

STATE OF THE ART REVIEWS ON MECHANISMS OF ALLERGIC DISEASE

What makes an allergen?

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Summary

Allergic diseases are an immune disorder reacting to certain type of allergen(s). Remarkably only a small number of proteins of the plant and animal proteome act as allergens. Therefore, allergens have been clustered according to their common structural, biochemical and functional features. Evidence has accumulated that some allergens possess intrinsic adjuvant properties to stimulate the innate immunity. The adjuvant properties appear to contribute to the allergenicity of the respective proteins, namely the ability to cause allergic sensitization in susceptible subjects or allergic reactions in sensitized individuals. Here, we discuss how allergens interact with the innate immune cells, in particular dendritic cells and epithelial cells, via binding to pattern recognition receptors, exhibiting proteolytic activities and/or inducing type 2 innate lymphoid cells (ILC2), thereby contributing to the sensitization and development of allergic diseases.

Introduction

IgE-mediated allergies affect up to 25% of the population in industrialized countries, and are characterized by a Th2-biased immune response to ubiquitous, innocuous environmental antigens, so-called allergens. Why only a small number of proteins cause aberrant Th2 immune responses is largely unanswered. It has been suggested that among other factors such as the route and level of exposure, allergens share specific features that contribute to the allergenicity. Allergenicity is defined as the property of antigens to induce humoral and cellular Th2 immune responses resulting in the formation of allergen-specific IgE and Th2 cytokines and subsequently in the induction of clinical symptoms.

Different approaches have been applied to identify common structural and functional features of allergens to predict allergenicity. Structural and physicochemical properties of allergenic proteins, such as functional domains and ligand binding relevant for biological function, as well as parameters such as post-translational modification, stability, hydrophobicity, solubility and pI values have been addressed. Even other factors such as allergen concentration, duration and time point of exposure and strength of the T cell activating signal may impact the development of allergic diseases in susceptible individuals. Nowadays, it becomes evident that aller-

genic may also depend on adjuvant components derived from allergenic sources and environmental factors such as the microbiome to which individuals are exposed in parallel to the allergen. Recently, an increasing number of reports provide evidence that allergenicity of certain allergens relies on their intrinsic properties to trigger innate immunity. Knowledge about the basis of protein allergenicity is of fundamental importance for understanding the pathogenesis and prevention of allergies, and furthermore for the development of optimized approaches to allergen-specific immunotherapy.

The present review article illustrates the allergenic properties of certain protein families and the underlying mechanisms of how allergens can stimulate innate immune responses and subsequently contribute to Th2-biased adaptive immune responses. Biochemical features as well as intrinsic adjuvant effects of allergens on the innate immune cells, in particular antigen-presenting cells and epithelial cells, are taken into consideration.

Biochemical and structural aspects of allergenicity

Most allergens are thought to be relatively small proteins or glycoproteins (5–100 kDa) which in most cases are water soluble. Recently, there is increasing evidence for hydrophobic or amphipathic proteins such as oleosins to act as allergens [1]. IgE-binding epitopes

are determined by the protein backbone (linear or conformational epitopes) or by carbohydrate residues (N- and O-glycosylated proteins), or both. Although non-mammalian α 1,3-fucose and β 1,2-xylose glycoproteins from plant and insects are immunogenic, IgE binding to the so-called cross-reactive carbohydrates (CCD) is considered to be clinically not significant in the majority of patients. In contrast, IgE reactivity to galacto-oligosaccharides (GOS) in sea squirt and GOS-supplemented milk formula and lactic acid beverages has been reported in a small subgroup of patients, mainly from Asia [2]. Moreover, IgE binding to galactose- α -1,3-galactose (α -Gal) residues in red meat has been shown to elicit severe (delayed) allergic symptoms in some patients which likely became previously sensitized by tick bites [2]. Allergens require at least two IgE-binding epitopes to facilitate IgE cross-linking and activation of mast cells and basophils. Remarkably, a considerable number of allergens naturally form dimers or oligomers which further enhance mediator release capacity [3]. In contrast, the aggregation of allergens was suggested to explain the enhanced Th1 immunogenicity of hypoallergenic Bet v 1d from birch pollen in mice [4]. Furthermore, allergenic properties are frequently attributed to the stability of proteins. This is of particular importance for food allergens which are subjected to thermal processing and exposed to gastrointestinal enzymes. Food allergens with high stability such as the peanut allergens Ara h 2 and Ara h 6 [5] are able to reach the gastrointestinal immune system in their IgE-reactive form and to elicit more severe reactions. Strong proteolytic stability might also be important for a delayed phagolysosomal digestion in murine and human antigen-presenting cells and sustained MHC class II/peptide presentation to T cells [6].

Remarkably, only a small number of proteins of the plant and animal proteome are known as allergens. Therefore, allergens have been clustered according to their common structural, biochemical and functional features to less than 2% of all known protein families [7–10]. It has been proposed that the sensitization capacity of allergens is dedicated to specific molecular features. In contrast, Albersee and Cramer suggested that almost all antigens can become an allergen, but some antigens are more likely to induce IgE than others [11]. There is a general consensus that the molecular basis of allergenicity probably is not defined on the epitope level. Common rules for the main allergen-IgE binding sites cannot be deduced because of the diverse physicochemical properties of the proteins [8]. In line with this, the IgE-epitope repertoire is too large to make specific IgE epitopes, a realistic target for allergenicity prediction [11].

