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New Insights Into Innate Immune Mechanisms Underlying Allergenicity

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Abstract

Allergic diseases, which have reached epidemic proportions, are driven by inappropriate immune responses to a relatively small number of environmental proteins. The molecular basis for the propensity of specific proteins to drive maladaptive, allergic responses has been difficult to define. Recent data suggest that the ability of such proteins to drive allergic responses in susceptible hosts is a function of their ability to interact with diverse pathways of innate immune recognition and activation at mucosal surfaces. This review highlights recent insights into innate immune activation by allergens—via proteolytic activity, engagement of pattern recognition receptors, molecular mimicry of TLR signaling complex molecules, lipid binding activity, and oxidant potential—and the role of such activation in inducing allergic disease. A greater understanding of the fundamental origins of allergenicity should help define new preventive and therapeutic targets in allergic disease.

Introduction

The prevalence of allergic diseases has increased dramatically over the last few decades, with population prevalence rates reaching 30% in the industrialized nations that have led the epidemic. The defining feature of allergic disorders is their association with aberrant levels, and targets, of immunoglobulin E (IgE) production. Allergy is thought to result from maladaptive immune responses to ubiquitous, otherwise innocuous environmental proteins, referred to as allergens[1]. Allergens, by definition, are proteins that have the ability to elicit

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powerful T helper lymphocyte type 2 (Th2) responses, culminating in IgE antibody production (atopy). While allergens represent a minute fraction of the protein universe that humans are routinely exposed to, allergenicity is a very public phenomenon, with the identical proteins behaving as allergens in different allergic patients. Why specific proteins drive such aberrant T cell and B cell responses is a basic mechanistic question that has remained largely unanswered.

Allergens derive from a variety of environmental sources such as plants (trees, grasses), fungi (*Alternaria alternata*), arthropods (mites, cockroaches), and other mammals (cats, dogs, cows). Allergens constitute a diverse range of molecules, varying in size from small to large multi-domain proteins. As they are derived from complex living organisms they serve a broad range of functions in their respective hosts, from structural to enzymatic. For example, the common house dust mite allergens include several cysteine proteases (Der p 1, Der p 3), serine proteases (Der p 3, Der p 6, Der p 9), chitinases (Der p 15, Der p 18), lipid-binding molecules (Der p 2), and structural molecules such as tropomyosin (Der p 10). Some are species specific; others are molecules with broad biochemical homology that are found in many species. Much work has centered on the study of the allergen epitopes recognized by T and B cells. However, as there is no compelling evidence for common structural characteristics among the diverse T and B cell epitopes recognized in allergic responses [2,3,4], it appears doubtful that the presence of such B cell and T cell epitopes are sufficient to endow a protein with allergenic potential. Other factors such as the size, glycosylation status, resistance to proteolysis, and enzymatic activity, have been suggested to play an important role in allergenicity. However, none of these factors have been consistently linked with allergenic potential. For example, glycosylation appears not to be a common critical determinant of allergenicity as both glycosylated and non-glycosylated proteins act as food allergens. It may be that there are many structural paths to allergenicity, but the absence of any common structural motif or conformational sequence pattern leaves open the possibility that proteins with allergic potential exhibit a necessary commonality of biological function. Indeed, it has recently been proposed that allergens are linked by their ability to activate the innate immune system of mucosal surfaces, triggering an initial influx of innate immune cells that subsequently drive Th2-polarized adaptive immune responses. It should be noted that, reductive experimental systems aside, natural exposure is not to single, purified proteins, but to complex mixtures of molecules. It may well be that the innate immune-activating molecules are not identical to the proteins recognized by allergic responses, although this would still beg the question as to why those particular proteins are so recognized among the many present during exposure. In this review, we will address recent advances in our understanding of the diverse innate immune-activating properties of allergens that appear to endow them with a propensity for driving Th2 immune responses.

