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The use of animal models to discover immunological mechanisms underpinning sensitization to food allergens

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In almost all countries, food allergy is of growing concern affecting all age groups. Given the increased prevalence of food allergies, current research focuses on developing new treatment strategies and to predict allergenicity of novel and modified food proteins. The recent use of animal models has significantly contributed to a better understanding of the complex immunological and pathophysiological mechanisms of food allergies. Central to the development of food allergy is the allergic cascade driven by cells of the innate and adaptive immune system. These models can now be integrated into the risk assessment of possible allergenic proteins. In this review, we discuss the role of the immune system as a qualitative readout for the sensitizing potential and risk assessment of food proteins.

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Introduction

Previously, a lack of suitable animal models and immunological techniques has made it difficult to grasp the role of the immune system in the sensitization to food allergens. However, since the early 90s, there have been several food allergy models investigating the role of the immune system in the pathogenesis of food allergy [1,2]. One key finding was that oral administration of a protein to an animal might result in sensitization or may induce oral tolerance [3,4]. A better understanding of the mechanisms leading to sensitization versus tolerance indicates that oral tolerance is probably the normal physiological response and that a breakdown of this process results in sensitization to food allergens. One possibility is that sensitization to food allergens actually occurs via other sites like airways or skin in contrast to the intestine, where oral tolerance is considered the default response. For example, alterations in skin barrier integrity due to filaggrin gene mutations were associated with increased rates of food sensitization [4]. However, studies on IgE responses and digestibility of food protein suggest that exposure via the oral route is also important for sensitization to food allergens [5].

Oral tolerance to food antigens requires the robust induction of regulatory T (Treg) cells within the mucosa. The gut

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micro-environment promotes expansion of Tregs through multiple mechanisms including the presence of retinoic acid and bacterial-derived metabolites such as short chain fatty acids [6]. The main mechanisms underpinning Treg cell function include production of inhibitory cytokines (IL-10, TGF-β and IL-35), effector cell cytolysis (via secretion of granzymes A and B), direct targeting of DCs via inhibitory PD-1 and CTLR4 cell surface molecules and metabolic disruption of effector cells (CD25, cAMP, adenosine, CD39, and CD73) [7]. However, why Tregs fail to suppress the sensitization and effector phases of allergic reactions remains incompletely understood. A recent study by Noval Rivas *et al.* demonstrated that uncontrolled IL-4 signaling blocks the generation of allergen-specific Treg cells and thus favors the pathogenesis of food allergy [8].

Most food proteins are largely digested by gastric acids in the stomach and intestinal enzymes after ingestion. The remaining intact food proteins and peptides are then transferred from the lumen to the mucosa via gut epithelial cells (IECs) by specialized M cells lining the Peyer's Patches or by direct sampling of mucosal dendritic cells (DCs) [9]. Activated epithelial cells can secrete type 2 promoting cytokines including TSLP, IL-25 and IL-33 to attract IL-4 competent innate immune cells such as eosinophils, basophils or group 2 innate lymphoid cells (ILC2) [10] that promote surface expression of Th2 permissive co-stimulatory molecules (e.g. OX40L) on DCs [11]. The activation of distinct DC subsets and expression of co-stimulatory molecules are important for determining the resulting immune response [12]. Activated DCs process proteins and peptides, move to T cell areas and present them on major histocompatibility complex (MHC) II where they can interact with naïve T cells to induce T helper (Th) 2 cell polarization [13] (Fig. 1). Migration and activation of IELs, including γδ T cells, also occurs in response to allergic sensitization in mice [14]. While innate immune cells contribute to the initiation of allergen specific Th2 responses [15]