Initially, it has been proposed that the lack of homology to bacterial proteins determines allergenicity [12]. Recently, a concept suggests that allergenicity of many

proteins is determined by homologous proteins in Th2-promoting helminths sharing similar determinants [13]. However, patients with helminth infection and potential cross-reactive memory Th2 cells do not exhibit increased susceptibility for allergies. Moreover, the increased incidence of allergic diseases in industrialized countries with less parasite infections does not support this hypothesis. In line with this, it has been suggested that allergens induce stronger specific T cell selection from the T cell repertoire than helminths leading to the production of high-affinity IgE [14].

More recently, attention has been drawn to the fact that several allergens comprise intrinsic adjuvant activity such as lipid-binding properties and protease activity, and can directly interact with the innate and adaptive immune system [3]. Likewise, allergenic proteases from mites have been described to possess Th2 adjuvant reactivity [15]. Remarkably, several allergenic (cysteine, serine, metallo-, aspartic) proteases from fungi, food, pollen, helminths, insects, yeast, mite and cockroaches are listed in the IUIS allergen nomenclature database (www.allergen.org).

Allergen classification into structural groups will allow to evaluate the risk of cross-reactivity rather than identifying the molecular basis of allergenicity. The allergenicity of proteins likely is determined by additional factors, such as the amount and duration of exposure to the immune system, environmental conditions including microbial exposure, immune-modulating components of allergenic sources facilitating Th2 immune responses, as well as the intrinsic effects of proteins on the innate and adaptive immune system.

Interaction of allergens with antigen-presenting cells

Antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages are central players in immune responses and essential to bridge innate and acquired immunity. APCs capture antigens for efficient processing and presentation, and undergo maturation (innate immunity). Activation of specific T cell effector mechanisms (acquired immunity) is then initiated and modulated by the APCs. To perform their function, APCs are equipped with a full array of specialized receptors, including co-stimulatory molecules (e.g. CD40, CD80 and CD86), and pattern-recognition receptors (PRR) such as Toll-like receptors (TLRs), C-type lectins (CTLs) and scavenger receptors (SR). Recent evidence has shown that some allergens interact with PRR and stimulate innate immunity.

Toll-like receptors

TLRs play an essential role in the mechanisms of host-defence against a wide range of micro-organisms [16].

In mammals, more than 10 TLRs have been identified, each recognizing distinct pathogen-associated molecular patterns (PAMP). Among the TLRs, LPS-mediated TLR4 signalling appears to be closely involved in sensitization and development of allergic diseases. The TLR4 signalling complex on the cell surface comprises TLR4, myeloid differentiation-2 (MD-2) and co-receptor CD14, a glycosylphosphatidylinositol (GPI)-anchored receptor. TLR4 is physically associated with MD-2. In the presence of LPS, the TLR4–MD-2 complex and CD14 are brought into close proximity [17] inside lipid rafts [18]. After translocation into the lipid rafts, TLR4 in the plasma membrane activates signalling pathways. Importantly, some allergens appear to interact with the TLR4 signalling complex by (i) mimicking MD-2, (ii) associating with TLR4 via binding to the ligand LPS or (iii) binding to the TLR4/MD-2 complex directly (see TLR4, MD-2 and CD14 in Fig. 1).

Mimicking MD-2. The house dust mite allergen *Derma-tophagoides pteronyssinus* group 2 (Der p 2) shares structural homology with MD-2 [19]. Der p 2 and MD-2 belong to a small family of lipid-binding proteins that have a β -sandwich or cup-type fold. Strikingly, Trompette et al. showed that Der p 2 could mimic the function of MD-2 and bind to TLR4 and LPS directly. Specifically, Der p 2 reconstituted LPS-mediated TLR4 signalling in CD14 and TLR4-transfected human embryonic kidney HEK293 cells in the absence of MD-2 [20]. Moreover, Der p 2 enhanced LPS-mediated TLR4 signalling in the presence of MD-2. Airway sensitiza-

tion and challenge with Der p 2 led to experimental allergic asthma in wild-type and MD-2-deficient, but not TLR4-deficient mice. These results suggest that Der p 2 has an auto-adjunct effect in stimulating TLR4 signalling. TLR4 is expressed in many immune and structural cells. Indeed, Der p 2 activates dendritic cells and respiratory EC to produce pro-inflammatory mediators [21, 22]. Der p 2 may trigger inflammatory responses by stimulating innate immune response in a TLR4-dependent manner and promote sensitization and development of allergic diseases. Remarkably, the MD-2-related lipid recognition domain is conserved in other mite aeroallergens Der f 2, Eur m 2, Gly d 2, Try p 2 and Lep d 2 [3, 23, 24]. Therefore, mimicry of the MD-2/TLR4 complex is suggested as a molecular basis for the allergenicity of inhaled group 2 allergens [3].

Binding to TLR4 ligand LPS and lipids. Some allergens may contribute to TLR4 signalling not by mimicking MD-2, but by binding to the ligand of the receptor, LPS. Herre et al. [25] showed that Fel d 1 from cat dander enhances lipoteichoic acid-induced TLR2, or LPS-induced TLR4 signalling by using transfected TLR2–CD14–MD-2 or TLR4–CD14–MD-2 transgenic HEK293 cells. Fel d 1 also synergistically enhanced LPS-induced pro-inflammatory cytokine production in peripheral blood mononuclear cells from healthy donors. However, this allergen alone was not capable of triggering TLR2, or TLR4 signalling, suggesting that the enhanced effect of Fel d 1 is based on its binding to lipid components. It has been postulated that Fel d 1

