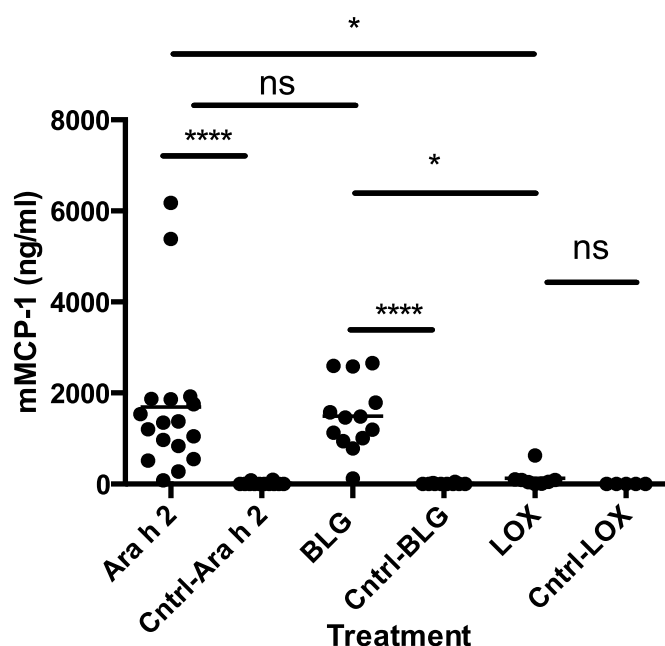


Fig. 4. Serum mMCP-1 levels post-challenge. Germ-free mice ~ 20 treatment mice and ~ 10 control mice per protein (combined results of two separate mouse experiments for each protein). Controls were immunized with alum alone and challenged with the indicated allergen. Results were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparison test **** $P < 0.0001$, * $P < 0.05$, ns = not significant. The detection limit for this assay was 11.7 ng/ml mMCP-1.

Table 3

Mortality rate of mice post-challenge. Mortality rate of germ-free mice post-challenge with three different proteins are reported as percentage and sample size (n) are indicated (deaths/n). Two experiments for each protein, with consistent results, are reported.

Allergen	Mortality Post-challenge
Ara h 2	28% (5/18)
BLG	31% (6/19)
LOX	0% (0/19)

sensitized and challenged with either BLG or LOX. The BLG sensitized mice had an intermediate mean temperature drop during specific challenge when compared to mice treated with the other two proteins (Fig. 5). It is important to note that both of the treatment groups that received a potent allergen experienced statistically significant hypothermia compared to their sham immunized and protein challenged control group. While the non-allergen, LOX, treatment group compared to LOX control group was not (Fig. 5).

3.6. Weak response observed in LOX sensitized mice

The responses to the LOX challenge in LOX sensitized and the LOX control mice was not significant in temperature drop (hypothermia), mMCP-1 release, or clinical scores. The lack of a significant allergic response to LOX while showing that it was able to sensitize (IgE-positivity) indicates that this model has the ability to differentiate proteins based on their intrinsic allergenicity. While an argument could be made that a non-allergen should result in no sIgE positive mice, we conclude that the low LOX-specific IgE positivity observed was due to a combinatory effect of adjuvant and lack of GI microbes, and the magnitude of the other markers and the relative sIgE positive mice compared to the potent allergens are indicative of the models ability to differentiate between potent, moderate, and non-allergens. As adjuvant has been