Protease Activity and Allergic Sensitization

Several allergens have cysteine or serine protease activity, including diverse allergens from arthopods [e.g., house dust mites [5-7], German cockroaches [8], fungi (*Alternaria alternata*)[9] and *Cladosporium herbarum* [10], mammals (e.g., *Felis domesticus*)[11], plants (e.g. pollens from ragweed [12]]. In addition, many forms of occupational allergy are associated with

encounters with proteolytic enzymes such as those used in the manufacture of detergents (alkaline detergents)[13], or in the food industry (papain)[14].

Several lines of evidence suggest that proteases may facilitate allergen sensitization. First, intrinsic protease activity appears to be linked with sensitization ability in several allergens. Removal of proteases from *A. fumigatus* [15], German cockroach frass [16], American cockroach Per a 10 antigen[17], Epi p1 antigen from the fungus *Epicoccum purpurascens* [18] or Cur 11 antigen from the mold *Curvularia Iunata* [19] was reported to decrease airway inflammation and airway hyperresponsiveness in mouse models of allergic asthma. Secondly, direct exposure of mice to proteolytic enzymes such as papain can induce allergic sensitization [20]. Moreover, co-administration of active proteases from *A. fumigatus* with the tolerogenic antigen, ovalbumin (OVA), resulted in allergic sensitization [15]. As co-exposure to a tolergenic protein with a protease can induce allergic sensitization, proteases found in ambient air derived from bacterial and viral species may play accessory roles. Lastly, subcutaneous injection of a serine protease inhibitor, nafamostat mesilate, during sensitization to house dust mite extracts blunted the development of allergic inflammation and airway hyperresponsiveness [21].

The exact mechanisms by which proteases can drive allergic sensitization are not well understood. Several mechanisms have been postulated. Firstly, protease activity may increase transepithelial access of allergens to critical cells of the innate immune response, such as dendritic cells (DCs). For example, the cysteine protease Der p 1, can alter epithelial permeability through disruption of epithelial tight junctions and a reduction in ZO-1 and occludin content [6]. Consistent with this, proteolytic enzymes from a number of tree and grass pollens have also been shown to degrade ZO-1 and disrupt tight junctions [22]. Moreover, Der p 1 has been shown to cleave α-1-anti-trypsin, inhibiting its ability to protect the respiratory tract against serine proteases such as Der p 3 and Der p 9. This may disrupt the protease-anti-protease balance in mucosal tissues, enhancing the activity of both endogenous and exogenous proteases and leading to enhanced tissue damage and immune activation.

Secondly, the cysteine protease activity of several mite allergens (Der p 1, Der f 1) may directly impair innate defense mechanisms in the lung by degrading and inactivating lung surfactant proteins (SP) −A and −D [23]. SP-A and AP-D are calcium-dependent carbohydrate-binding proteins with multiple innate immune functions, including bacterial agglutination and modulation of leukocyte functions. Importantly, SP-D and SP-A have been shown to protect against *Aspergillus fumigatus*-induced allergic inflammation in mice [24], [25], likely via binding to glucan moieties of inhaled allergens and facilitation of their clearance.

Proteases may have more direct immunomodulatory actions as well. Proteases from mites, cockroaches, and fungi can increase the expression of cytokines, including interleukin (IL)-6, IL-8 and GM-CSF [5], [6], [8], [9], which may lead to the recruitment, activation and/or enhanced survival of DCs at the mucosal surface. Additionally, Der p 1 has been shown to influence the expression of costimulatory molecules such as CD40 on DCs. Der p 1 can cleave CD40 on human monocyte-derived DCs, resulting in inhibition of the production of

