

REVIEW ARTICLE

Protease allergens as initiators–regulators of allergic inflammation

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Abstract

Tremendous progress in the last few years has been made to explain how seemingly harmless environmental proteins from different origins can induce potent Th2-biased inflammatory responses. Convergent findings have shown the key roles of allergens displaying proteolytic activity in the initiation and progression of the allergic response. Through their propensity to activate IgE-independent inflammatory pathways, certain allergenic proteases are now considered as initiators for sensitization to themselves and to non-protease allergens. The protease allergens degrade junctional proteins of keratinocytes or airway epithelium to facilitate allergen delivery across the epithelial barrier and their subsequent uptake by antigen-presenting cells. Epithelial injuries mediated by these proteases together with their sensing by protease-activated receptors (PARs) elicit potent inflammatory responses resulting in the release of pro-Th2 cytokines (IL-6, IL-25, IL-1 β , TSLP) and danger-associated molecular patterns (DAMPs; IL-33, ATP, uric acid). Recently, protease allergens were shown to cleave the protease sensor domain of IL-33 to produce a super-active form of the alarmin. At the same

Abbreviations: ADAM, A disintegrin and metalloprotease; ADI, Allergen delivery inhibitor; AEC, Airway epithelial cell; ATP, Adenosine 5'-triphosphate; CCL, C-C chemokine ligand; CD, Cluster of differentiation; CDC2, Type 2 Myeloid/conventional dendritic cell; CXCL, C-X-C chemokine ligand; DAMP, Danger-associated molecular pattern; DC, Dendritic cell; DC-SIGN, Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; EGFR, Epidermal growth factor receptor; GM-CSF, Granulocyte macrophage-colony stimulating factor; GPCR, G protein-coupled receptor; HDM, House dust mite; IDO, Indoleamine 2,3-dioxygenase; IL, Interleukin; ILC2, Type 2 innate lymphoid cell; MD2, Myeloid differentiation factor 2; MDA-5, Melanoma differentiation-associated protein-5; MRGPR, Mas-related G-protein-coupled receptor; PAR, Protease-activated receptor; PRR, Pattern-recognition receptor; RIG-I, Retinoic acid-inducible gene-I; RiPIL-33, Ripoptosome IL-33 maturation; SPINK, Serine peptidase inhibitor Kazal type; Th17, Type 17 T-helper cell; Th2, Type 2 T-helper cell; TJ, Tight junction; TLR, Toll-like receptor; TRPV, Transient receptor potential vanilloid-type; TSLP, Thymic stromal lymphopoietin; ZO-1, Zonula occludens-1.

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time, proteolytic cleavage of fibrinogen can trigger TLR4 signaling, and cleavage of various cell surface receptors further shape the Th2 polarization. Remarkably, the sensing of protease allergens by nociceptive neurons can represent a primary step in the development of the allergic response. The goal of this review is to highlight the multiple innate immune mechanisms triggered by protease allergens that converge to initiate the allergic response.

KEYWORDS

IL-33, protease allergen; epithelial cell, protease-activated receptor, sensory neuron

1 | INTRODUCTION

Allergic inflammation was once simply described by the cross-linking of allergen-specific IgE and activation of CD4⁺ T cells to produce Th2 cytokines (e.g., IL-4, IL-5, or IL-13), and is now known to be dominated by more complex and nuanced interactions between the immune cells and barrier tissues. Recent insights have exposed key roles for skin, airway (nasal, bronchial), and gastrointestinal (esophageal and intestinal) epithelial cells, as well as sensory neurons in allergen sensing.¹ These observations have underlined the role of allergen bioactivity and function in the initiation and propagation of an allergic immune response. However, our knowledge of the intrinsic proinflammatory properties of allergens remains limited. One functional attribute of allergens known to determine their allergenicity is proteolytic activity. Proteolytic allergens are found in major airborne sensitizers, but are a restricted feature in the entirety of the known allergome.² Despite their uncommonness, the isolation and characterization of IgE binding capacity of all the proteolytic enzymes (termed proteases or proteinases) from allergenic sources remain an incomplete and challenging task.