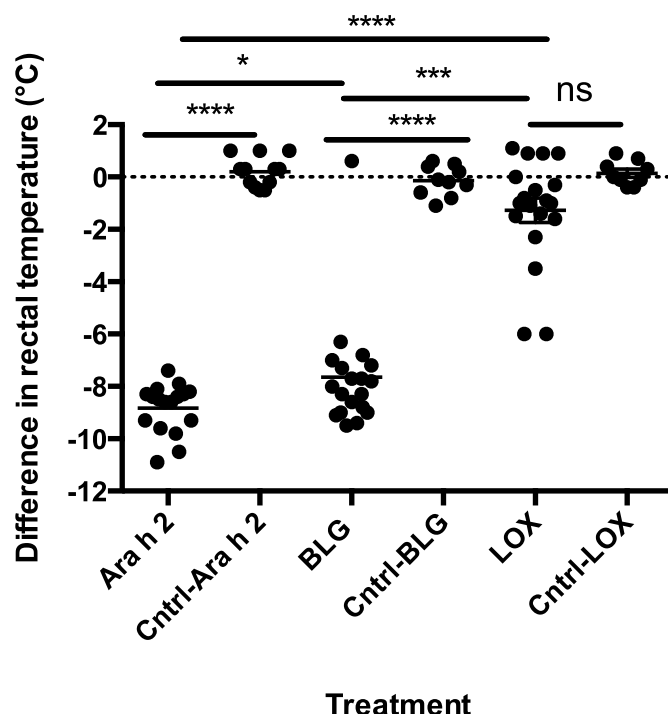


Fig. 5. Temperature drop post-challenge. Germ-free C3H/HeN mice ~20 treatment mice and ~10 control mice per protein (combined results of two separate experiments for each protein). Controls were immunized with alum alone and challenged with the indicated allergen. Y-axis calculated by subtracting the temperature pre-challenge from the temperature post-challenge, negative value indicates drop in temperature. Results analyzed using Kruskal-Wallis test and Dunn's test for multiple comparisons **** $P < 0.0001$, *** $P < 0.0002$, * $P < 0.05$, or NS non-significant.

discussed to be a potential source of false positivity (Buehler, 1996), the importance of weak clinical symptoms, mild hypothermia, low sIgE, and low mMCP-1 response to a control non-allergen (LOX) compared to potent allergens demonstrated the weak or non-allergic properties of the protein.

4. Conclusions

This animal model is able to differentiate between purified potent and non-allergens. As far as ranking the two potent allergens in this study, hypothermia was the only indicator that Ara h 2 was more potent than BLG. The other indicators mMCP-1, sIgE positivity, and clinical scores were similar between the two allergens. The responses to both known allergens, Ara h 2 and BLG were significantly greater than responses observed in mice sensitized and challenged with LOX.

In published studies Ara h 2 has not been shown to cause anaphylaxis in mice with a functional toll-like receptor 4 (TLR4). The high mortality observed for Ara h 2 in this study is likely due to the fact that the mice were germ-free and therefore did not have LPS signaling through TLR4, which presumably would skew the immune response to a Th1 profile. Reports of anaphylaxis to Ara h 2 in conventional C3H/HeJ mice with a non-functional TLR4 (Oyoshi et al., 2014), supports this theory.

Characterizing both clinical and serological responses is important for evaluating the allergic response in an animal model. When looking at the various markers it seems clear that this model is able to differentiate between known allergens based on potency. As pure protein challenges are not performed in humans some informed assumptions are necessary. We assume Ara h 2 is more potent than BLG because more severe reactions are observed in a larger number of consumers who are allergic to peanut rather than milk, although occasional life-

threatening or fatal reactions can be elicited by both allergenic foods. These specific proteins are indicated as allergens in humans based on sera testing for IgE binding, protein characteristics, and skin prick tests. We assume LOX is a non-allergen based on a lack of reported IgE binding to LOX from soybean allergic patients. That being said, the observed responses correlate with the overall allergenicity response to these proteins in humans, which is essential if this model will be useful in assessing the allergenic risk of novel proteins in the future.

LOX-specific IgE was detected in LOX sensitized mice showing that C3H/HeN were able to produce antibodies to LOX, and also indicating that compared to other proteins there were less sIgE positive mice (Fig. 3). The response was much weaker than to known potent allergens when all markers are assessed (Figs. 2, 4, and 5).

A robust predictive animal model of food allergy requires a strong response to potent allergens and little or no response to non-allergens, as seen in this study. Additionally, it has proven to be reproducible in our facility. Sample sizes, 18–19 treatment mice and 10 control mice per protein, reported were the combination of two separate experiments for each test protein repeated at different times, in different isolators. Were this model to be widely adopted, the absence of the gut microbiota in GF mice will likely allow for a level of reproducibility between labs not previously seen. By using GF mice, we believe we have taken out one of the important environmental variables that may be responsible for the variation reported in conventional animal models that have tested the same proteins before (Ladics et al., 2010).