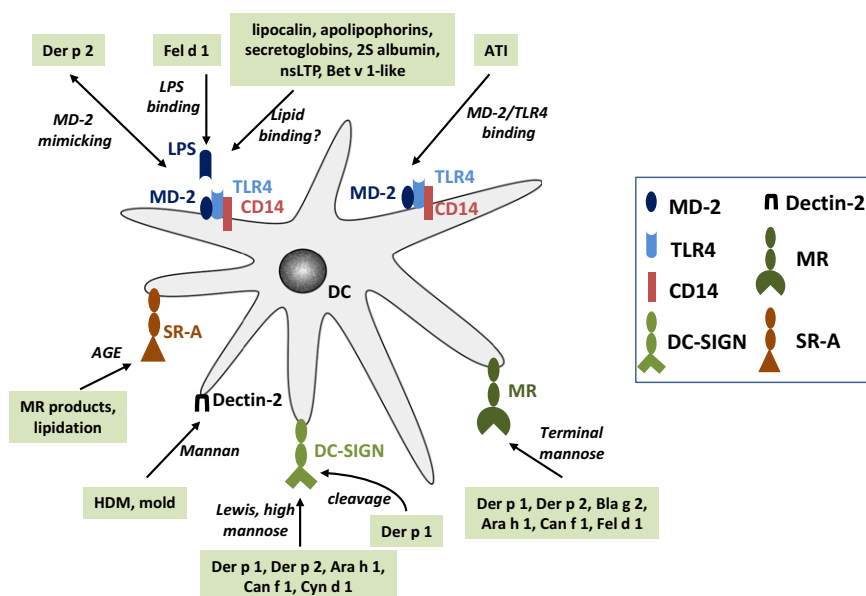


Fig. 1. Interaction of allergens with APCs. **Upper part:** Allergen-induced TLR4 signalling by mimicking MD-2, associating with TLR4 via binding to the ligand LPS, or binding to the TLR4/MD-2 complex directly. **Lower part:** C-type lectin (CTL)-mediated activation and internalization of glycosylated allergens. ATI, alpha-amylase/trypsin inhibitors; HDM, house dust mite; nsLTPs, non-specific lipid transfer proteins; AGE, advanced glycation end products; MR, maillard reaction.

clusters together with LPS to form larger complexes that then promotes greater clustering of TLR4-bearing lipid rafts, leading to increased receptor activation [25]. Fel d 1 belongs to the secretoglobin family of proteins with a putative lipid-binding pocket, where lipid components may interact [26, 27]. Notably, there are many other allergens that belong to protein families with lipid-binding properties such as lipocalins, apolipoprotein, secretoglobins, 2S albumins, non-specific lipid-binding proteins (nsLTPs) and Bet v 1-like proteins. For instance, Can f 6 (dog), belonging to the protein family of lipocalin [27], has similar TLR4-stimulatory properties as Fel d 1, but also shows some MD-2-independent effects [25]. Lipid-binding properties were attributed to allergenic 2S albumins and nsLTPs (e.g. Par j 1 and Par j 2 binding monoacylated negative lipids), which are plant food allergens belonging to the prolamin superfamily. Furthermore, it has been speculated that Bet v 1-like proteins are also able to associate with lipid structures (binding hydrophobic ligands such as fatty acids and flavonoids) [24, 28]. It would be important to see whether lipid-binding properties promote Th2 immune responses, likewise via TLR4 signalling by binding to LPS.

Binding to TLR4/MD-2 complex. Alpha-amylase/trypsin inhibitors (ATIs) facilitate pest resistance in wheat and other cereals and grains. It has been suggested that ATIs bind to the TLR4–MD-2 complex directly and thereby activate TLR4 signalling. Junker et al. [29] found that wheat ATIs in a water-soluble gliadin extract induce cytokine production in TLR4–MD-2–CD14-transfected HEK293 cells. Co-immunoprecipitation analysis showed that ATIs bind to the TLR4 and MD-2 complex directly. Blocking antibodies against CD14 inhibited ATI-induced cytokine production in human monocyte-derived DCs, suggesting ATIs also need this co-receptor to trigger TLR4 signalling. ATIs induced up-regulation of maturation markers and production of inflammatory cytokines in peripheral monocyte-derived DCs from both coeliac and non-coeliac subjects. This suggests that ATIs stimulate innate immunity via activation of TLR4 signalling in both steady and inflammatory states. Notably, ATIs comprise allergenic members belonging to the prolamin family [28] and are involved in food and respiratory allergies [30]. These cereal and grain components alone may not trigger the onset of disease directly, but reduce the amount of allergens required for sensitization and promote disease status by inducing inflammatory responses via interaction with TLR4.

C-type lectins

Several C-type lectins are expressed abundantly on the surface of DCs and macrophages [31, 32]. C-type lectins

bind carbohydrate ligands in a calcium-dependent manner using highly conserved carbohydrate recognition domains (CRDs). Recent studies have shown that natural carbohydrate residues of allergens could bind to C-type lectins on the cell surface of APCs and subsequently influence both innate and adaptive immunity. Many C-type lectins are expressed on the cell surface of APCs, such as the mannose receptor (MR), DEC205, DC-SIGN, DC-SIGNR, DC-ASPGR, BCDA-2, dectin-1 and dectin-2 that all recognize various distinct carbohydrate-containing antigens [32]. Among them, MR, DC-SIGN, DC-SIGNR and Dectin-2 have been reported to bind to several allergens (see MR, DC-SIGN and Dectin-2 in Fig. 1).