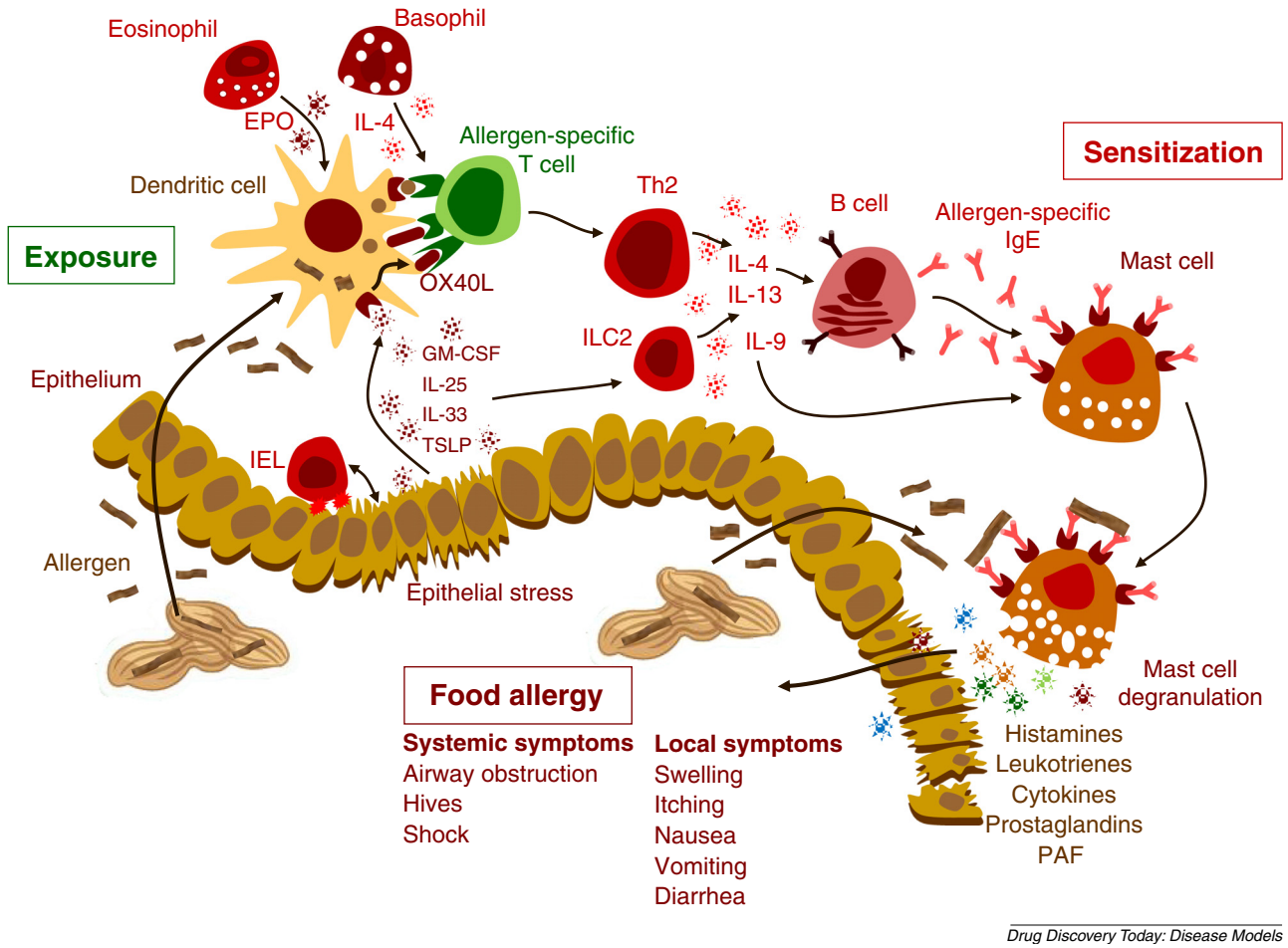

Figure 1. Humoral and cellular mechanisms of food allergy. Allergens pass the epithelium and are captured by DCs. Epithelial stress, under control of IELs, promotes the transfer of allergen and the release of GM-CSF, IL-25, IL-33 or TSLP. These mediators upregulate the co-stimulatory molecule OX40L on DC, followed by the differentiation of naïve allergen specific T cells into Th2 type responses under the influence of eosinophils (via eosinophilic peroxidase) or basophils (via IL-4). In addition, IL-33 induces ILC2s. ILC2 and Th2 cells promote class switching of B-cells into IgE via IL-4 and IL-13. These cells also secrete IL-9, involved in the propagation of mast cells. Mast cell bound IgE induces mast cell degranulation and release of mast cell mediators which induce local and systemic food allergic responses.