A distinguishing feature of protease allergens is their intrinsic ability to overcome host tolerance. Without additional adjuvants, allergenic house dust mite (HDM) extracts, papain or fungal allergens induce allergen-specific IgE, initiate gene expression and mediator release, and recruit eosinophils and antigen-presenting

cells to challenged airways or skin.^{3–6} However, this process is prevented by specific^{6,7} and non-specific protease inhibitors,^{6–12} or inhibitors of signaling pathways activated by protease allergens.^{13–18} Furthermore, the addition of a protease to an enzymatically inactive protein can render that protein allergenic.^{4,10,19–21} Thus, not only does protease activity endow an allergen with its adjuvant activity, but it also acts as an exogenous allergic adjuvant for other inert or otherwise not allergenic proteins. For those reasons, protease allergens could be defined as initiator allergens promoting allergy development.

The broad substrate specificity of protease allergens means that a clear identification of their immune biosensors is a challenging task. HDM proteases and papain can directly activate mast cells and basophils. However, while mast cell function may promote sensitization, neither mast cells nor basophils are necessary for the allergic sensitization.^{8,22–24} That said, mild eosinophilia following protease delivery to the airways of HDM-naïve wild type or Rag-2 deficient mice indicates the presence of IgE-independent sensing mechanisms for protease allergens.^{6,8,25,26} Despite these complexities, fundamental advances have been made in understanding allergic immune sensitization by protease allergens to clarify the process of protease allergen sensing. The purpose of this review is to characterize the different molecular mechanisms triggered by protease allergens which promote the development of allergic diseases (Box 1). Particular attention is given to the effects of protease allergens on epithelial cells and sensory neurons.

BOX 1 Major milestone discoveries.

- The protease activity of allergens is sufficient to elicit pro-Th2 innate immune signaling pathways
- Protease allergens can alter epithelial integrity, permitting their entry where they can maintain activity by altering antiprotease defenses.
- Cleavage of fibrinogen by protease allergens or by thrombin matured by protease allergens elicits allergic responses through Toll-like receptor-4 (TLR4) activation
- Serine protease allergens trigger protease-activated receptor 2 (PAR2) activation
- Protease allergens induce the release of the alarmin IL-33, as well as cleavage to its highly active state, although this response is not limited to protease allergens
- Protease allergens directly activate nociceptive neurons, enriched in transient receptor potential vanilloid-type 1 (TRPV1)⁺ neurons, which promote the migration of Th2-skewing dendritic cells

2 | MAIN PROTEASE ALLERGENS

In December 2022, the updated list of allergens from the WHO IUIS allergen database (official list of allergens issued by the International Union of Immunological Societies Allergen Nomenclature Subcommittee) (<http://www.allergen.org>) included 1089 entries. Of these, only 57 were classified as proteases or peptidases based on their protein sequence/structural similarities with archetypal serine, cysteine, aspartic or metallo-proteases (Table S1). A limited number of allergenic sources contain protease allergens: HDMs, cockroaches, fungi, pollens, fruits, and bee venoms. However, as only few allergens have yet been characterized for their proteolytic activity and propensity to stimulate innate immune signaling pathways, the present review will be focused mainly on the protease allergens listed in Table 1. These endopeptidases display cysteine or serine protease activity, according to their active site residues, respectively, cysteine-histidine-asparagine or serine-Histidine-Aspartic acid (Figure 1). Although the physiological roles of these proteases in their corresponding allergenic source remain to be elucidated, it is reasonable to assume their involvement in protein turnover, nutrient production through proteinaceous substrate digestion (in the digestive gut for HDM and cockroach or externally for fungi), fungal/plant defense system, and also immune evasion.^{27–29} Nearly all proteases are produced as enzymatically inactive zymogens and require spatiotemporal activation either by auto-catalytic processing or activation by other proteases. A typical example is the natural self-maturation of the HDM allergen Der p 1 under acidic conditions.³⁰ This process also plays a key role in the maturation of serine protease allergens Der p 3, Der p 6, and Der p 9.³¹

Protease allergens are usually stable and abundant proteins in their allergenic sources (up to several hundreds of µg/g of extracts for the highly expressed ones).^{11,32} Their presence in mite fecal pellets, cockroach frasses or their extensive secretion by fungi can greatly facilitate their airborne transmission. Collectively, these properties could explain, at least in part, why the identified protease allergens are major sensitizers, with IgE binding frequencies higher than 50% (www.allergen.org).

