Toxicology Letters 262 (2016) 62–69

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Evaluation of the sensitizing potential of food proteins using two mouse models

Joost Smit[a,b,*], Mary-lène de Zeeuw-Brouwer[b,c], Manon van Roest[a,b], Govardus de Jong[b,c], Jolanda van Bilsen[b,c]

[a] Institute for Risk Assessment Sciences, University Utrecht, Utrecht, The Netherlands
[b] Utrecht Center for Food Allergy, Utrecht, The Netherlands
[c] TNO, Zeist, The Netherlands

HIGHLIGHTS

- Most assays used for the risk assessment of novel proteins show false positive results for low/non-allergens.
- A DC-T cell assay and a mouse model were able to distinguish 5 known allergens from 5 low/non-allergens.
- This method to identify the allergenic risk of new or modified proteins. This method may also elucidate the mechanism of sensitization to food proteins or to improve diagnostics and immunotherapy of food allergy.

ARTICLE INFO

Article history:
Received 24 June 2016
Received in revised form 30 August 2016
Accepted 11 September 2016
Available online 20 September 2016

Keywords:
Allergenic proteins
Dendritic cells
Food allergy
Mouse models
Allergenicity
Sensitization
T cells

ABSTRACT

The current methodology to identify allergenic food proteins is effective in identifying those that are likely to cross-react with known allergens. However, most assays show false positive results for low/non-allergens. Therefore, an *ex vivo/in vitro* DC-T cell assay and an *in vivo* mouse model were used to distinguish known allergenic food proteins (Ara h 1, β-Lactoglobulin, Pan b 1, bovine serum albumin, whey protein isolate) from low/non allergenic food proteins (soy lipoxygenase, gelatin, beef tropomyosin, rubisco, Sola t 1).

CD4+ T cells from protein/alum-immunized mice were incubated with corresponding protein-pulsed bone marrow-derived DC and analyzed for cytokine release. All known allergens induced Th2 responses *in vitro*, whereas soy lipoxygenase, gelatin or beef tropomyosin did not. Sola t 1 and rubisco induced a more generalized T cell response due to endotoxin contamination, indicating the endotoxin-sensitivity of the DC-T assay. To analyze responses *in vivo*, mice were orally sensitized on days 0 and 7. Known allergens induced IgE and mMCP-1 release upon oral challenge at day 16, whereas the low/non-allergens did not.

Both the DC-T cell assay and the mouse model were able to distinguish 5 known allergens from 5 low/non-allergens and may be useful to identify novel allergenic food proteins.

1. Introduction

Sustainable food production, including exploring alternative protein sources such as insects and algae, is receiving a lot of attention and exploration of these sources is widely supported by international organizations. However it is unknown if these proteins pose any health risks, including the potential to induce food allergy. Most of the existing methods for allergenicity prediction are based on (a) the stability to gastric digestion, assuming that proteins that are able to survive passage through the stomach are more likely to be available for subsequent immune responses within or beyond the intestinal mucosa (Berin and Mayer, 2009; Bol-Schoenmakers et al., 2011; Smit et al., 2011a); and (b) the structural similarity of novel proteins to known allergens. So the identification of a novel, structurally distinct

Abbreviations: BLG, beta lactoglobulin; BSA, bovine serum albumin; CT, cholera toxin; DC, dendritic cell; LPS, lipopolysaccharide; mMCP-1, murine mast cell protease-1; TM, tropomyosin WPI whey protein isolate.

* Corresponding author at: Institute for Risk Assessment Sciences, University Utrecht, Utrecht, The Netherlands.
E-mail address: j.j.smit@uu.nl (J. Smit).

http://dx.doi.org/10.1016/j.toxlet.2016.09.005
0378-4274/

allergen may not be predicted by these methods since limited amount of data may be available. Therefore the question on how to assess the allergenicity of new protein sources arises to properly judge their potential as safe new food sources.

The induction of sensitization to food proteins, is a poorly understood and complex process. It seems that most allergens will only lead to sensitization in combination with intrinsic or endogenous innate immune-activating signals, as reviewed previously (Berin and Mayer, 2009; Bol-Schoenmakers et al., 2011; Smit et al., 2011a). In order to trigger sensitization, numerous innate immune cells, including epithelial cells, dendritic cells, basophils, innate lymphoid cells and others all interact to elicit ultimately Th2 immune responses leading to IgE induction. Since this is a complex interplay of many factors, there is a clear need to use models that take the multifactorial aspects of the sensitization phase into account. Not only host factors are involved in this process, quantity and frequency and site of allergen contact are of importance. It is currently matter of debate whether sensitization for food allergens occurs *via* the intestine or whether other sites of exposure (*e.g.* airways or skin) are of importance (Lack, 2012; Wavrin et al., 2014, 2015).

Dendritic cells (DCs) are professional antigen-presenting cells that critically link innate and adaptive immunity and actively participate in allergic sensitization *via* the gut (Rescigno et al., 2001; Worbs et al., 2006). In order to trigger sensitization, an allergen must have the capacity to trigger inflammation at both the epithelial and the DC level (Salazar and Ghaemmaghami, 2013), thereby generating a micro-environment that promotes DC activation and migration to the lymphoid tissue, and finally T cell priming. The quality of this activation will dictate whether the organism becomes sensitized or becomes tolerant. Analyzing these early mechanisms of sensitization, may provide tools to predict whether food proteins may eventually lead to functional changes in the Th2 cytokine profile.

Next to analyzing early events of sensitization *in vitro/ex vivo*, animal models may be useful to gain a more holistic insight into the mechanisms of sensitization to food proteins and development of food allergy with clinical manifestations.

Several attempts have been made to develop rodent and non-rodent animal models to test for potential food allergens (Berin and Mayer, 2009; Bol-Schoenmakers et al., 2011; Smit et al., 2011a). The ideal model should mimic human disease in terms of exposure route, disease mechanisms and symptomology. Skin exposure has been proposed to be a driving factor in sensitization to foods while tolerance development would occur *via* oral consumption, particularly in allergy to peanut (Lack, 2012). However, the mechanisms leading to food sensitization are still unknown, including the route of exposure. Nevertheless, the oral route might be considered the most appropriate route of exposure when considering an animal model. This is particularly relevant in light of the clearly established role of oral tolerance in regulating systemic IgE responses as well as the ambiguous relationship between allergenicity and digestibility. In laboratory animals the predominant response to ingestion of soluble proteins is usually oral tolerance. Therefore an adjuvant, such as cholera toxin (CT) is most used in these models to overcome oral tolerance. The mechanism for the adjuvant function of CT is not known, but an important aspect is the disruption of the intestinal epithelial cell layer by interruption of tight junction molecules *via* a cAMP-driven mechanism (Guichard et al., 2013).Cholera toxin disrupts barrier function by inhibiting exocyst-mediated trafficking of host proteins to intestinal cell junctions. This again fits closely with the immunological concept that sensitization requires a danger signal, such as epithelial damage.