CRediT authorship contribution statement

Nathan L. Marsteller: Conceptualization, Methodology, Data curation, Validation, Writing - original draft, Writing - review & editing. **Richard E. Goodman:** Conceptualization, Writing - review & editing. **Kwame Andoh-Kumi:** Methodology, Investigation. **Fulei Luan:** Methodology, Investigation. **Katrine L. Bøgh:** Methodology, Writing - review & editing. **Joseph Baumert:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Aalberse, R.C., 2000. Structural biology of allergens. *J. Allergy Clin. Immunol.* 106, 228–238.
- Ahrens, B., et al., 2014. Development of an animal model to evaluate the allergenicity of food allergens. *Int. Arch. Allergy Immunol.* 164, 89–96.
- Berlin, M.C., Sampson, H.A., 2013. Mucosal immunology of food allergy. *Curr. Biol.* 23, R389–400.
- Birmingham, N., et al., 2003. An ELISA-based method for measurement of food-specific IgE antibody in mouse serum: an alternative to the passive cutaneous anaphylaxis assay. *J. Immunol. Methods* 275, 89–98.
- Bogh, K.L., et al., 2016. Current challenges facing the assessment of the allergenic capacity of food allergens in animal models. *Clin. Transl. Allergy* 6, 21.
- Bossios, A., et al., 2011. Effect of simulated gastro-duodenal digestion on the allergenic reactivity of beta-lactoglobulin. *Clin. Transl. Allergy* 1, 6.
- Bowman, C.C., Selgrade, M.K., 2008. Differences in allergenic potential of food extracts following oral exposure in mice reflect differences in digestibility: potential approaches to safety assessment. *Toxicol. Sci.* 102, 100–109.