3 | PROTEASE ALLERGENS AND TISSUE BARRIER PERMEABILITY

In the airway, skin, and esophageal epithelium, tight junctions (TJs) have an important role in forming the dynamic protective barriers at these surfaces.³³ Disruption of these barriers perturbs tissue homeostasis and enables external agents such as allergens to gain increased contact with the immune system and sensory neurons.^{5,24}

The principal adhesion proteins of TJs are the claudin family, MARVEL domain proteins such as occludin, and junction-associated molecules (JAMs). TJs are theoretically capable of permitting passage to some low molecular weight allergens (Stokes' radius < 2 nm). In reality, the transepithelial flow of the ~14kDa group 2 HDM

allergens is low unless catalytically competent group 1 allergens are also present, indicating that shape, oligomerization, and desolvation are additional influencers of allergen permeation, and underscoring the importance of protease allergens in accelerating the flux of unrelated allergens through direct and indirect means.⁷

HDM fecal pellets and HDM allergen extracts both trigger the cleavage of TJs in bronchial epithelial cells with a concomitant rise in barrier permeability,^{6,34} facilitating the sensing of allergens by dendritic cells and sensory neurons (Figure 2). These effects are replicated by Der p 1 or the serine peptidase HDM allergens.⁶ Predictably, the actions of Der p 1 are prevented by Der p 1-selective inhibitors, but these agents also substantially inhibit the effects of HDM allergen extracts suggesting that group 1 allergens spearhead the proteolytic attack.⁶

Disruption of junctional proteins was also observed in airway epithelial cells (AECs) treated with the fungal (Pen c 13) or the cockroach (Per a 10) serine protease allergen.^{35,36} Similar barrier erosion occurs in primary cultures of human sinonasal cells exposed to Der p 1³⁷ or serine protease activity of *Alternaria* extracts,³⁸ in the skin of mice exposed to Der f 1,³⁹ keratinocyte models and mouse skin exposed to papain.^{10,40,41} Occludin and claudins are target substrates of HDM protease allergens, and specific cleavage sites have been identified in extracellular domains. Notably, occludin and claudin proteins (e.g., -1, -3, -4, -5, -7, and -18) which regulate paracellular permeability conserve a Leu-Leu dyad cleavage "hotspot" for group 1 HDM allergens.^{7,26} For mites, junctional complexes in the airway epithelium also undergo indirect proteolysis which may be PAR-dependent.^{6,7,15,34} A recent study showed that the fungal allergen Asp f 13 damaged airway epithelial TJ and E-Cadherin (AJ). In bronchiolar club cells, a distinct subset of epithelial cells,⁴² this junction injury triggered a calcium influx sensed by the mechanosensor TRPV4 leading to allergic inflammation. In addition to airways and skin, other barriers may be affected by protease allergens (Figure 2). Although conventionally considered as being encountered via the airways, Der p 1 was detected in human gut and HDM extracts were shown to augment colonic barrier permeability in a cysteine protease-dependent manner.⁴³ Moreover, HDM extracts can degrade occludin and ZO-1 in the human intestinal epithelial Caco-2 cell line. Collectively, these findings suggest the possibility of oral sensitization to HDM allergens through protease-dependent events.⁴³ Analogously, kiwi fruit actinidin (Act d 1) increases intestinal permeability in tandem with the expression of the Th2-promoting cytokines IL-25, IL-33, and TSLP in vivo⁴⁴ and cleavage of occludin and ZO-1 has been observed in Act d 1-treated intestinal epithelial cells.^{45,46}

Protease allergen effects on TJs are highly localized, temporary and can be fully reversible. However, these permeability changes, for a time, allow any allergen an increased probability of contact with antigen-presenting cells and sensory neurons. This process may be aggravated in human disorders where the homeostatic antiprotease systems, normally found in barrier tissues, are lost. In the skin and esophagus, epithelial differentiation is accompanied by expression of high levels of serine protease inhibitors such as

TABLE 1 Protease allergens identified as stimulators of innate immune pathways.