Importantly, a food allergy model should be able to demonstrate differences between known allergenic and non-allergenic foods. None of the food allergy animal models using adjuvants that have been used so far (Berin and Mayer, 2009; Bol-Schoenmakers et al., 2011; Smit et al., 2011a) have been thoroughly tested with a wide range of allergens and low/non-allergens. Previously Bowman et al. described a promising model in which a small panel of extracts from 3 known allergenic foods (peanut, egg white, brazil nut) and 2 low-allergenic foods (spinach, turkey) was tested (Bowman and Selgrade, 2008). It was suggested that it may be possible to distinguish allergenic foods from non/low allergenic foods in C3H/HeJ mice using a protocol in which food extracts are administered orally with CT, provided the exposure is short term (2 weeks) and the exposure dose does not exceed 2 mg.

So despite decades of research looking for the ideal model that would predict the sensitizing potential of novel or native proteins, to date no single predictive model is available which incorporates all necessary multifactorial aspects of the sensitization phase. Therefore we propose a two-step model in which we combine (a) *in vitro/ex vivo* testing of stimulatory effects of food proteins on the innate immune and the adaptive immune system with (b) an animal model where antibody sensitization and reactions to clinical challenges act as outcome parameters.

Previously Pochard et al. (2010) described an *in vitro/ex vivo* system to characterize the effect of TLR-activation on DCs of peanut-induced responses of DCs and T cells. We adapted this method to investigate whether high and low allergenic proteins activate DCs differently, resulting in differences in the quality of protein-specific T cell responses, elucidating the early events of possible sensitization.

Using an *in vivo* model, we tested a panel of high and low allergenic proteins in C3H/HeOuJ mice a strain which has been shown also to be able to produce specific IgE and IgG1 and mount strong allergic effector responses, such as mast cell degranulation and systemic anaphylaxis (Smit et al., 2011b). The presented models provide a qualitative answer with respect to sensitizing potency of food proteins.

2. Materials and methods

2.1. Food proteins

BSA (A7906, ≥98% protein) and Gelatin type A (G-6144, 95% protein) were obtained from Sigma Aldrich. Whey protein isolate (WPI) (Bipro, 91% protein) was obtained from Davisco and contained BLG (±65%), alpha-lactalbumin (±25%), bovine serum albumin (±8%). Ara h 1 (peanut 7S vicilin-like globulin) was purified from de-fatted unroasted peanut flour, as described previously (de Jong et al., 1998). Rubisco was purified from spinach as described previously (Martin et al., 2014). BLG was purified from whey protein isolate (Bipro, Davisco) using an anion exchange chromatography (Source Q, GE Healthcare). Whey proteins were eluted using a gradient from 0 to 0.5 M NaCl in 20 m Tris/HCl pH 8.0. BLG containing fractions were desalted using a Sephadex G25 column (GE Healthcare) equilibrated and eluted with Milli Q. The final sample was lyophilized. Pan b 1 (shrimp tropomyosin) was isolated from Pandalus borealis by boiling shrimp for 1 h in 10 mM sodium phosphate pH 7.0. After centrifugation (20 min, 15,000*g*), the supernatant was used in ammonium sulphate precipitation. The 60% pellet after centrifugation was solubilized in Milli Q and dialyzed overnight against Milli Q. The dialysate was set at pH 7 using 20 mM TRIS pH 8 and used for anion exchange (Source Q, GE Healthcare). A gradient of 0–0.6 M NaCl in 20 mM TRIS pH 8 was used to elute the tropomyosin. Fractions containing tropomyosin were concentrated using Millipore steered ultrafiltration cells and dialyzed against water using diafiltration. Samples were stored frozen at – 80 °C. Beef tropomyosin was prepared from raw beef by Gary Bannon (Knippels and Penninks, 2003).

Sola t 1 (patatin) was isolated from cultivar Vivaldi, new harvest Egypt. Peeled potatoes were cut and put into ice cold 50 mM NaCl pH 6.5 containing 2% sodium metabisulphite. After 30 min incubation juice was produced using an Angel Juicer followed by centrifugation (20 min 215,000*g*) to remove the insoluble starch and the supernatant was used in ammonium sulphate precipitation. The 40–70% pellet after ammonium sulphate precipitation and centrifugation was solubilized in PBS containing 0.2% sodium metabisulphite and further purified using size exclusion in PBS (Superdex 75, GE Healthcare). Fractions containing Sola t 1 were concentrated using Millipore steered ultrafiltration cells. Samples were stored frozen at – 80 °C. Lipoxygenase was isolated from defatted soy flour extracted with 50 mM acetate buffer pH 5. The extract was filtered and centrifuged (20 min 15,000*g*) and applied to a 120 ml SP Sepharose column (GE Healthcare) with elution using a 440 ml linear salt gradient of 0–200 mM NaCl in 50 mM sodium acetate buffer pH 5.5. The collected lipoxygenase fractions were pooled and desalted using a 720 ml Sephadex G25 column (GE Healthcare) in MilliQ. The protein contents of the isolated proteins are >95%.

2.2. Endotoxin testing

Endotoxin contamination of proteins was determined in a bioassay in which the ability of proteins to stimulate tumour necrosis factor (TNF)-α production in RAW 264.7 macrophages (ATCC, USA) was compared with effects of different concentrations of LPS. For this purpose 1.25×10^5 cells were plated for 24 h, followed by exposure to proteins (1–100 μg/ml) or LPS (0.1 ng–100 μg/ml). After 24 h, TNF-α production was measured by ELISA (eBioscience, Austria).