- Bozzetto, S., Carraro, S., Giordano, G., Boner, A., Baraldi, E., 2012. Asthma, allergy and respiratory infections: the vitamin D hypothesis. *Allergy* 67, 10–17.
- Breiteneder, H., Mills, E.N., 2005. Molecular properties of food allergens. *J. Allergy Clin. Immunol.* 115, 14–23 quiz 24.
- Brix, S., Bovetto, L., Fritsche, R., Barkholt, V., Frokiaer, H., 2003. Immunostimulatory potential of beta-lactoglobulin preparations: effects caused by endotoxin contamination. *J. Allergy Clin. Immunol.* 112, 1216–1222.
- Buehler, E.V., 1996. Nonspecific hypersensitivity: false-positive responses with the use of Freund's complete adjuvant. *Contact Dermatitis* 34, 111–114.
- Cerecedo, I., et al., 2008. Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J. Allergy Clin. Immunol.* 122, 589–594.
- Chatel, J.M., Bernard, H., Orson, F.M., 2003. Isolation and characterization of two complete Ara h 2 isoforms cDNA. *Int. Arch. Allergy Immunol.* 131, 14–18.
- Ganeshan, K., et al., 2009. Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. *J. Allergy Clin. Immunol.* 123, 231–238 e234.
- Gonipeta, B., Parvataneni, S., Tempelman, R.J., Gangur, V., 2009. An adjuvant-free mouse model to evaluate the allergenicity of milk whey protein. *J. Dairy Sci.* 92, 4738–4744.
- Goodman, R.E., 2006. Practical and predictive bioinformatics methods for the identification of potentially cross-reactive protein matches. *Mol. Nutr. Food Res.* 50, 655–660.
- Goodman, R.E., 2008. Performing IgE serum testing due to bioinformatics matches in the allergenicity assessment of GM crops. *Food Chem. Toxicol.* 46, S24–S34.
- Goodman, R.E., Tetteh, A.O., 2011. Suggested improvements for the allergenicity assessment of genetically modified plants used in foods. *Curr. Allergy Asthma Rep.* 11, 317–324.
- Goodman, R.E., et al., 2008. Allergenicity assessment of genetically modified crops - what makes sense? (vol 26, pg 73, 2008). *Nat. Biotechnol.* 26 241–241.
- Kimber, I., et al., 2001. Skin sensitization testing in potency and risk assessment. *Toxicol. Sci.* 59, 198–208.
- Ladics, G.S., et al., 2010. Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. *Regul. Toxicol. Pharmacol.* 56, 212–224.
- Ladics, G.S., et al., 2014. Allergic sensitization: screening methods. *Clin. Transl. Allergy* 4, 13.
- Mandal, S., Dahuja, A., Kar, A., Santha, I.M., 2014. In vitro kinetics of soybean lipoxigenase with combinatorial fatty substrates and its functional significance in off flavour development. *Food Chem.* 146, 394–403.
- Metsala, J., et al., 2013. Mother's and offspring's use of antibiotics and infant allergy to cow's milk. *Epidemiology* 24, 303–309.
- Mirsky, H.P., Cressman Jr., R.F., Ladics, G.S., 2013. Comparative assessment of multiple criteria for the in silico prediction of cross-reactivity of proteins to known allergens. *Regul. Toxicol. Pharmacol.* 67, 232–239.
- Nordlee, J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A., Bush, R.K., 1996. Identification of a Brazil-nut allergen in transgenic soybeans. *N. Engl. J. Med.* 334, 688–692.
- Nowak-Węgrzyn, A., 2007. Food allergy to proteins. *Nestle Nutrition workshop series. Paediatric programme* 59, 17–31 discussion 31–16.
- Nwaru, B.I., et al., 2014. Prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy*.
- Oyoshi, M.K., Oettgen, H.C., Chatila, T.A., Geha, R.S., Bryce, P.J., 2014. Food allergy: insights into etiology, prevention, and treatment provided by murine models. *J. Allergy Clin. Immunol.* 133, 309–317.
- Palmer, D.J., Prescott, S.L., 2012. Does early feeding promote development of oral tolerance? *Curr. Allergy Asthma Rep.* 12, 321–331.
- Palmer, G.W., et al., 2005. Comparative potency of Ara h 1 and Ara h 2 in immunochemical and functional assays of allergenicity. *Clin. Immunol.* 115, 302–312.
- Prescott, A.G., John, P., 1996. DIOXYGENASES: molecular structure and role in plant metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 245–271.
- Rodriguez, B., et al., 2011. Germ-free status and altered caecal subdominant microbiota are associated with a high susceptibility to cow's milk allergy in mice. *FEMS Microbiol. Ecol.* 76, 133–144.
- Rona, R.J., et al., 2007. The prevalence of food allergy: a meta-analysis. *J. Allergy Clin. Immunol.* 120, 638–646.
- Selgrade, M.K., Bowman, C.C., Ladics, G.S., Privalle, L., Laessig, S.A., 2009. Safety assessment of biotechnology products for potential risk of food allergy: implications of new research. *Toxicol. Sci.* 110, 31–39.
- Sen, M., et al., 2002. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J. Immunol.* 169, 882–887.
- Sicherer, S.H., Munoz-Furlong, A., Godbold, J.H., Sampson, H.A., 2010. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J. Allergy Clin. Immunol.* 125, 1322–1326.
- Smit, J.J., et al., 2011. Contribution of classic and alternative effector pathways in peanut-induced anaphylactic responses. *PLoS One* 6, e28917.
- Smit, J., Zeeuw-Brouwer, M.L., van Roest, M., de Jong, G., van Bilsen, J., 2016. Evaluation of the sensitizing potential of food proteins using two mouse models. *Toxicol. Lett.* 262, 62–69.
- Stanley, J.S., et al., 1997. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Arch. Biochem. Biophys.* 342, 244–253.
- Sun, J., et al., 2007. Impact of CD40 ligand, B cells, and mast cells in peanut-induced anaphylactic responses. *J. Immunol.* 179, 6696–6703.
- Taylor, S.L., et al., 2010. Threshold dose for peanut: risk characterization based upon diagnostic oral challenge of a series of 286 peanut-allergic individuals. *Food Chem. Toxicol.* 48, 814–819.
- Tranbarger, T.J., Franceschi, V.R., Hildebrand, D.F., Grimes, H.D., 1991. The soybean 94-kilodalton vegetative storage protein is a lipoxygenase that is localized in paraveinal mesophyll cell vacuoles. *Plant Cell* 3, 973–987.
- Waggoner, M.R., 2013. Parsing the peanut panic: the social life of a contested food allergy epidemic. *Soc. Sci. Med.* 90, 49–55.