Source type	Name	Allergen source	Protease family	Physiological role	MEROPS classification/ Uniprot/PDB	Ref.
Arthropod	Der p 1	<i>Dermatophagoides pteronyssinus</i>	Cysteine protease (Papain-like)	Digestive function	C01.073/P08176/1XKG	188,189
	Der f 1	<i>D. farinae</i> (HDM)		Maturation of Der p 3, 6, 9		
	Der p 3	<i>D. pteronyssinus</i>	Serine protease	Digestive function	S01.234/P39675/N.A.	28
	Der f 3	<i>D. farinae</i>	(Trypsin-like)		S01.234/P49275/N.A.	
	Tyr p 3	<i>Tyrophagus putrescentiae</i> (HDM)			S01.234/C6ZDB5/N.A.	
	Der p 9	<i>D. pteronyssinus</i> (HDM)	Serine protease	Digestive function	S01.031/Q7Z163/N.A.	110
Fruit	Per a 10	<i>Periplaneta americana</i> (American cockroach)	Serine protease (Trypsin-like)	Digestive function	S01.510/Q1M0X9/N.A.	27
	Papain	<i>Carica papaya</i> (Papaya)	Cysteine protease	Protein turn-over; Plant defense response	C01.001/P00784/2CIO	190
	Act d 1	<i>Actinidia deliciosa</i> (Kiwi)	Cysteine protease	Protein turn-over; Plant defense response	C01.007/P00785/2ACT	190
Fungi	Ana c 2 (Bromelain)	<i>Ananas comosus</i> (Pineapple)	Cysteine protease	Protein turn-over; Plant defense response	C01.028/O23791/6YCB	190
	Asp f 13	<i>Aspergillus fumigatus</i> , <i>flavus</i> ,	Subtilisin-like Alkaline	Degradation of environmental proteins for	S08.053/P28296/N.A.	191,192
	Asp fl 13	<i>oryzae</i> (Common mold)	serine protease	metabolic growth; Complement evasion	S08.053/N.A./N.A.	
	Asp o 13			mechanism	S08.053/P12547/N.A.	
	Pen ch 13	<i>Penicillium chrysogenum</i> ,	Subtilisin-like Alkaline	External digestion of macromolecular nutrients	S08.025/Q9URR2/N.A.	35,193
	Pen c 13	<i>citrinum</i>	serine protease		S08.025/N.A./N.A.	

Note: MEROPS: Peptidase Database (<https://www.ebi.ac.uk/merops/>), Uniprot: Protein sequence database (<https://www.uniprot.org/>), PDB: Protein Data Bank (<https://www.rcsb.org/>).

Abbreviation: N.A., not available.