2.3. Mice

This study was conducted with female C3H/HeOuJ mice which were obtained from a colony maintained under SPF conditions at Charles River, Sulzfeld, Germany. At commencement of the sensitization (day 0), the mice were 7 weeks old. Mice were maintained on a 12-h light/dark cycle and allowed access to food (cereal-based VRF1 diet; SDS Special Diets Services, Whitham, England; certified free of test proteins) and water *ad libitum*. The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive 2010/63/EU) and Dutch legislation (The Experiments on Animals Act, 1997). This included approval of the study by TNO's and Utrecht University's Animal experimental committees (DEC-numbers 3640, 2013. III.01.003).

2.4. Dendritic cell/T cell assay

This assay was used as described previously (Pochard et al., 2010). In short, 2–3 C3H/HeOuJ mice were immunized by intraperitoneal injection with 100 μg protein or WPI-proteins bound to imject-alum (Pierce, USA) on day 0 and 14. After 29 days, the CD4+ T cells were isolated from spleen single cell suspensions using magnetic beads (Stemcell Technologies, France). Bone marrow cells from C3H/HeOuJ mice were cultured for 6 days with GM-CSF (R&D, United Kingdom), after which the bone marrow-derived dendritic cells (DC) were pulsed overnight with 50 or 100 μg/ml protein. The CD4+ T cells were incubated for 72 h with protein-pulsed dendritic cells. Hereafter, IL-5, IL-13 and IFN-γ production was analyzed by sandwich ELISA (eBioscience, Austria). IL-4 production was below the detection limit of ELISA.

2.5. Sensitization and challenge

Mice were gavaged two times at a weekly interval with 2 mg of food proteins or 20 mg WPI in PBS with 10 μg cholera toxin (CT; List Biological Laboratories, Inc., Campbell, CA). Control animals received PBS and CT only. The sensitization dose was based on Bowman et al. (Bowman and Selgrade, 2008) and Van Esch et al. (van Esch et al., 2013). Protein-exposed groups consisted of six animals (n = 6) whereas control groups consisted of 3 animals (n = 3). On day 16, all mice, except mice from the rubisco and beef TM group, received an oral challenge by gavage with 50 mg food protein or 100 mg WPI in PBS. It was not possible to challenge mice with rubisco and beef tropomyosin because of stock concentration and viscosity issues. Thirty minutes after oral challenge, blood samples were collected. Blood samples were centrifuged at room temperature for 15 min at 13,500 rpm and sera were stored at −20 °C until further analysis for protein-specific antibodies and mouse mast cell protease-1 (mMCP-1) as a reflection of mast cell activity. From human studies it is known that threshold distribution levels to elicit clinical symptoms differ between different allergenic foods (Kruizinga et al., 2008; Spanjersberg et al., 2007). So for the challenge, a high dose was chosen to ensure that the dosing was high enough to overcome the threshold dose to elicit elicitation.

2.6. Measurement of protein-specific IgE and IgG1

Concentrations of protein-specific IgE and protein-specific IgG1 were determined in serum collected at day 16 by means of ELISA. All volumes added were 100 μl unless otherwise noted. Flat-bottom MaxiSorp plates (VWR, 735-0038) were coated overnight at 4 °C with 10 μg/ml protein or 20 μg/ml WPI, dissolved in carbonate-bicarbonate buffer (Thermo Scientific,28382). The plates were washed three times with 250 μl/well washing buffer (PBS/0.1% Tween20). Following blocking with 200 μl/well blocking buffer (PBS/4%BSA/0.1%Tween20), serial dilutions of serum (in PBS/1%BSA/0.05% Tween20) were incubated in duplicate (2 h, 37 °C). In the BSA-specific ELISA, the BSA in the wash and blocking buffer was replaced by fish gelatin (same%). Starting dilutions for IgG1 and IgE were 1/32 and 1/8 respectively (two times dilution-steps, 7 dilutions in total). After washing, plates were incubated with biotinylated detection antibody (rat anti-mouse IgG1 or IgE; BD Biosciences; 553441/553419; 45 min, 37 °C). Hereafter, plates were washed and incubated with poly-horseradish peroxidase-conjugated streptavidin (Sanquin, M2032; 30 min 37 °C) prior to a final wash and addition of tetramethylbenzidine substrate (TMB; Sigma-Aldrich, T0440; 2–15 min at room temperature in the dark). Reactions were stopped with 1 M H2SO4 (Sigma-Aldrich) and absorbance at 450 nm wavelength was measured using a Synergy™ HT Microplate Reader (BioTek, USA). Each plate contained blanc wells and serum (or serum pool) collected from the corresponding PBS-treated control group. Samples were considered positive for the presence of protein-specific antibody, when the optical density recorded for that dilution was at least 2-fold higher than the optical density recorded for serum collected in the PBS-treated control group (orally challenged with the same protein) at the same dilution. Titers were expressed as 2log values.

2.7. Measurement of mouse mast cell protease-1 after oral challenge

Serum concentrations of mMCP-1 were determined according to the manufacturer's protocol using a commercially available ELISA kit (eBioscience, Austria).

2.8. Statistical analysis

Data are presented as means ± standard error of the mean (SEM) and analyzed using GraphPad Prism software. Cytokine levels were logarithmic transformed followed by a one-way ANOVA and Bonferroni as a post-hoc test. Antibody and MMCP-I levels were analyzed by Mann Whitney *U* test, followed by Dunn's Multiple comparison test.

3. Results

3.1. Sola t 1 and rubisco are contaminated with endotoxin

First, we determined the biological endotoxin activity of the food proteins used. For this purpose we measured TNF-α production after protein exposure of RAW macrophages, which are exquisitely sensitive to endotoxin. In this assay, Sola t 1 and rubisco spiked TNF-α production (Fig. 1), indicative of high endotoxin contamination. The other used food proteins: Ara h 1, BLG, BSA, WPI, shrimp and beef tropomyosin, soy lipoxygenase and gelatin were considered low or negative endotoxin contaminated.