Mannose receptor. The MR (CD206) structurally comprises an N-terminal cysteine-rich domain, a domain containing fibronectin type II repeats, eight C-type lectin-like domains, a transmembrane domain and a short cytoplasmic tail [33]. The cysteine-rich domain recognizes sulphated sugars, whereas the recognition of mannose, fucose and *N*-acetylglucosamine is mediated by multiple CRDs [34]. Several allergens such as Der p 1 and Der p 2 (house dust mite, HDM), Bla g 2 (cockroach), Ara h 1 (peanut), Can f 1 (dog) and Fel d 1 (cat) appear to bind to CRDs of MR through their carbohydrate moieties [35–37]. MR may be involved not only in delivering allergens to the antigen-presenting pathway [38], but also in directing DC conditioning for Th2 cell priming. Deslée et al. [36] showed that the expression of MR on DCs was up-regulated in patients with HDM allergy. The interaction of the MR with glycosylated allergens may facilitate DC activation and Th2 cell polarization, and enhance sensitization and development of allergic diseases.

DC-SIGN and DC-SIGNR. Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209) is a type II transmembrane protein that contains a short, cytoplasmic N-terminal domain with several intracellular sorting motifs, an extracellular stalk of seven complete and one partial tandem repeat, and a CRD [39]. DC-SIGN interacts specifically with fucose-containing Lewis antigens and high-mannose-type oligosaccharides, but not with single terminal mannose residues, which are recognized by the MR [40]. DC-SIGN shares 77% amino acid sequence identity, similar overall structure and ligand-binding characteristics with the human receptor, DC-SIGNR (L-SIGN; CD299). Ara h 1, Der p 1, Der p 2, Can f 1 and Cyn d 1 (Bermuda grass pollen) have been reported as ligands for DC-SIGN and/or DC-SIGNR [41, 42]. DC-SIGN has been suggested to modulate human DC activation for Th1 cell polarization, or IL-10-mediated regulatory responses [40, 43, 44]. Interestingly, Furmonaviciene

et al. [45] showed that Der p 1 cleaves DC-SIGN and DC-SIGNR by its proteolytic activity and releases these receptors from the cell surface in human DCs, thereby promoting Th2 cell polarization. However, a recent study showed that fucose-stimulated DC-SIGN directly induces human DC modulation for Th2 cell polarization [46]. The expression of DC-SIGN on the cell surface of human DC is up-regulated in the presence of IL-4 [39]. Further studies are necessary to define the role of DC-SIGN on T cell polarization in allergic patients.

Dectin-2. Dectin-2 is a type II transmembrane protein of the C-type lectin family, with a single CRD in their extracellular region. Dectin-2 recognizes α -mannans (high-mannose structures), becomes activated and transduces its signal through association with the ITAM-containing Fc receptor γ chain. A recent study showed that, upon stimulation with extracts from the house dust mite or from the mould, Dectin-2 triggered the production of pro-inflammatory cytokines and lipid mediators, including the cysteinyl leukotrienes (CysLTs) in murine bone marrow-derived DCs [47]. Furthermore, Dectin-2 activation elicited by the house dust mite extract triggered Th2 pulmonary inflammation through the specific actions of CysLTs in a murine model of allergic asthma [48]. These results suggest that Dectin-2 also plays a role in inducing allergic asthma by triggering inflammatory responses in DCs.

Scavenger receptors

Macrophage scavenger receptors (SR) were originally defined as receptors which could bind to low-density lipoproteins (LDL) modified by acetylation (AcLDL) or oxidation (OxLDL), but not unmodified LDL. The family is divided into eight classes (Class A-H) according to their multi-domain structure [49]. Among the receptors, SR class A (SR-A) was identified as a receptor for natural hen's egg ovalbumin (OVA, Gal d 2) [38]. We recently found that SR-A also mediated murine DC uptake of glycated OVA, a product of the Maillard reaction between OVA and glucose, via interaction with glycation structures in addition to natural carbohydrate residues (see SR-A in Fig. 1) [50]. The Maillard reaction is a non-enzymatic reaction between reducing sugars and compounds with free amino groups such as amino acids and proteins, and takes place during thermal processing and storage of foods. The reaction leads to the modification of proteins with various types of glycation structures. Importantly, glycated OVA induced higher allergen-specific CD4⁺ T cell responses and IgE production in mice, compared to native OVA [51], suggesting that SR-A transfers its ligands to MHC class II loading pathway in APCs to promote CD4⁺ T cell activation. SR-A structurally comprises the transmembrane

domain, a spacer region, a helical coiled coil domain, a collagenous domain and a C-terminal cysteine-rich domain [52]. To this date, the collagenous domain has been identified as a ligand-binding domain. Notably, some scavenger receptors including SR-A and SR-B bind to a wide range of anionic ligands including lipid-modified components such as LPS [52]. It could be hypothesized that SR takes up glycosylated or glycated allergens, or a complex of allergen with lipid component, which in turn enhance T cell responses against allergens.