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Th2 cells are the main intermediate effector cells of disease. Studies in experimental food allergy models have demonstrated the importance of Th2 cells as depletion of CD4 T cells protects mice from food allergic responses while transfer of allergen specific CD4 T cells into naïve mice can transfer disease upon exposure to allergen [16].

B cells have an essential role in humoral immune responses via their secretion of antigen-specific antibodies. B cell secretion of immunoglobulin E (IgE) is a fundamental mediator in atopic diseases and a hallmark of allergic sensitization [17]. Following help from Th2 cells, B cells proliferate, undergo immunoglobulin isotype class switch recombination (CSR) toward IgE and differentiate into antibody-secreting plasma cells. IgE mediates immediate phase reactions like mast cell and basophil degranulation. In addition to antibody secretion, B cells can limit aggressive immune reactivity. B cells regulate immune responses mainly via IL-10, which has been shown in experimental models of infection, allergic inflammation and tolerance [18].

Together, despite the significant advances that have been made to understand cellular and molecular pathways associated with the pathogenesis of food allergy we do not fully understand why default immune responses to food proteins deviate from induction of tolerance to Th2-biased immune responses that promote food allergy. One prominent hypothesis is that the observed increase in the prevalence of food allergy in the Western world strongly correlates with changes in our lifestyle [19].

Food allergy models

To study immune mechanisms driving the pathogenesis of food allergy and the sensitizing potency of food allergens, researchers have established numerous *in vivo* rodent models (Table 1). Some large animal models in pig, dogs or sheep have been used, and might be more relevant for modeling human responses [20]. However, the availability, ethical concerns, high costs and extensive practical considerations have limited the use of these models. Notably, the Brown Norway rat model, which was first established in the 90s [21], develop food-specific IgE in the absence of an adjuvant, after a high frequency of intragastric dosing. However, this model is hampered by the variable number of IgE responders and practical disadvantages including the daily dosing for a long period with a relatively high amount of protein. Thus, the mouse as a food allergy model system has gained momentum due to the need for less protein allergen and the immunological tools available.

Adjuvants in food allergy models

In most mouse models, feeding the protein alone induces oral tolerance. Therefore, adjuvants such as alum or cholera toxin (CT) are frequently used to induce allergic sensitization to co-administered proteins. Alum is administered systemically, by intraperitoneal injection and boosts adaptive immunity by inflammatory mediators and activating inflammatory DCs [22]. CT is administered by intragastric administration and induces innate immune changes that trigger allergen-specific T- and B-cell responses, leading to an allergic phenotype. These innate immune changes induced by CT involve activation of epithelial cells (ECs), IELs, DCs and induction of co-stimulatory molecules, such as OX40L [14,23–26]. Understanding the mechanisms involved in the disruption of tolerance by mucosal adjuvants is highly relevant because the same pathways may be operative in the pathogenesis of human disease. For instance, molecular stress imposed on gut epithelial cells by CT or other mucosal adjuvants are a principal trigger for IEC and IEL to subsequently activate DCs, T- and B-cells during allergic sensitization [27]. In addition, the IEC-mediated intestinal barrier function also plays a fundamental role in mucosal allergic responses, which is illustrated by studies in mice following oral administration of alcohol during allergen sensitization [28] that increases small and large intestinal permeability thus facilitating sensitization and allergic effector responses.