3.2. Endotoxin-free high allergenic food proteins induce Th2 responses ex vivo *in contrast to low/non allergenic proteins*

To determine the induction of T cell responses to food proteins we analyzed the cytokine production of DC-T cell co-cultures. Mice immunized intraperitoneally by food proteins showed comparable levels of specific IgG (data not shown) demonstrating that T cell responses to these proteins did develop *in vivo*. Restimulation of CD4+ T cells derived from Ara h 1, BLG, BSA, WPI and shrimp tropomysin immunized mice with corresponding protein-pulsed DC resulted in production of IL-5 and IL-13 but no or little IFN-γ (Fig. 2a + b). Soy lipoxygenase, gelatin and beef tropomyosin did not induce these cytokines. Restimulation of CD4+ T cells from the endotoxin contaminated food proteins rubisco and Sola t 1 immunized mice induced high levels of IL-5, IL-13 and IFN-γ (Fig. 2c), showing a more generalized T cell response to these proteins.

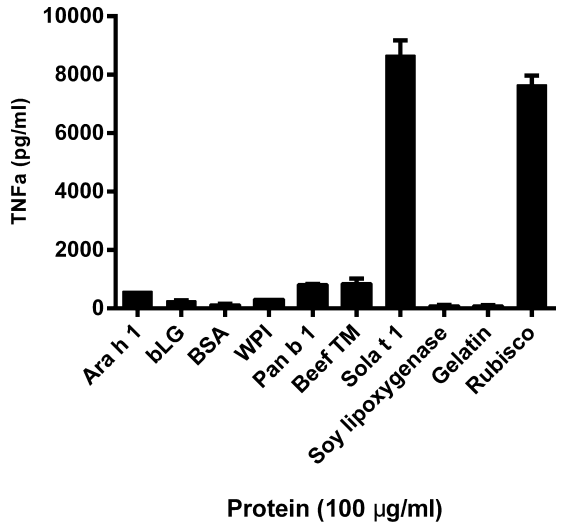

Fig. 1. Determination of endotoxin contamination of proteins in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with 1 (not shown) or 100 µg/ml of purified proteins or LPS (1 fg–10 µg/ml; data not shown). After 24 h, TNFα production was analyzed in the supernatant by ELISA. Each protein-condition was assayed in duplicate; data represent the mean TNFα concentration ± SEM.

3.3. High allergenic food proteins induce ag-specific IgG1 and IgE and mast cell degranulation whereas low allergens do not

To study allergic sensitization *in vivo*, mice were intragastrically exposed to all food proteins and adjuvant. Exposure to Ara h 1, BLG, BSA, WPI and Pan b 1 led to induction of protein specific IgG1 and IgE as compared to PBS-sensitized animals (Fig. 3).

In contrast, all tested non/low allergenic proteins Sola t 1, soy lipoxygenase, gelatin, rubisco and beef tropomyosin, did not elicit protein-specific IgG1 and IgE as compared to PBS-sensitized animals.

Finally, to investigate effector mucosal mast cell responses, all groups were orally challenged 50 mg of protein, including their PBS-control groups. Unfortunately, it was not possible to dose mice with rubisco and beef tropomyosin at this concentration because of stock concentration and viscosity issues. Oral challenge with known allergenic proteins Ara h 1, BLG, BSA, WPI and Pan b 1 resulted in a significant mMCP-1 response in the serum, indicative of mucosal mast cell degranulation, in contrast with the PBS-sensitized control groups (Fig. 4). In contrast, oral challenge with the known non/low allergenic proteins Sola t 1, soy lipoxygenase and gelatin did not induce an mMCP-1 release.

4. Discussion

In recent years, we have seen a huge increase in knowledge about food allergens, with more information on chemical and physical characteristics of individual allergens. Interestingly, food allergens belong to a small number of protein families. Only 2% of protein families known so far contain allergens (Radauer et al., 2008). This certainly has consequences for the risk assessment of novel proteins, whether (genetically or chemically) modified or conventional/native. However, it is still unknown why certain proteins are allergenic, compared to the large majority of food proteins which do not display any allergenicity. This suggests that subtle differences in physical or biological properties or in other external factors modulate the allergic response. It is therefore of importance to be able to distinguish allergenic proteins from the indefinitely present non-allergenic food proteins. In this study, we investigated with a panel of food proteins if we can distinguish known allergenic proteins from low or non-allergenic proteins. In the two complementary models used, we observed that the known allergens Ara h 1, BLG, BSA, WPI and Pan b 1 indeed induced Th2 responses, IgE and mast cell degranulation, while low/non-allergens soy lipoxygenase, gelatin and beef tropomyosin did not.

In silico pre-screening of putative allergens can be very useful to narrow down the number of proteins in follow-up *in vitro*/*ex vivo*/*in vivo* experiments. It has been described that certain protein structures and sequences are more allergenic than others: animal proteins that resemble their human homologue proteins (sequence identity of >62%), rarely lead to allergenicity (Jenkins et al., 2007). In contrast, proteins that resemble known allergens, are more likely to be an allergen, by inducing cross reactivity. For transgenic proteins, IgE cross reactivity is considered by FAO/WHO when there is >35% identity over a sliding 'window' of 80 amino acids (FAO/WHO, 2001). However, the sliding window methodology is not suitable to identify de novo sensitization.

An overall mechanistic model of how food proteins may sensitize the human immune system is not available. Certainly, not every molecule will have the properties to induce sensitization, either lacking the required intrinsic pro-allergenic properties or being presented in the wrong context (Ladics et al., 2014; McClain et al., 2014; Poulsen et al., 2014).

Several aspects have been shown to influence sensitization or tolerance to food proteins, such as (a) exposure routes; (b) frequency and dose of exposure; (c) dose–response relationships;