Interaction of allergens with the epithelium

Several allergens and allergen extracts have been shown to possess an intrinsic immunogenicity by interacting with structural cells, such as epithelial cells (EC). The epithelium of the respiratory and gastrointestinal tract is the first barrier for exogenous antigens such as pathogens and allergens. The epithelium is an active component of the immune system at which EC interact with allergens and instruct immune cells to maintain homeostasis or to induce immune response, respectively. In line with this, some allergens can specifically activate EC and are transported across EC to orchestrate with immune cells. Hence, allergen uptake into mucosal tissue of the airways and the gut affects the sensitization capacity of proteins. Allergens are generally taken up by transcytosis via enterocytes, para-cellular transport and dendritic cells (Fig. 2). However, in sensitized individuals CD23 (Fc ϵ RII)-dependent transcytosis of IgE allergen immune complexes across intestinal and airway EC plays a critical role in the perpetuation of allergic diseases. In addition, the small intestine is endowed with M (microfold) cells which preferentially transport particles and digested proteins which became insoluble and/or form aggregates under neutral pH conditions. In contrast, undigested soluble and non-aggregated antigens can pass through intestinal epithelial cells (IEC) by para-cellular or transcellular transport. Therefore, protein solubility and aggregation can define the route of mucosal transport of allergens. In addition to the transcellular transport, antigen processing and presentation can play further role in IEC, as shown by using Caco-2, HT29, MoS13 and IPEC-J2 cell lines [53]. Hence, the specific activation of EC and enhanced allergen uptake, by either genetic or disease-mediated increased permeability and by intrinsic properties of the allergens, is thought to be critical for the induction of allergies.

Effect of proteases on the epithelial barrier

Although protease activity is not a general feature of allergens, proteolytic activity of several allergens and non-allergenic proteases released by allergenic sources

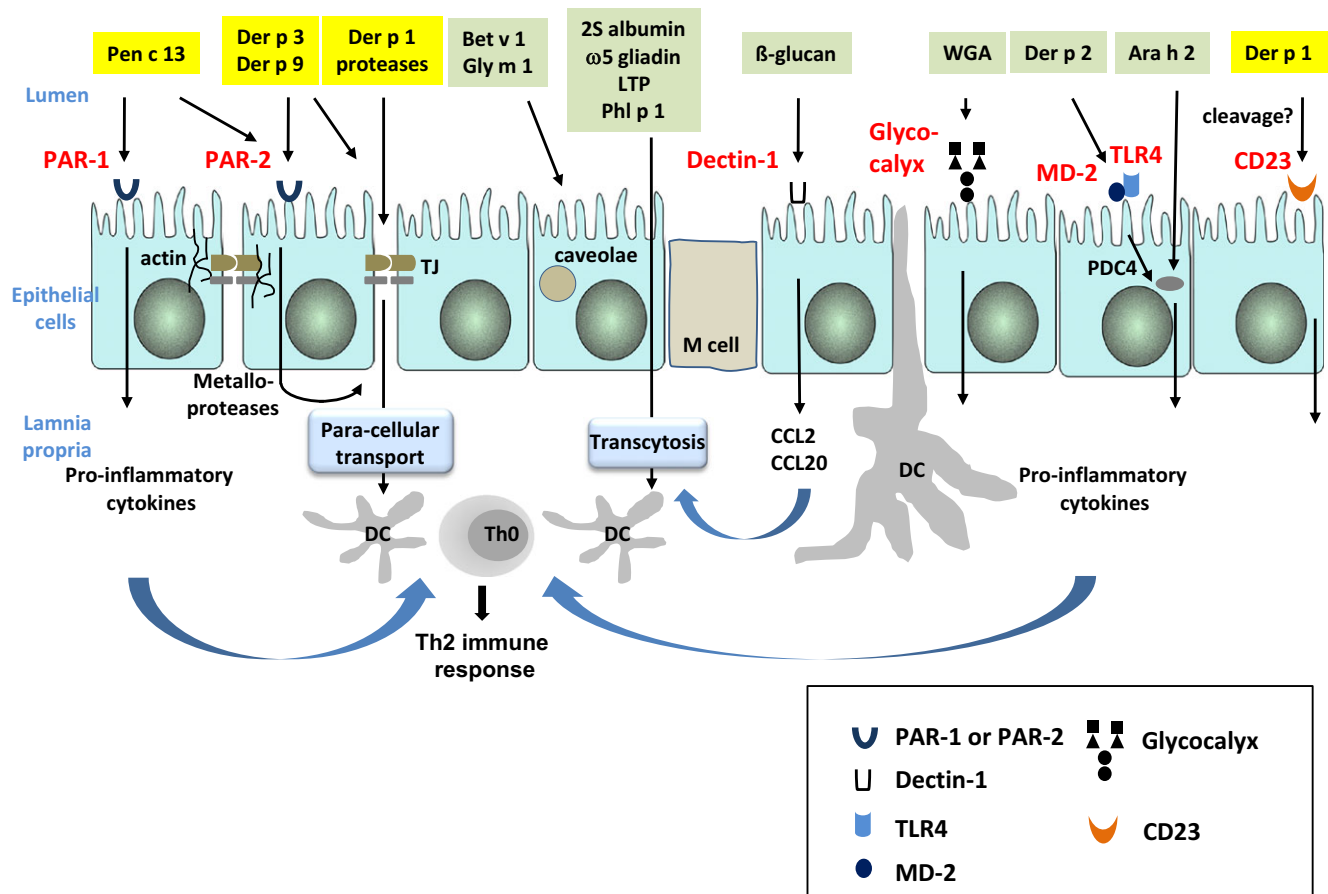


Fig. 2. Interaction of allergens with epithelial cells (EC). Allergenic proteases (yellow) activate EC by binding of PAR (protease-activated receptor), disrupt tight junctions (TJ), or might cleave low-affinity IgE receptor CD23. Non-proteolytic allergens can interact with EC via pathogen recognition receptors (e.g. dectin-1, MD-2/TLR4) or binding to the glycocalyx. EC activation triggering pro-inflammatory cytokines and DC attractants (CCL2, CCL20), and increased mucosal uptake by para-cellular transport, transcytosis (via EC and M cells) or internalization by DC promote Th2 immune responses. PDC4, programmed cell death protein 4.