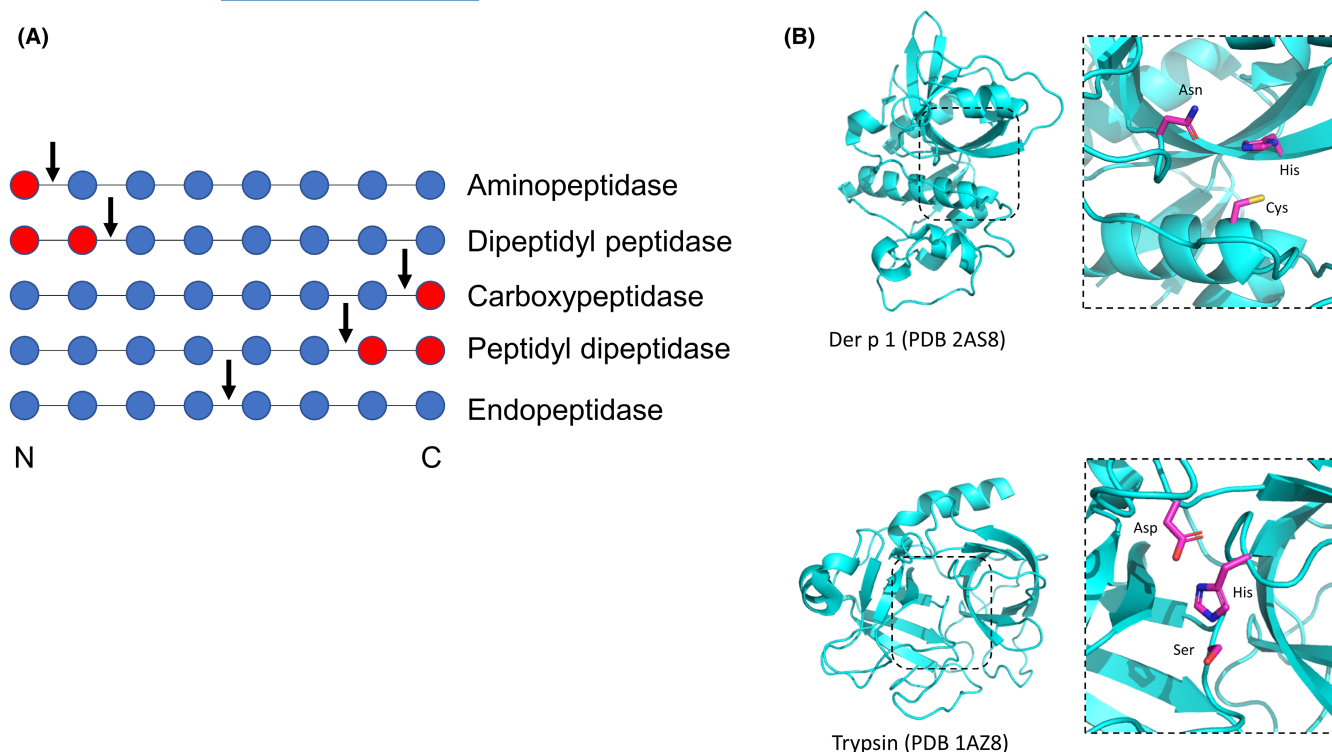


FIGURE 1 The identified protease allergens inducing innate immune activation are cysteine or serine proteases. Proteases can be classified according to the location of the cleavage site in their putative substrates (A) and to the nature of their active site residues (B). With the exception of bee venom dipeptidyl peptidase allergens, all the identified protease allergens are endopeptidases (Table S1). The arrows point to the cleavage site on the protein substrate and the red circles the terminal amino acids or dipeptides released by the proteolysis. Most of cysteine and serine protease allergens share structural homologies with Der p 1 and Trypsin, respectively. The insets of panel B shows the key residues of their respective active site (Cysteine–Histidine–Asparagine or Serine–Histidine–Aspartic acid for Der p 1 and trypsin, respectively). Structures taken from the Protein Data Bank (PDB, <https://www.rcsb.org/>) and figure generated using Pymol.

SPINK5 or SPINK7 (serine peptidase inhibitor kazal type 5 and 7, respectively).⁴⁷ Genetic or acquired loss of expression of these protease inhibitors results in uncontrolled serine protease activity involving kallikrein family members which trigger PAR2 and subsequent transcriptional activation of the TSLP gene leading to subsequent type 2 immune responses.^{48–50} The contribution of endogenous versus exogenous proteases in this type 2 inflammation remains to be determined.

Pollen extracts or fresh pollen from various sources (e.g., *Ambrosia trifida*, *Poa pratensis*, *Betula verrucosa*, *Lilium logifolium*, *Chenopodium album*, *Plantago lanceolata*, *Eucalyptus globulus*, *Olea europaea*, *Dactylis glomerulosa*, *Cupressa sempervirens*, and *Pinus sylvestris*) cleave TJs by disrupting claudin-1, occludin, ZO-1 and the AJ protein E-cadherin in a manner reminiscent of HDM protease allergens, increasing permeability in AECs. Cysteine, serine, and metalloprotease activity has been presumptively linked to these effects.^{51–53} Of note, IgE-reactive cysteine proteases have been reported in the coat of *Cynodon dactylon*, *Sorghum halepense*, and *Phleum pratense* pollen.⁵⁴ Although the effects of these pollen coat cysteine proteases on TJs were not elucidated, the coat cysteine protease from *Bermuda Grass* pollen increased permeability and cell detachment of human AECs. To date, it must be pointed out

that only the cysteine protease Amb a 11 has been recognized as an allergen.⁵⁵ Recently, a IgE-reactive cysteine protease allergen displaying 95% sequence identity with Am b 11 was isolated from *A. trifida*.⁵⁶ Moreover, a potential Der p 1 homologue has been reported in *Betula verrucosa* and *Pinus sylvestris*.⁵⁷

4 | ACTIVATION OF STRUCTURAL AND IMMUNE CELLS BY PROTEASE ALLERGENS

Although degradomic methodologies have not been fully applied to protease allergens, several protein substrates targeted by allergenic proteases have been identified (Figure 2). Common among these targets is linkage to mechanisms promoting and/or regulating Th2 immune responses^{6,26,58} or skewing to Th17 responses.^{5,18} The activation of PAR2 by protease allergens is separately described in the next section.