the pivotal Th1-differentiating cytokine, IL-12 [26]. The suppression of CD40 signaling and IL-12 production may induce a shift towards Th2 responses. A similar effect has been observed with the mold *Aspergillus* (Asp). Exposure of healthy human monocyte-derived DCs to Asp induced their maturation and enhanced their ability to prime Th2 immune responses in allogeneic naïve T cells as compared with naive T cells primed with LPS-activated DCs [27]. When the proteolytic activity of Asp was neutralized by chemical inactivation, Asp failed to up-regulate costimulatory molecules on DCs, and these DCs did not prime a Th2 response in naive T cells. The skewed Th2 response was thought to occur as a result of suppressed IL-12 production by Asp-primed DCs. Interestingly, although the exact mechanisms by which allergen-derived proteases influence the decision making capability of DCs are not well understood, recent studies suggest that protease containing allergens such as Der p 1 can target two C-type lectins, DC-SIGN and DC-SIGNR [28]. Loss of DC-SIGN expression following Der p 1 treatment led to a reduction in its binding to its ligand, ICAM-3, on naïve T cells, which is thought to be important in Th1 signaling [28]. Thus the combined proteolytic activities of Der p 1 on surface expression of molecules such as CD40, and DC-SIGN could have profound effects on the decision making capability of DCs, biasing adaptive immunes response towards a Th2 pattern of response.

Recently, study of the occupational allergen, papain (commonly used in the food industry), has led to the novel hypothesis that proteases can directly prime Th2 immune responses through actions on basophils [20]. Data suggest that papain can cleave a yet-to-be identified host sensor which, in turn, activates basophils to produce IL-4, and thymic stromal lymphopoietin (TSLP)—driving Th2 differentiation. This intriguing work supports the concept that host detection of protease activity associated with allergens may provide a unique pathway of innate immune activation. The generality of this pathway, as well as its molecular identification, remain to be defined.

Allergen-derived proteases can have direct effects on adaptive immune responses as well, through cleavage of molecules such as CD25, and CD23. Specifically, Der p 1 has been shown to be able to cleave the α chain of the IL-2 receptor (CD25) on human T cells [29] (Figure 1). As a result, T cells exposed directly to Der p 1 display markedly reduced Th1 cytokine production and enhanced Th2 cytokine production, something dependent on the protease activity or Der p 1. Cleavage of CD25 might also, of course, alter regulatory function, as IL-2 stimulation is required for the maintenance of regulatory T cells in the periphery. The overall effect may be to shift the balance of immune responses from a tolerogenic response to one favoring a Th2 pattern of response. Allergenic proteases such as Der p 1 have also been shown to be able to cleave the low affinity receptor for IgE, CD23, from the surface of human B cells, releasing the soluble form of the receptor [30] (Figure 1). As the membrane-bound form of the IgE receptor is thought to act as a negative regulator of IgE synthesis, Der p 1 cleavage of CD23 could potentially disrupt the negative feedback signal and enhance IgE synthesis, thereby amplifying the allergic response. Anti-trypsin can inhibit this effect of Der p 1 on CD23 cleavage, suggesting that disruption of the balance between proteases and protease inhibitors might play a role in allergic sensitization. It should be noted that whether intact proteases such as Der p 1 actually gain functional access to lymphocytes *in vivo* remains an open question.

The biological effects of some allergenic proteases may also be mediated through activation of the Protease-Activated Receptor 2 (PAR2). PARs (1,2,3,4) are a family of proteolytically activated G-protein coupled receptors. Proteases cleave within the N-terminus of the receptors and expose a tethered ligand domain that binds and activates the cleaved receptor. Several lines of evidence suggest that PAR2, in particular, may be important in allergic sensitization. It is expressed by many cells in the lung, including airway epithelial cells [31], fibroblasts [32], macrophages [33] and mast cells [34], and, importantly, patients with asthma have been shown to exhibit increased expression of PAR2 on respiratory epithelial cells [35]. Several house dust mite allergens (Der p1, (30), Der p 3, Der p 9), along with German cockroach extract [36], have been shown to be able to cleave and activate PAR2 (Figure 1). Several reports have shown that activation of PAR2 by house dust mite extract [7], German cockroach [37], or the mold allergen Pen c 13 [38] leads to increased cytokine production by airway epithelia. Specifically, airway epithelial cells were shown to increase the expression of TSLP through the activation of PAR2 when treated with papain, trypsin or the fungus *Alterneria* [9]. As TSLP drives DC polarization of naïve T cells to a Th2 phenotype, these results suggest that PAR2 activation may serve as a link between innate and adaptive immune responses.