have been reported to interact with EC and promote allergic sensitization. Several studies suggested the exposure to inhaled allergens with proteolytic activity affects the airway epithelium, either by increasing the permeability or by direct activation leading to enhanced inflammation. In particular, HDM, cockroach, fungi and moulds possess strong proteolytic activity [54]. Binding of proteases to the protease-activated receptor (PAR)-2 (see PAR-2 in Fig. 2), likewise expressed on human bronchial EC or murine P815 mastocytoma cells, and subsequent disruption of the epithelial barrier function have been shown to promote the translocation of cockroach allergens and elicits allergic diseases [55]. So far, four PARs have been identified, to be expressed on virtually all cells involved in the allergic response. PAR becomes activated by cleavage of an extracellular peptide at which the cleavage site for activation is specific for each PAR. Finally, PAR stimulation opens tight junctions (TJ) by the secretion of metalloproteinase 9

[56]. Furthermore, it was described that some pollen grains release proteases with serine and/or amino peptidase activity which cleave transmembrane adhesion proteins facilitating para-cellular allergen delivery across Calu-3 human EC line and likely contributing to allergic sensitization [57]. Interestingly, in a recent study the protease activity of allergens was directly linked to TLR4 activation. Like thrombin, a yet unknown fungal protease derived from *Aspergillus oryzae* cleaves clotting factor fibrinogen, yielding cleavage products that bind and activate TLR4 on airway EC in mice and subsequently elicit allergic immune responses [58]. Noteworthy, *Aspergillus oryzae* is described as allergenic mould expressing allergic proteases such as Asp o 13, an alkaline serine protease (www.allergen.org). Finally, it can be concluded that the allergenic potency of an inhaled antigen in an epithelium environment will be substantially modulated by simultaneous exposure with endogenous proteases.

However, several allergens themselves are proteases and display intrinsic adjuvant activity. The mite allergens, Der p 1 (cysteine protease) and Der p 3 (serine protease), were shown to induce IL-8 secretion from human alveolar EC A549, at which cytokine secretion by Der p 3 (and Der p 9, a serine protease) but not by Der p 1 depends on PAR-2 [56, 59]. Proteolytic active mould allergen Pen c 13 (*Penicillium citrinum*), a serine protease, activates both PAR-1 and PAR-2 and induces IL-8 from human airway EC [60] (see PAR-1 and PAR-2 in Fig. 2). Later, using a murine model and NCI-H441 human lung EC, Pen c 13 has been reported to induce increased permeability of the epithelial barrier by structural changes of the TJ upon actin rearrangement [61]. Disruption of the TJ allows the para-cellular transport of allergens. Like papain, the papain-like cysteine protease Der p 1 and serine proteases from HDM destruct epithelial integrity and TJ by the cleavage of occludin and ZO-1 resulting in an increased para-cellular permeability of human bronchial Calu-3 and 16HBE14o-EC [62, 63]. Protease activity was attributed to an increased sensitization potency of Der p 1. It needs to be elucidated whether other cysteine proteases from food (e.g. Car p 1 from papaya, Act d 1 from kiwi), pollen (e.g. Amb a 11 from short ragweed) or mites (e.g. Blo t 1, Der f 1 or Eur m 1) exhibit similar properties. Remarkably, papain degrades TJ from human keratinocytes *in vitro* and induces epicutaneous inflammation by recruiting neutrophils and mast cells and by the induction of a Th2-biased immune response, independent of its enzymatic activity or TLR4 activation [64]. Although Der p 1 is capable to cleave the α -subunit of the IL-2 receptor CD25 from T cells and CD23 on human B cells which promotes IgE synthesis [65], the effect of Der p 1 on CD23 expressed on EC remains largely unknown (see CD23 in Fig. 2).

Interaction of non-proteases with EC

Furthermore, the transcellular transport (transcytosis) of certain allergens has been investigated by using distinct EC lines. Integrity of the monolayer is monitored by the transepithelial resistance (TEER). A maintained TEER upon administration of allergens indicates functional TJ and relates to the transepithelial passage of proteins. Using human colon cancer cell line (Caco-2) monolayers, purified wheat allergen w5-gliadin and nsLTPs have been described to cross the barrier by the transepithelial route [66]. Moreover, the transepithelial uptake across Caco-2 cell has been described for 2S albumins [67], Ber e 1 (brazil nut) and Ses i 1 (sesame) [68]. The major respiratory soya bean allergen Gly m 1 (P34) was shown to be endocytosed by the intestinal EC line IPECJ-2 derived from neonatal piglet by the involvement of caveolae/lipid raft microdomains [69]. Similar

properties to enter the nasal epithelium by active caveolar transport were shown for Bet v 1 in allergic patients, but not in healthy subjects [70, 71]. Transport of pollen allergen through the respiratory epithelium is proposed to be specific, as birch allergic patients transport Bet v 1 but not grass allergens and *vice versa*. It was hypothesized the mechanism likely is antibody dependent [72]. Using an *in silico* approach, the authors proposed that Bet v 1 has a hydrophobic binding site for different ligands including caveolar amphiphilic and lipid ligands, for example glycolipids and gangliosides enriched in caveolae. Remarkably, pathogenesis of birch pollen allergy was associated with the hypo-responsiveness of EC derived from allergic donors [73]. Natural purified major timothy grass pollen allergen Phl p 1 was able to induce pro-inflammatory cytokines secretion from alveolar A549 cells [74]. The authors excluded any proteolytic activity and speculated the induction of TGF- β and p38 MAPK to have an indirect effect on epithelial integrity. In an independent study, the unidirectional transcytosis of Phl p 1 across A549 cells (and to lower extend across bronchial NCI-H7272 and Calu-3 cells) via non-acidic compartments bypassing lysosomal digestion and subsequent exocytosis of intact allergens has been reported [75].