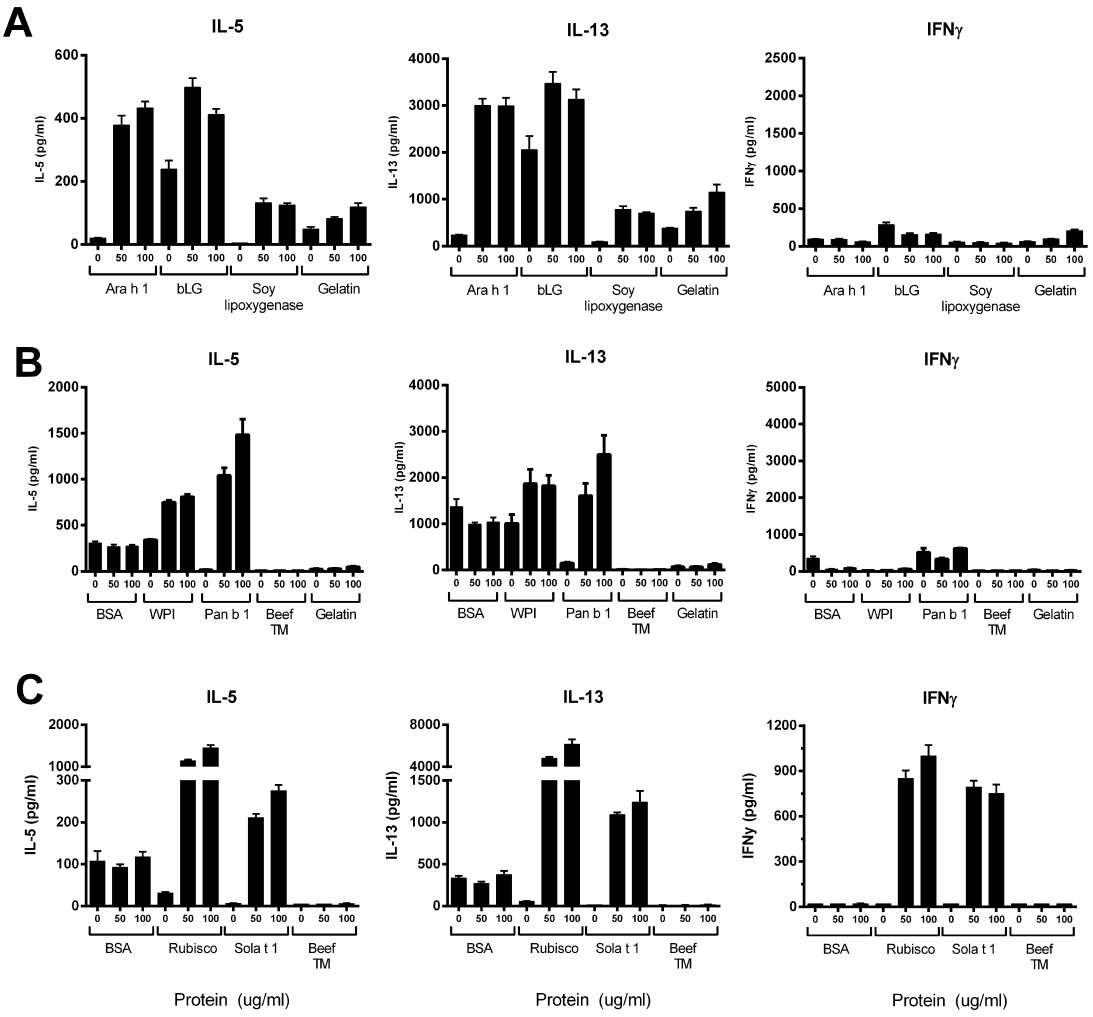

Fig. 2. Cytokines production of DC-T cell co-cultures. CD4+ T cells from protein/alum-immunized mice were incubated for 72 h with protein-pulsed bone marrow derived DC. Cell supernatants were analyzed using ELISA. All food proteins were tested at least two times in independent experiments. Representative experiments are depicted in (A + B) experiments testing low-endotoxin containing proteins and (C) experiment testing low- (BSA and beef TM) and high-endotoxin (Sola t 1 and rubisco) containing proteins. Data represent the mean IL-5, IL-13 or IFN-γ concentration ± SEM.

(d) role of digestion, food processing, and the food matrix; (e) role of infection; (f) role of the gut microbiota; (g) influence of the structure and physicochemical properties of the protein; and (h) the genetic background and physiology of the consumers, as recently reviewed by the Protein Allergenicity Technical Committee (PATC) of the International Life Sciences Institute's Health (ILSI) and Environmental Science Institute (HESI) (Ladics et al., 2014; McClain et al., 2014; Poulsen et al., 2014)

In mouse models *in vivo*, the mucosal adjuvant cholera toxin (CT) is generally used to induce allergic sensitization to co-administered protein *via* the gastrointestinal route. The display of allergic parameters from sensitization to clinical manifestations depends on the used mouse strain (Smit et al., 2011b). CT induces innate immune changes that trigger allergen-specific T- and B-cell responses, leading to an allergic phenotype. These innate immune changes involve among others activation of epithelial cells, intraepithelial lymphocytes, dendritic cells, and induction of co-stimulatory molecules (Berin and Mayer, 2009; Bol-Schoenmakers et al., 2011; Smit et al., 2011a). It is important to realize that, although the principal allergic response in the used allergy model is driven by CT, the properties of the administered protein may be even more decisive. Prolonged exposure (more than 28 days) may elicit responses to most administered proteins (allergen and non-allergen) (Van Bilsen, unpublished results). However, short exposure (14 days) as used before (Bowman and Selgrade, 2008) and in the present study leads to clear discriminative differences in the allergic response to food proteins.

It is still matter of debate whether it is feasible to perform a real quantitative analysis on food protein allergenicity, or whether allergenicity, is an all-or-nothing response. Importantly, the use of *in vivo* and *in vitro* models for predicting allergenicity have addressed the issue of discriminating potent allergenic from weak or non-allergenic proteins. Published data so far demonstrate a lack of reproducible and predictive allergenicity assessment for low, or even more important, intermediate allergenic proteins. In order to develop such models further, a wide range of well-characterized, purified, and endotoxin free weak or non-allergens are required. Moreover, in order to determine quantitatively the protein allergenicity, criteria are needed to classify and thereby identify the different classes of low-intermediate-high allergenic proteins (Houben et al., 2016).