Several mouse models of allergic inflammation have also underscored a potential role for PAR2 in allergic sensitization. For example, one recent study showed that tolerance to inhaled OVA could be overcome by co-administration of PAR2-activating peptides to the airways, promoting allergic sensitization [39]. Other studies have shown that overexpression of PAR2 in mice renders them susceptible to allergic airway inflammation when sensitized and challenged locally with OVA, as compared to wildtype controls [40]. In contrast, PAR2 deficient mice were protected against allergic inflammation. While these data strongly support the concept that protease activity may lead to airway allergic sensitization via PAR2 activation, there are a few reports suggesting that PAR2 activation may also reduce airway inflammation. For example, despite the fact that TLR4 (*vide infra*) and PAR2 signaling have been shown to exhibit cooperativity [41], PAR2-activating peptides have been reported to inhibit lipopolysaccharide(LPS)-induced neutrophil influx into mouse airways [42]. In a rabbit model of experimental asthma, sensitization to the pollen *Parietaria judaica*, followed by allergen challenge in the presence or absence of a PAR2-activating peptide, led to PAR2-mediated attenuation of the development of airway hyperresponsiveness and airway eosinophilia [43]. It is possible that the discrepancies in these results are due to the timing of PAR2 activation, with activation by exogenous proteases during generation of immune responses having different effects than that occurring in the midst of an ongoing inflammatory response.

Toll-like Receptors, Lipid-Binding Activity and Allergic Sensitization

Recent recognition of the critical roles played by innate pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs), in activation and instruction of antigen-presenting cells (APCs)[44] has led to the exploration of the role of these pathways in allergic responses. It will be noted that, while TLR-driven activation of Th1 responses by DCs is

well-studied and -understood, the receptors and pathways driving Th2 immune responses have been considerably less tractable to experimental investigation.

Numerous studies have probed the role of TLR4 signaling in allergic inflammation. Epidemiological studies have reported an inverse correlation between high levels of bacterial products such as LPS in the ambient environment during very early life and the subsequent development of atopy and allergic disease [45-47]. It has been postulated, *pace* the hygiene hypothesis, that such exposures drive robust counter-regulatory tone in the developing immune system [48]. LPS exposure can also exacerbate established asthma, however, probably by direct stimulation of airway pro-inflammatory responses [49]. Experimental mouse models have provided mechanistic insight into the ability of LPS exposure to regulate the development of allergic asthma. As predicted by the hygiene hypothesis, LPS dose appears to be a critical variable. While airway sensitization with OVA along with “very low dose” (<1 ng) LPS was reported to induce tolerance, sensitization in the presence of “low dose” (100 ng) LPS drove TLR-dependent, Th2 inflammation, and sensitization in the presence of “high dose” (100 ug) LPS led to a Th1 (and likely a regulatory) response [50, 51]. Recent studies using bone marrow chimeras indicate that TLR4 signaling in radioresistant cells, not radiosensitive hematopoietic cells, are necessary and sufficient for DC activation and priming of allergic effector T helper responses in the lung in response to dust mite extracts [52]. As for other TLRs, TLR2 ligands have been shown to be able to drive [53] or inhibit [54] Th2 differentiation and allergic inflammation in the lung.