Like cells of the innate immune system, EC express pattern-recognition receptors (PRR), such as C-type lectin receptors (CLR), Toll-like receptors (TLR) and protease-activated receptors (PAR) (see TLR4, PAR and Dectin-1 in Fig. 2). In line with this binding of β -glucan moieties in *Aspergillus fumigatus*, pollen, HDM and animal dander to CTL, dectin-1 was shown to mediate the activation of human bronchial EC 16HBE14o- [76, 77]. Remarkably, β -glucan-mediated secretion of DC attractant CCL-20 was specific for HDM extract and independent of endotoxin and protease activity, as Der p 1 and other aeroallergens fail to induce this chemokine [76]. It remains interesting to investigate whether allergens carrying β -glucans mediate these effects. Moreover, non-proteolytic Der p 2 was shown to activate human bronchial BEAS-2B EC (but not alveolar A549 cells) and to induce pro-inflammatory immune response [21]. Der p 2 acts as a homologue of MD-2, a lipid-binding protein associated with TLR4 also expressed on EC [21]. Binding of recombinant Ara h 2 to human intestinal Caco-2 EC was reported which was further enhanced by the heating of Ara h 2 [78]. Whereas native Ara h 2 induced IL-33 and TSLP mRNA, the cytokine response was changed by heat-treated Ara h 2. Authors suggested an adjuvant effect of Ara h 2 resulting from the structural similarity with mouse programmed cell death protein 4 (PDC4) which is involved in TLR4 signalling. Upon TLR4 activation in mice and human blood mononuclear cells, PDC4 promotes pro-inflammatory immune response

and suppresses IL-10 [79]. Actually, the involvement of allergens in the MD-2/TLR4 and PDC4 signalling in EC needs to be further verified. Further intrinsic adjuvant property of some allergens was attributed to their capacity to bind carbohydrate structures. Wheat germ agglutinin (WGA) is a plant lectin which binds to *N*-acetyl-glucosamine (GlcNAc), an *N*-acetyl-D-neuraminic acid of the glycocalyx, and which is translocated across enterocytes (Caco-2) (see glycocalyx in Fig. 2). Therefore, WGA was also suggested as a carrier for oral drugs [80]. Although there is a controversial discussion whether WGA displays IgE-binding capacity, it was denominated as wheat allergen Tri a 18 (www.allergen.org). Nevertheless, plant lectins including hevein and agglutinin from several legume foods are described as potential allergens [81, www.allergome.org].

In summary, several studies provided evidence for EC activation and translocation of allergens mediated by lipid- and carbohydrate-binding properties. However, whether these mechanisms are attributed to the allergenicity of a broad panel of allergens needs to be addressed in future studies.

Allergens drive EC to activate type 2 innate lymphoid cells

Allergen-mediated EC activation is also implicated in disease pathogenesis through the secretion of thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, IL-25 and IL-33. IL-25 and IL-33 (an alarmin) have been shown to activate group 2 innate lymphoid cells (ILC2) in the lung (Fig. 3). The innate lymphoid cell (ILC) family includes group 1 ILCs that predominantly express interferon- γ (IFN- γ), ILC2s that predominantly express IL-5, IL-9 and IL-13, and ILC3s that predominantly express IL-22 and/or IL-17. Among ILCs, ILC2 likely are involved in the early sensitization phase and the reactivation of effector or memory T cells upon re-exposure to allergens. In contrast to mucosal ILC2 from lung and gut-associated lymphoid tissue, ILC2 from skin respond to TSLP and are suggested to contribute to atopic dermatitis [82].

Several inhaled allergens (e.g. HDM, *Aspergillus*, *Alternaria*) as well as glycolipid antigens found in HDM and pollen have been shown to induce recruitment and proliferation of ILC2 in the lung, in particular in murine allergy models [83]. Unlike Th2 cells, ILC2s do not express receptors recognizing specific antigens. Hence, ILCs are non-antigen specific so that the mechanisms by which ILC2 become activated by allergens were investigated. Allergen-induced inflammation is related to EC stress or damage which are crucial for the activation of ILC2. It has been explored that proteases itself cause the damage of human and murine EC which