The first studies comparing food proteins and allergenicity proposed that stability to gastric digestion *in vitro* significantly discriminated known allergens from non-allergens (Astwood et al., 1996). However, following studies with a wider range of proteins did not find such a relationship (Fu et al., 2002). This elicited the

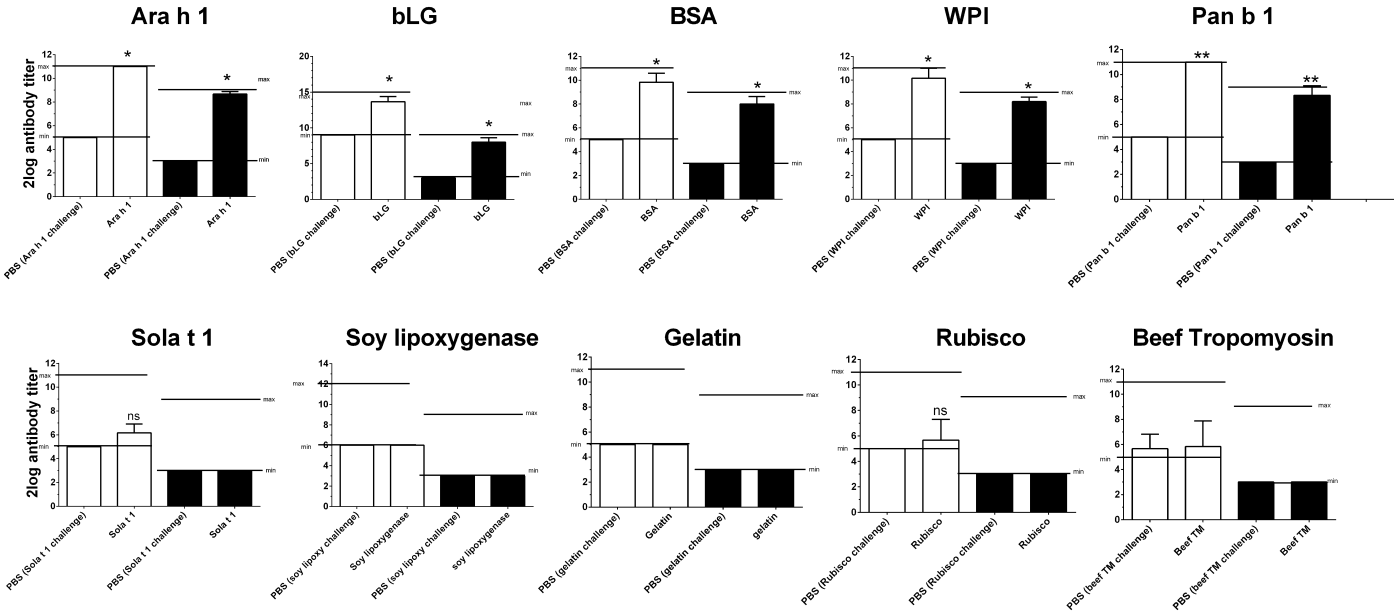

Fig. 3. Protein-specific antibody responses to food proteins *in vivo*. Mice were gavaged two times at a weekly interval with food proteins in PBS with CT. Control animals received PBS and CT only. On day 16, protein-specific IgE (black bars) and IgG1 (white bars) responses in serum were determined. Data represent the mean 2log antibody titer ±SEM. (*$p < 0.05$ or **$P < 0.01$ compared to PBS-sensitized mice; ns: not significant). Horizontal lines represent the detection limits of the assays (min/max). All food proteins were tested at least two times in independent experiments.

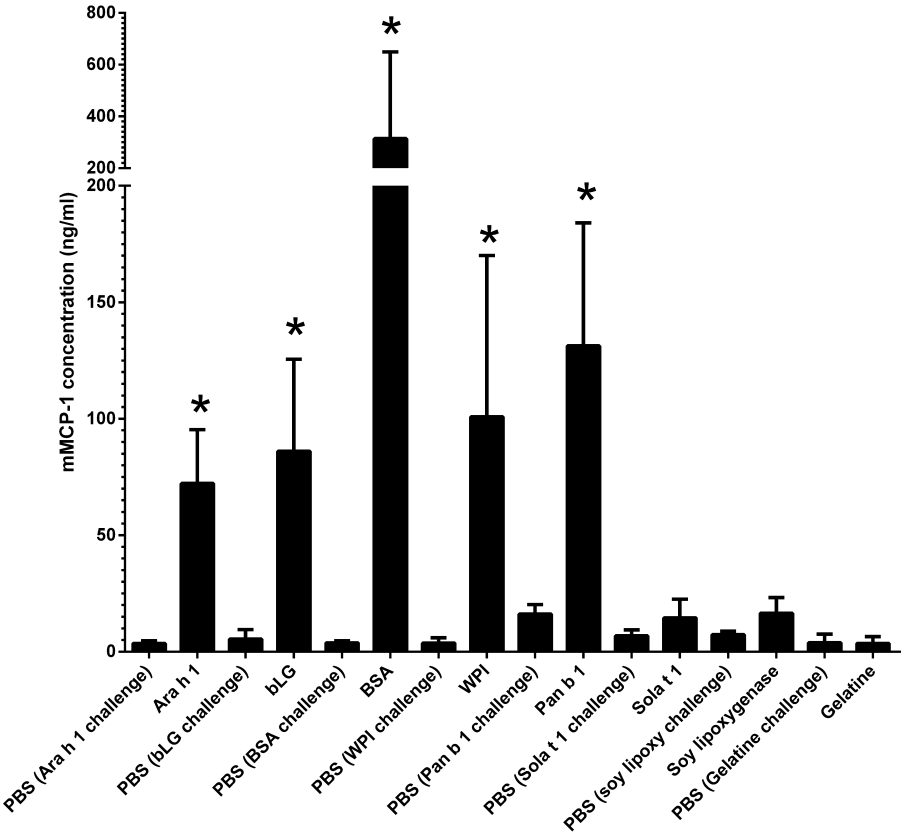

Fig. 4. Allergic manifestations of food proteins *in vivo*. Mice were gavaged two times at a weekly interval with food proteins in PBS with CT. Control animals received PBS and CT only. On day 16, all mice received an oral challenge by gavage with food protein in PBS. Thirty minutes after oral challenge, blood was taken for mMCP-1 analysis, as measurement of mucosal mast cell degranulation. Due to technical issues, animals were not challenged with beef TM or rubisco. Data represent the mMCP-1 concentration (ng/ml) ±SEM. (*$p < 0.05$ compared to PBS-sensitized mice; ns: not significant). All food proteins were tested at least two times in independent experiments.

need of other parameters, including *in vivo* models, to asses the allergenic or non-allergenic potential of proteins. Reports by Dearman and Kimber (Dearman and Kimber, 2001) showed that known allergenic proteins (peanut agglutinin, ovalbumin) induced protein specific IgE upon intraperitoneal injection of mice, while assumed non-allergenic proteins (potato agglutinin and potato acid phosphatase) were immunogenic but only produced low titer IgE responses. In contrast, a multi laboratory study was not able to accurately differentiate between known allergens and putative non-allergens, including rubisco (from spinach) and lipoxygenase (from soy) using the systemic route of administration (Ladics et al., 2010). Complementary to our studies, it was shown that oral exposure to allergens under appropriate experimental conditions (*e.g.* the use of an adjuvant) was able to distinguish allergenic from non-allergenic food extracts while systemic exposure did not (Bowman and Selgrade, 2008). Important in this respect is to mention that in our studies and those of others before, especially plant-derived allergens are highly contaminated with endotoxin, hereby complicating the above mentioned studies. Strikingly, in human T cells it was shown that even low levels of endotoxin enhance allergen-stimulated proliferation and reduce the threshold for activation (Velickovic and Jankov, 2008). This was observed in our studies as well, endotoxin contaminated proteins were highly immunostimulatory *in vitro*. This was not observed *in vivo*, most likely since endotoxin in the intestine is already highly present.