Sensitization to food proteins is a prerequisite for induction of effector immune responses upon allergen re-exposure (Table 1). During the sensitization phase, an increase in serum allergen-specific IgE and Th2-type responses in lymphoid organs is evident. Subsequent allergen challenges lead to manifestations of food allergy. For example, intradermal or intragastric sensitization leads to local manifestations including itching, redness and swelling of skin or diarrhea, respectively. Systemic exposure to allergens via intraperitoneal or intravenous routes can result in anaphylactic reactions measured by reduced body temperature. The milk allergen beta-lactoglobulin causes anaphylaxis after intragastric exposure in relatively low amounts (JJ Smit, unpublished data), while for peanut allergens doses over 200 mg are necessary to induce anaphylaxis [28]. For peanut and other allergens, multiple dosing of the allergen and additional treatments, such as alcohol administration, may be necessary to induce allergic responses.

Mouse food allergy models

There are many different types of mouse and rat food allergy models, which influence disease outcomes and make comparisons between different models difficult. For instance, the sensitizing material may be a protein extract or an isolated individual protein [20,29]. The dose of allergen and the frequency of allergen administration during sensitization and challenge and the type and dose of adjuvant used (CT, SEB) may influence the response to the specific allergen [27,30,31]. In addition, factors that damage the epithelial barrier (e.g. alcohol or toxins), route of exposure [20,21,27,28,30], matrix effects (e.g. lipids, sugars, aggregated proteins in protein extracts) [32], microbial contamination of

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Table 1. Summary of adaptive rodent models for food allergy.

Reference	Species; strain	Allergen	Sensitization				Challenge			Parameters
			Route	Frequency	Dose	Adjuvant	Route	Frequency	Dose	
[29] (review)	Rat; BN	OVA PN CM HEW Ara h1, Sol t1, Pen a1, Ber e1	IG	42× (daily for 6 weeks)	1–10 mg	None	IG	1×	10–100 mg	Allergen-specific IgG, IgE Gut permeability
[42]	Mouse; BALB/c	OVA CM WPE	IP	2× (2-weekly)	10–100 μg	Alum	IG	6–10× (every 3 days)	10–50 mg	Allergen-specific IgG, IgE Anaphylaxis (score + temperature) Diarrhea MMCP-1, histamine Intestinal histology Cell population counts Cytokines
[43,44]	Mouse; C3H/HeJ, BALB/c	OVA CPE Soy ALA Ara h1 Ara h2	EP	6× (weekly)	0.1–1 mg	None	IG IP	1× 1×	50 mg 100 μg	Allergen-specific IgG, IgE Anaphylaxis (score + temperature) Cytokines
[45]	Mouse; BALB/c	WPE Cashew	TD	4–6× (weekly)	1 mg	None	IG	1×	15 mg	Allergen-specific IgG, IgE Anaphylaxis (score + temperature) Cytokines
[28,46,47]	Mouse; C3H/HeJ	CPE CM BLG	IG	4–6× (weekly)	0.2–10 mg	CT or CT + Vodka	IG	1×	10–200 mg	Allergen-specific IgG, IgE Anaphylaxis (score + temperature) MMCP-1, histamine Cytokines Cell population counts
[27,35,38,48,49]	Mouse; C3H/HeJ, C3H/HeOuJ, BALB/c, C57BL/6	CPE HEW WPE OVA Ara h 1-6 Spinach Turkey Brazil Nut among others	IG	4–8× (weekly)	0.25–20 mg	CT SEB DON	IP ID	1× 1×	0.1–5 mg 50 μg	Allergen-specific IgG, IgE Anaphylaxis (score + temperature) MMCP-1, histamine Cytokines Cell population counts Ear swelling

BN: Brown Norway, OVA: ovalbumin, PE: peanut extract, CM: cow's milk, WPE: whey protein extract, HEW: hen's egg white, ALA: alfa-lactalbumin, BLG: beta-lactoglobulin, IG: intragastric, IP: intraperitoneal, EP: epicutaneous, TD: transdermal, CT: cholera toxin, SEB: Staphylococcal enterotoxin B, DON: deoxynivalenol.

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protein extracts [33], composition of the microbiota [34] and the animal strain used in the experiments [30,35] can all influence the outcome of the model.