HDM allergens Der p 1 and Der f 1 can degrade airway surfactant protein-A and protein-D (part of the soluble innate immune system collection) predisposing to compromised epithelial barrier defences.⁵⁹ In addition, Der p 1 also degraded α_1 -antitrypsin, secretory leukocyte protease inhibitor, and elafin which are

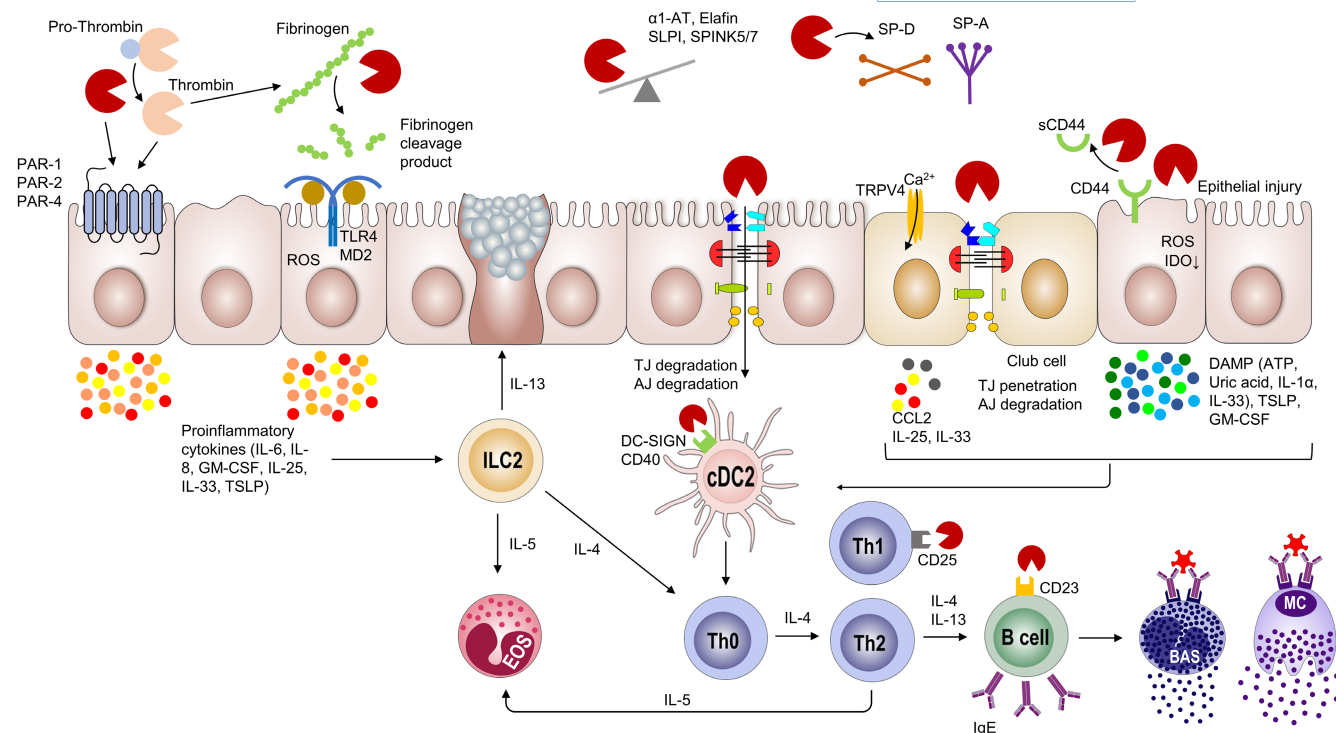


FIGURE 2 General representation of non-IgE mediated innate inflammatory pathways mediated by protease allergens on exposed epithelial and immune cells. Protease allergens can alter epithelial integrity through degradations of tight (TJ) or adherens junctions (AJ) which facilitates their penetration into submucosal tissues. The junction injuries of bronchiolar club cells are sensed by mechanosensor TRPV4. Epithelial damages are initiated by protease allergens (red-colored enzyme symbol) through their capacity to alter the antiprotease network and to degrade surfactant proteins. Serine protease allergens or thrombin (peach-colored enzyme symbol) trigger protease-activated receptor (PAR) activation. Cleavage of fibrinogen by protease allergens or by thrombin matured by protease allergens elicits TLR4 activation. Activated and/or damaged epithelium produces intracellular ROS leading to the release a large collection of proinflammatory cytokines (IL-6, GM-CSF, TSLP, IL-25), DAMP (IL-1 α , IL-33, Uric acid, ATP), and chemokines (CCL2) which activate ILC2 and cDC2 cells. IL-4 produced by activated ILC2s initiates the Th2 differentiation. The production of IL-4, IL-5, IL-13 by Th2 cells and ILC2s induces the production of allergen-specific IgE, the associated sensitization/degranulation of basophils (BAS)/mast cells (MC) and the recruitment of eosinophils. ILC2-derived IL-13 drives goblet cell hyperplasia. The contacts of protease allergens with immune cells, facilitated by the epithelial barrier degradation, allow the cleavage of surface receptors (CD23, CD25, CD40, DC-SIGN/DC-SIGNR) on these target cells which optimizes the Th2-biased inflammatory response. AJ, Adherens Junction; AT, antitrypsin; CCL2, C-C Motif Chemokine Ligand 2; cDC, conventional dendritic cell; cDC2c, type 2 conventional DC; IDO, indoleamine 2,3-dioxygenase; MD2, myeloid