Another confounding factor in previous experiments has been the use of highly purified proteins versus semi-purified or crude extracts in this respect. We used well defined and highly purified protein isolates to measure the actual responses to a single protein, compared to the more complex and less defined responses to whole food extracts. It is known that the matrix of food proteins can influence responses to individual proteins (van Wijk et al., 2005) dependent on the route of administration (Wavrin et al., 2015). Even so, proteins from the same source display different allergenic properties while being ingested in the same matrix (*e.g.* not all proteins in peanut are allergenic and allergenic peanut proteins induce significantly different allergic responses (Smit et al., 2015)), so also protein-specific factors are likely to be involved. Moreover, for risk assessment purposes, the individual food proteins need to be evaluated, therefore assays testing purified proteins without their matrix are a necessity.

Despite the discriminative responses seen in our 2 models there are practical considerations and (dis)advantages using the described *in vivo* and *ex vivo*/*in vitro* models. The *in vivo* model as used in our studies is insensitive to endotoxin contamination but the amount of protein needed is relatively high (approx. 500 mg). Furthermore, studying a large panel of proteins *in vivo* would probably require a high number of mice. Moreover, it may not always be possible to administer the appropriate dose since it is sometimes impossible to dose highly concentrated viscous protein-fractions, as we observed for rubisco and beef tropomyosin. In our study, rubisco and beef tropomyosin did not induce an increased IgE response, therefore, despite the absence of an oral challenge, it is highly unlikely that these proteins are allergenic in our *in vivo* system. The *in vitro/ex vivo* model used in our studies can be used to study a large panel of proteins, requires less protein amount but is very sensitive to endotoxin contamination.

In conclusion, both the DC-T cell assay and the *in vivo* mouse model were able to distinguish 5 known allergens from 5 low/non-allergens. This approach may prove to be a promising method to identify new allergenic proteins which have not been experienced previously or to identify the risk of modified proteins in the diet. This requires a further validation with more (food) proteins. In addition, the described models may also be used to elucidate the mechanism of sensitization to food proteins in the intestine.

Conflict of interest

The authors have declared no conflicts of interest.

Author contributions

MZB has carried out the animal studies, MR has carried out the DC-T assay, GJ has purified and analyzed the test proteins, JB has supervised the study, JS and JB have performed data analysis and have written the manuscript.

Acknowledgment

The work was financially supported by Dutch governmental Research Cooperation Funds.

References

Astwood, J.D., Leach, J.N., Fuchs, R.L., 1996. Stability of food allergens to digestion in vitro. Nat. Biotechnol. 14, 1269–1273. doi:http://dx.doi.org/10.1038/nbt1096-1269.

Berin, M.C., Mayer, L., 2009. Immunophysiology of experimental food allergy. Mucosal Immunol. 2, 24–32. doi:http://dx.doi.org/10.1038/mi.2008.72.

Bol-Schoenmakers, M., Marcondes Rezende, M., Bleumink, R., Boon, L., Man, S., Hassing, I., Fiechter, D., Pieters, R.H., Smit, J.J., 2011. Regulation by intestinal gammadelta T cells during establishment of food allergic sensitization in mice. Allergy 66, 331–340. doi:http://dx.doi.org/10.1111/j.1398-9995.2010.02479.x.

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. doi:http://dx.doi.org/10.1093/toxsci/kfm288.

de Jong, E.C., Van Zijverden, M., Spanhaak, S., Koppelman, S.J., Pellegrom, H., Penninks, A.H., 1998. Identification and partial characterization of multiple major allergens in peanut proteins. Clin. Exp. Allergy 28, 743–751.

Dearman, R.J., Kimber, I., 2001. Determination of protein allergenicity: studies in mice. Toxicol. Lett. 120, 181–186 S0378-4274(01)00276-4[pii].

FAO/WHO, 2001. Report of Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, 22–25 January. .

Fu, T.J., Abbott, U.R., Hatzos, C., 2002. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid-a comparative study. J. Agric. Food Chem. 50, 7154–7160 jf020599h [pii].

Guichard, A., Cruz-Moreno, B., Aguilar, B., van Sorge, N.M., Kuang, J., Kurkciyan, A.A., Wang, Z., Hang, S., Pineton de Chambrun, G.P., McCole, D.F., Watnick, P., Nizet, V., Bier, E., 2013. Cholera toxin disrupts barrier function by inhibiting exocyst-mediated trafficking of host proteins to intestinal cell junctions. Cell Host Microbe 14, 294–305. doi:http://dx.doi.org/10.1016/j.chom.2013.08.001.

Houben, G., Burney, P., Chan, C.H., Crevel, R., Dubois, A., Faludi, R., Klein Entink, R., Knulst, A., Taylor, S., Ronsmans, S., 2016. Prioritisation of allergenic foods with respect to public health relevance: report from an ILSI Europe Food Allergy Task Force Expert Group. Food Chem. Toxicol. 89, 8–18 S0278-6915(15)30138-1[pii].

Jenkins, J.A., Breiteneder, H., Mills, E.N., 2007. Evolutionary distance from human homologs reflects allergenicity of animal food proteins. J. Allergy Clin. Immunol. 120, 1399–1405 S0091-6749(07)01585-0[pii].

Knippels, L.M., Penninks, A.H., 2003. Assessment of the allergic potential of food protein extracts and proteins on oral application using the brown Norway rat model. Environ. Health Perspect. 111, 233–238.