Allergic responses can be assessed by measuring changes in cellular subsets, release of mediators and the appearance of disease symptoms. Cytokine production upon re-stimulation of mesenteric lymph nodes (MLN) and/or spleen cells determine whether Th2-type allergic mediators are upregulated. Flow cytometry of isolated cell populations from intestinal tissues including MLN, lamina propria and Peyer's Patches can be used to identify allergen-specific or Th2-associated cell subsets. Measurement of allergen-specific IgG1, IgG2a, IgE or IgA antibody levels can help establish the class of the immune response that was elicited. Histological analysis of the intestine or lymphoid organs is useful to quantify the severity of the inflammatory response. *In vivo*, mast cell degranulation can be assessed by measuring histamine or mouse mast cell protease-1 release. Functional parameters include gut permeability and airway reactivity in response to ingested or inhaled allergen challenges. Anaphylaxis is measured by monitoring changes in body temperature and using an anaphylaxis scoring system [36]. Ear swelling after intra-dermal challenge and passive cutaneous anaphylaxis can be assessed by extravasation of Evans Blue dye [37]. Together, numerous quantitative and semi-quantitative measurements can be assessed in murine food allergy models to assess severity of allergic manifestations. However, these manifestations depend upon multiple exogenous and endogenous factors (reviewed in [20,29,30]. The pros and cons of each animal model is extensively reviewed recently elsewhere [29,31].

Predictability of food allergy models

There is extensive information on the chemical and physical characteristics of food allergens, which belong to only 2% of protein families. It remains unknown why certain proteins are allergenic, compared to the large majority of food proteins, which are not allergenic. Animal models that discriminate between low or non-allergenic proteins from high-allergenic proteins would be ideal for understanding food allergy mechanisms and for allergenicity risk assessment of novel proteins. Dearman *et al.* showed that known allergenic proteins induced protein-specific IgE upon systemic exposure in mice, while non-allergenic proteins produced low IgE titers [33]. By contrast, using the same route of administration, it was not possible to differentiate between known allergens and putative non-allergens, for example, rubisco and soy lipoxygenase [29]. However, oral protein administration allowed researchers to distinguish allergenic from non-allergenic food extracts. Peanut, egg white and Brazil nut allergens were distinguished from low-allergenic spinach and turkey after 2 weeks of feeding a dose of 2 mg [38]. Additionally, using an *ex vivo*/*in vitro* DC-T cell assay and an *in vivo* mouse model, it was possible to distinguish known allergenic food proteins (Ara h1, beta-lactoglobulin, shrimp tropomyosin, bovine serum albumin, whey protein isolate) from low/non allergenic food proteins (soy lipoxygenase, gelatin, beef tropomyosin, rubisco, patatin) [39]. However, in this model prolonged exposure (>28 days) may elicit responses to both allergen and non-allergen proteins. Importantly, in these models, there is the possibility that allergens are contaminated with endotoxin, which will enhance allergen-stimulated proliferation and reduce the threshold for T cell activation [40].

The use of highly purified proteins versus raw food extracts is another important factor in food allergy models. The food matrix influences responses to individual proteins dependent on the route of administration [32]. For example, proteins from the same source display different allergenic properties while being ingested in the same matrix, that is, not all proteins in peanut are allergenic and allergenic peanut proteins induce significantly different allergic responses [41], thus suggesting that protein-specific factors are possible.

Summary

Animal models have allowed us to uncover many of the cellular responses and molecular mediators involved in the induction of oral tolerance or allergic sensitization to food antigens. Determination of regulatory T cell activity and induction of Th2 lymphocyte polarization and B cell IgE class switching are important parameters. In addition, *in vitro* or *ex vivo* model systems should be complementary to animal models and may provide information on cellular processing and presentation of food proteins. Many different known and unknown factors can influence the outcome of a food allergy model and the development of a reference protein toolbox (with validated high- and low-allergenic proteins) is essential and would help standardize animal model responses across different laboratories.

Conflict of interest

The authors have no conflict of interest to declare.

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