A recent study reported a more direct link between TLR signaling and allergic sensitization. Among defined dust mite antigens, Der p 2 and Der f 2 have the highest rates of skin test positivity in atopic patients [55]. Notably, sequence homology places these allergens in the MD-2-related lipid-recognition (ML) domain family of proteins [56,57]—MD-2 being a secreted protein that is the LPS-binding member of TLR4 signaling complex. As the crystal structures of Der p 2 and MD-2 exhibit structural homology, Trompette *et al.* [58] examined whether Der p 2 exhibited functional homology as well. Indeed, they reported that Der p 2 can facilitate TLR4 signaling through direct interactions with the TLR4 complex, reconstituting LPS-driven TLR4 signaling in the absence of MD-2 and facilitating such signaling in the presence of MD-2 (Figure 1). They further found that Der p 2 could facilitate LPS signaling in primary APCs, with or without MD-2 being present. Finally, they reported that the *in vitro* functional and biochemical activity of Der p 2 mirrors its *in vivo* allergenicity—Der p 2 drives experimental allergic asthma in a TLR4-dependent manner, retaining this property in mice with a genetic deletion of MD-2. These data suggest that Der p 2's propensity to be targeted by the adaptive immune response is a function of its autoadjuvant properties. In this light, it should be noted that efficient generation of effector T cell responses has been shown to depend on the presence of TLR ligands in the specific DC phagosome that contains the antigen [59]. In the case of Der p 2, antigen and TLR ligand are, perforce, co-localized. These data also suggest the possibility that Der p 2-mediated facilitation of TLR4 signaling under conditions of bacterial product exposure— those associated with increasing rates of aeroallergy in the urban, Westernized world— may shift the LPS-response curve from the tolerizing into the Th2-inducing range. Der p 2 may also

promote exacerbation of established asthma by facilitating TLR4 signaling by airway epithelial cells— cells reported to express TLR4, but little or no MD-2, in the basal state [60].

Several other members of the MD-2-like lipid-binding family are major allergens[4], suggesting generality for these findings. More broadly, however, greater than 50% of defined major allergens are thought to be lipid-binding proteins [4], something that suggests that intrinsic adjuvant activity by such proteins and their lipid cargo is likely to have wide generality as a mechanism underlying the phenomenon of allergenicity. Further studies defining the lipids normally bound by these allergens, the receptors thereby activated, and the pathways of innate and adaptive immune response driven by such activation are awaited. It should be noted that, in addition to activation of TLRs, lipid ligands are known to be important drivers (and targets) of innate lymphocyte responses.

Carbohydrate Structures and Allergic Sensitization

Recent data also suggest an important role for complex carbohydrates in driving Th2 immune responses. Helminth-derived carbohydrates such as lacto-*N*-fucopentaose III (LNFPIII) have been shown to promote Th2 responses via their ability to activate DCs in vivo [61]. In addition, LNFPIII has been shown to be able to promote Th2 responses to a co-administered, unrelated antigen such as human serum albumin [62]. Though the mechanisms underlying DC activation by LNFPIII glycoconjugates has not been fully elucidated, it involves ligation of C-type lectins on the DC, leading to subsequent antagonism of TLR signaling. Support for a broad role for complex carbohydrates, in particular glucans, in allergen-associated Th2 immune responses is emerging. Glucans are a diverse class of naturally occurring glucose polymers, which can be short or long, branched or unbranched, exist as α or β isomers, and be soluble or particulate. For the purposes of this discussion, we are mostly concerned with the β-glucans, which contain a polyglucose, (1-->3)-beta-D-glucan, and are commonly found in the cell walls of fungi, pollens, and certain bacteria. In plants, polymers of β-glucans are thought to protect the developing pollen during meiosis, and are later destroyed by the enzyme (1→3)-β-d-glucanase to liberate the microspores. Although β-glucans are widely expressed, they are not found in mammalian cells. As such, they can act as PAMPs, triggering immune responses through activation of specific PRRs.

The immunostimulatory properties of β-glucans have been recognized for decades, since their identification as the immunoactive component of mushrooms [63]. More recently, β-glucans have been shown to be able to drive Th2 responses. For example, it has been reported that β-glucan structures present in the peanut glycoallergen Ara h 1 have Th2 inducing characteristics [64] native, but not deglycosylated, Ara h 1 was shown to activate human monocyte-derived DC and induce IL-4 and IL-13 secreting Th2 cells.