Kruizingaa, A.G., Briggsb, D., Crevelb, R.W.R., Knulstc, A.C., van den Boscha, L.M.C., Houbena, G.F., 2008. Probabilistic risk assessment model for allergens in food: sensitivity analysis of the minimum eliciting dose and food consumption. Food Chem. Toxicol. 46, 1437–1443.

Lack, G., 2012. Update on risk factors for food allergy. J. Allergy Clin. Immunol. 129, 1187–1197. doi:http://dx.doi.org/10.1016/j.jaci.2012.02.036.

Ladics, G.S., Knippels, L.M., Penninks, A.H., Bannon, G.A., Goodman, R.E., Herouet-Guicheney, C., 2010. Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. Regul. Toxicol. Pharmacol. 56, 212–224. doi:http://dx.doi.org/10.1016/j.yrtph.2009.09.018.

Ladics, G.S., Fry, J., Goodman, R., Herouet-Guicheney, C., Hoffmann-Sommergruber, K., Madsen, C.B., Penninks, A., Pomes, A., Roggen, E.L., Smit, J., Wal, J.M., 2014. Allergic sensitization: screening methods. Clin. Transl. Allergy 4 doi:http://dx.doi.org/10.1186/2045-7022-4-13 13-7022-4-13.

Martin, A.H., Nieuwland, M., de Jong, G.A., 2014. Characterization of heat-set gels from RuBisCO in comparison to those from other proteins. J. Agric. Food Chem. 62, 10783–10791. doi:http://dx.doi.org/10.1021/jf502905g.

McClain, S., Bowman, C., Fernandez-Rivas, M., Ladics, G.S., Ree, R., 2014. Allergic sensitization: food- and protein-related factors. Clin. Transl. Allergy 4 doi:http://dx.doi.org/10.1186/2045-7022-4-11 11-7022-4-11.

Pochard, P., Vickery, B., Berin, M.C., Grishin, A., Sampson, H.A., Caplan, M., Bottomly, K., 2010. Targeting Toll-like receptors on dendritic cells modifies the T(H)2 response to peanut allergens in vitro. J. Allergy Clin. Immunol. 126 doi:http://dx.doi.org/10.1016/j.jaci.2010.04.003 92–7.e5.

Poulsen, L.K., Ladics, G.S., McClain, S., Doerrer, N.G., van Ree, R., 2014. Sensitizing properties of proteins: executive summary. Clin. Transl. Allergy 4 doi:http://dx.doi.org/10.1186/2045-7022-4-10 10-7022-4-10.

Radauer, C., Bublin, M., Wagner, S., Mari, A., Breiteneder, H., 2008. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J. Allergy Clin. Immunol. 121, 847–852.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., Ricciardi-Castagnoli, P., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol. 2, 361–367. doi:http://dx.doi.org/10.1038/86373.

Salazar, F., Ghaemmaghami, A.M., 2013. Allergen recognition by innate immune cells: critical role of dendritic and epithelial cells. Front. Immunol. 4, 356. doi:http://dx.doi.org/10.3389/fimmu.2013.00356.

Smit, J.J., Bol-Schoenmakers, M., Hassing, I., Fiechter, D., Boon, L., Bleumink, R., Pieters, R.H., 2011a. The role of intestinal dendritic cells subsets in the establishment of food allergy. Clin. Exp. Allergy 41, 890–898. doi:http://dx.doi.org/10.1111/j.1365-2222.2011.03738.x.

Smit, J.J., Willemsen, K., Hassing, I., Fiechter, D., Storm, G., van Bloois, L., Leusen, J.H., Pennings, M., Zaiss, D., Pieters, R.H., 2011b. Contribution of classic and alternative effector pathways in peanut-induced anaphylactic responses. PLoS One 6, e28917. doi:http://dx.doi.org/10.1371/journal.pone.0028917.

Smit, J.J., Pennings, M.T., Willemsen, K., vanRoest, M., vanHoffen, E., Pieters, R.H., 2015. Heterogeneous responses and cross reactivity between the major peanut allergens Ara h 1, 2,3 and 6 in a mouse model for peanut allergy. Clin. Transl. Allergy 5. doi:http://dx.doi.org/10.1186/s13601-015-0056-9 13-015-0056-9, eCollection 2015.

Spanjersberg, M.Q., Kruizinga, A.G., Rennen, M.A., Houben, G.F., 2007. Risk assessment and food allergy: the probabilistic model applied to allergens. Food Chem. Toxicol. 45, 49–54.

van Esch, B.C., van Bilsen, J.H., Jeurink, P.V., Garssen, J., Penninks, A.H., Smit, J.J., Pieters, R.H., Knippels, L.M., 2013. Interlaboratory evaluation of a cow's milk allergy mouse model to assess the allergenicity of hydrolysed cow's milk based infant formulas. Toxicol. Lett. 220, 95–102. doi:http://dx.doi.org/10.1016/j.toxlet.2013.04.008.

van Wijk, F., Nierkens, S., Hassing, I., Feijen, M., Koppelman, S.J., de Jong, G.A., Pieters, R., Knippels, L.M., 2005. The effect of the food matrix on in vivo immune responses to purified peanut allergens. Toxicol. Sci. 86, 333–341 kfi187[pii].

Velickovic, T.C., Jankov, R.M., 2008. Design and modifications of allergens for improving specific immunotherapy. Inflamm. Allergy Drug Targets 7, 270–278.

Wavrin, S., Bernard, H., Wal, J.M., Adel-Patient, K., 2014. Cutaneous or respiratory exposures to peanut allergens in mice and their impacts on subsequent oral exposure. Int. Arch. Allergy Immunol. 164, 189–199. doi:http://dx.doi.org/10.1159/000363444.

Wavrin, S., Bernard, H., Wal, J.M., Adel-Patient, K., 2015. Influence of the route of exposure and the matrix on the sensitisation potency of a major cows' milk allergen. Clin. Transl. Allergy 5 doi:http://dx.doi.org/10.1186/s13601-015-0047-x 3-015-0047-x. eCollection 2015.

Worbs, T., Bode, U., Yan, S., Hoffmann, M.W., Hintzen, G., Bernhardt, G., Forster, R., Pabst, O., 2006. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. J. Exp. Med. 203, 519–527 jem.20052016[pii].