differentiation factor 2; PAR, protease-activated receptor; ROS, reactive oxygen species; SLPI, secretory leukocyte protease inhibitor; SP-A/D, surfactant protein-A/D; SPINK 5/7, serine protease inhibitor Kazal type 5 or 7; TJ, tight junction; TLR4, Toll-like receptor 4; TRPV4, Transient receptor potential vanilloid-type 4; TSLP, thymic stromal lymphopoietin.

components of the lung antiprotease network⁶⁰ Der p 1 instigates the canonical, thrombin-dependent activation of PAR1 and PAR4 in AECs by its capacity to mature prothrombin into thrombin.^{61–63} The proteolysis of fibrinogen by thrombin releases fibrinogen cleavage products that activate Toll-like receptor (TLR4) (with clot formation)⁶⁴ (Figure 2). Fungal protease allergens and HDM extracts can selectively promote the fibrinogen cleavage product-TLR4 axis.⁶⁵ Thrombin was recently shown to activate the maturation of interleukin-1 α (IL-1 α), a central pleiotropic proinflammatory cytokine.⁶⁶ In addition, this mechanism entrains other signaling pathways (epidermal growth factor receptor (EGFR) signaling, activation of myosin motors, gating of pannexons, ATP release, ligation of P₂Y₂ and P₂Y₇ purinoceptors, activation of a disintegrin and metalloprotease 10 (ADAM10), ligation of TLR4, intracellular ROS generation) leading to IgE-independent cytokine

production (Figure 3). Of note, the *Aspergillus* proteases-mediated proinflammatory responses in primary bronchial epithelial cells were dependent on the mitochondrial ROS production.⁶⁷

Der p 1-induced ADAM10 activation mediates E-Cadherin shedding for the untethering of ILC2.⁶⁸ ADAM10 also displays “shedase” activity on CD23 and chemokines (CCL20, CCL2, CCL5, CX3CL1, CXCL8, CXCL16).^{69–71} Of note, a cysteine protease (presumptively Der p 1) in an HDM extract directly induced CX3CL1 chemokine shedding in cells and confirmed in a cell-free assay using a recombinant extracellular domain of CX3CL1.⁷¹ Cleaved soluble CX3CL1 was shown to promote survival of Th2 cells⁷² and proliferation of airway smooth muscle cells leading to asthmatic airway remodeling.⁷³

Interestingly, Der p 1 and Der f 1 induce cytokine release in primary human keratinocytes by an unknown mechanism separate