Exposure to β-glucans has also been shown to induce airway hyperresponsiveness in allergic humans [65]. Studies in guinea pigs have shown that direct delivery of (1—3)-β-D-glucan to the airways can induce the recruitment of lung eosinophils and lymphocytes [66]. In mice, exposure to soluble β-glucan isolated from *Candida albicans* [67] markedly exacerbated OVA-induced eosinophilic airway inflammation, concomitant with enhanced lung expression of Th2 cytokines and IL-17A. Exposure to β-glucans plus OVA increased the number of cells bearing MHC class II and the expression of APC-related molecules such as

CD80, CD86, and DEC205 on bone marrow derived DCs. In support of a role of β-glucans in the recruitment and activation of DCs at mucosal surfaces, recent studies have shown that β-glucans contained in house dust mite extracts and in moulds may initiate immune responses at the mucosal surface. House dust mite extract-mediated induction of the release of the chemokine, CCL20, which recruits immature DCs, by human airway epithelial cells in culture, was shown to occur through β-glucan and Syk-dependent signaling pathways [68] (Figure 1). Although the exact lectin receptor mediating these effects was not identified in these studies, the results suggested that β-glucan moieties contained in house dust mite extracts might mediate early processes leading to immature DC recruitment to the airways. This concept is supported by another recent study that showed that dectin-2 receptor signaling pathways (Dectin-2/FcRgamma/Syk) mediated the production of cysteinyl leukotrienes in bone marrow-derived DCs following stimulation with house dust mite extracts or Aspergillus [69] (Figure 1). Taken together, these findings identify the dectin-2/FcRgamma/Syk axis as a novel receptor mediated pathway by which several potent allergens are recognized by innate immune cells at the airway surface, linking them with the development of Th2-skewed adaptive immune responses.

Consistent with a role for lectins in driving Th2 immune responses, blockade of the mannose receptor, an endocytic C-type lectin receptor, significantly reduced Der p 1 uptake by DCs [70]. These findings are consistent with previous findings suggesting that engagement of the mannose receptor by selected ligands on human DCs leads to the induction of a DC phenotype favoring Th2 polarization [71]. Although the study of the role of glucans as Th2-inducing PAMPs is only in its infancy, data to date suggest that carbohydrate moieties contained in common allergens act as strong Th2 inducers via activation of variety of C-type lectin receptors on DCs.

Oxidative activity and Allergen Sensitization

It has recently been shown that common allergenic pollen grains contain nicotinamide adenine dinucleotide phosphate (reduced) [NAD(P)H] oxidase activity as well as allergens [72]. Such pollen grains have been shown to significantly increase the levels of reactive oxygen species (ROS) in cultured cells, and to be able induce allergic airway inflammation in experimental animals [73] (Figure 1). Pretreatment of these pollen grains with NAD(P)H oxidase inhibitors attenuated their capacity to increase ROS levels in airway epithelial cells and subsequent airway inflammation. Similarly, pre-treatment of mice with antioxidants has been shown to prevent the development of pollen-driven asthma in mice. Interestingly, delaying anti-oxidant treatment until after pollen challenge was ineffective, suggesting that the oxidase activity is of critical importance during the period of innate immune activation. Although the mechanisms remain to be defined, it has been speculated that NADPH oxidase activity initiates immune activation through its ability to recruit inflammatory cells, possibly through the induction of IL-8 by p38 MAPK [74]. Of interest, genetic polymorphisms in genes regulating oxidative stress have been shown to be associated with susceptibility to asthma in several populations [75].

Conclusions

Although allergens are a diverse group of molecules, it is becoming increasingly clear that their allergenicity likely resides in their ability to activate various innate immune pathways at mucosal surfaces, rather than in any structural similarities. Complex allergens contain multiple innate immune activating components, which trigger the initial mucosal influx of innate immune cells that subsequently drive Th2-polarized adaptive immune responses. Although the study of innate activating properties of allergens is in its infancy, it is clear that a better molecular understanding of the fundamental origins of allergenicity may well lead to the development of new therapeutic strategies to effectively block allergen recognition and the ensuing inflammatory cascade.