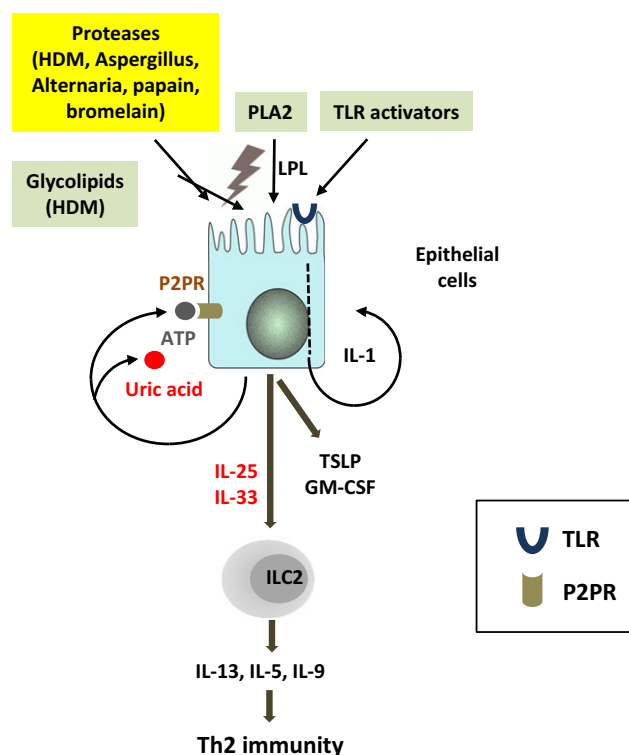


Fig. 3. Allergens drive EC to activate type 2 innate lymphoid cells (ILC2). Stimulation of EC by allergenic proteases (yellow) and HDM-derived glycolipids, phospholipase A2 (PLA2) or pathogen recognition receptors (PRR) induces stress and cell damage and secretion of IL-25 and IL-33 (danger signal) which are amplified by EC-derived uric acid, IL-1 and ATP. IL-33 is the main inducer of ILC2 which secrete Th2 cytokines such as IL-13 which is the key player in the early sensitization phase. TSLP, thymic stromal lymphopoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor; P2PR, P2 purinergic receptor; LPL, lysophospholipids.

release IL-33, and subsequently activate ILC2 which are the early source of IL-5 and IL-13 in allergen-induced airway diseases [82, 84–88]. Protease-induced release of IL-33 was suggested to be mediated by EC-derived danger signals. *Alternaria* proteases induce the release of danger signal ATP which promotes IL-33 release by the activation of P2 purinergic receptor in naïve human bronchial airway EC (see P2PR in Fig. 3) [89]. Using cysteine proteases such as bromelain and papain (from papaya) as model allergens, it has been shown that the release of uric acid from murine EC promotes the secretion of IL-33 [90].

In addition to proteases, the bee venom phospholipase A2 (PLA2) was capable of inducing Th2 and ILC2 responses that were dependent on its enzymatic activity. Mouse studies suggest that PLA2 cleaves membrane phospholipids to generate lysophospholipids which induce disruption of the cell membrane, cell death and the release of IL-33. IL-33 induces Th2 differentiation by signalling via ST2 on T cells [91]. It was proposed

that PLA2 activity represents a Th2-inducing activity that is conserved among a class of allergens, similar to protease activity, and the feature of PLA2 interaction with innate immune system is reminiscent of PAMP. Nevertheless, the translation of findings to the human system remains to be determined. Furthermore, allergen binding to PRR on EC induces IL-1 secretion which triggers IL-25 and IL-33 secretion in an autocrine feedback mechanism [92].

Finally, different mechanisms are involved in the activation of ILC2s. As ILC2s respond to helminths, virus and fungi, it remains interesting to investigate whether a specific Th2 immune response to non-proteolytic allergens can be facilitated by this pathogen-induced mechanism. Although ILC2s were initially discovered in the gut, the role of ILC2 in gastrointestinal allergy is less investigated.

Summary and conclusions

Allergens have been found to belong to a relatively small number of protein families. It has been suggested that the allergenicity of proteins is defined by typical characteristics of these protein families. Nevertheless, not all members of a certain protein family are allergens, and many allergens do not exhibit any known physicochemical, functional or structural properties that would account for their allergenicity. Therefore, it remains difficult to develop a general paradigm for the prediction of allergenicity on the basis of the biological function of a given protein. Also, allergenicity cannot be defined on common rules for allergen-IgE binding sites. Considering that the proposed attributes of allergenicity are shared by minor allergens and non-allergenic proteins, none of these features are reliable to discriminate between allergenic and non-allergenic proteins.

Today, the increasing knowledge about innate immune system has shifted the paradigm in our understanding of protein allergenicity. Pathways of innate immune activation appear central to contribute to allergenicity. Growing evidence arises that allergenicity is attributed to the intrinsic properties of allergens to activate the innate (and also the adaptive) immune system. These adjuvant properties are mainly

mediated by protease activity, carbohydrate residues and lipids which interact with the innate immune system to promote allergic Th2 responses. Proteases can (1) facilitate the access of allergens to the innate immune system, (2) activate PAR on EC and DC and (3) cleave receptor molecules on adaptive cells important for immune regulation. Allergens decorated with carbohydrates such as β -glucans and mannans or chitin are engaged by CLRs and can act as Th2-polarizing signal. Finally, lipid cargos associated with allergens likely act as Th2 adjuvants. Moreover, chemically modified allergens are taken up by CLRs and SR, which lead to enhanced T cell stimulation by APCs. Nevertheless, with the exception of group 2 aeroallergens, the responsible receptors and the mechanisms that control Th2 polarization need to be further investigated [24]. In summary, structurally and functionally different allergens interact in a different manner with immune cells.

The specific properties to interact with the innate (and adaptive) immune system allow to explain the allergenicity of several allergens, but probably cannot be generalized. Along with the intrinsic properties of allergens to activate the immune system, immune-modulating components of allergenic sources such non-allergenic proteases, pollen-associated lipid mediators (PALMs) or pollutants [93, 94], or environmental determinants such as contaminating bacteria or commensal bacteria of the gut and the lung [95] can substantially contribute to allergic diseases.

The question 'What makes an allergen as an allergen?' likely relies not only on features defined by the protein itself. Finally, the propensity to act as allergens in susceptible subjects is a complex phenomenon and cannot be predicted by a common rule. In line with this, the relationship between physicochemical and structural properties, the complexity of allergen exposure conditions, environmental cofactors and the intrinsic immune-modulating properties of proteins need to be addressed to assess the potential allergenicity of proteins.

Conflict of interest

The authors declare no conflict of interest.

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