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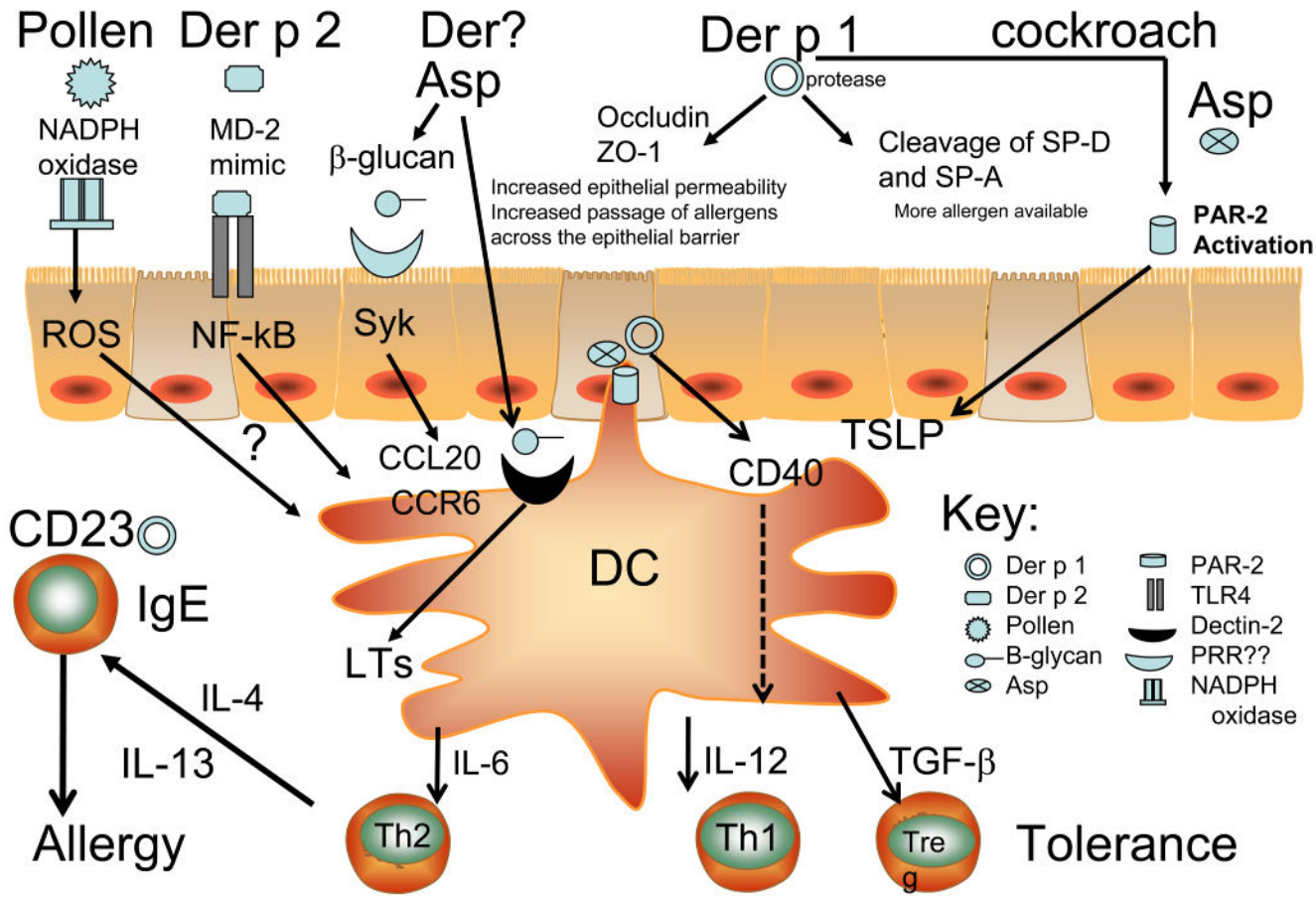

Figure 1. Schematic of innate immune mechanisms activated by allergens