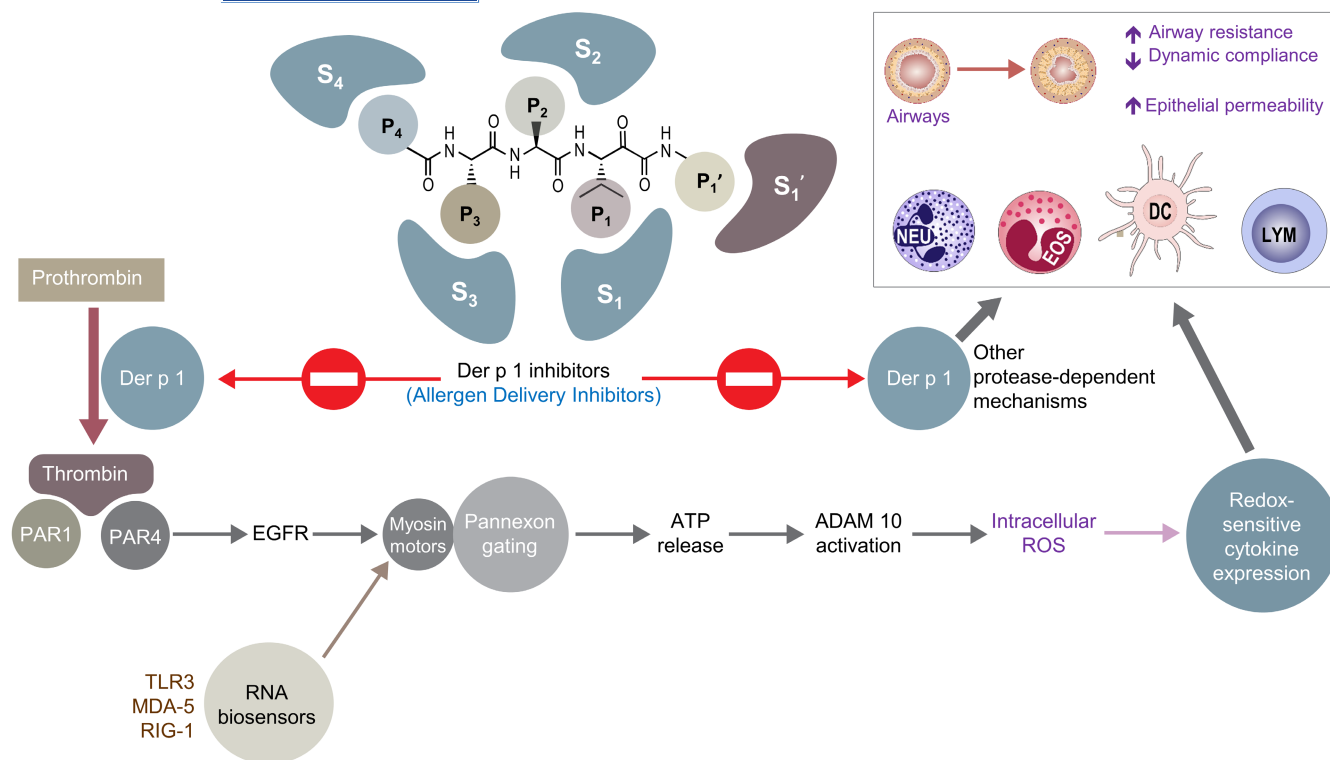


FIGURE 3 Activation of redox-dependent signaling by the prothrombinase activity of Der p 1 and its inhibition by Der p 1 class allergen delivery inhibitors. Thrombin generation enables the canonical activation of PAR1 and PAR4. In addition to their canonical GPCR signaling, this initiates a signaling network involving epidermal growth factor receptor (EGFR), pannexons and the TLR4-dependent intracellular generation of reactive oxidant species (ROS). This network also receives activating inputs from sensors detecting viral RNA. All these effects can be attenuated by compounds which have been designed to inhibit group 1 allergens potently and selectively. Illustratively, a favored molecular scaffold for inhibitor design is shown. Variation of substituents at positions P1'–P4 (Schechter–Berger nomenclature) tunes the interaction with the binding pockets (S1'–S4') of the protease allergen and confers other essential developability credentials. ADAM10, activation of a disintegrin and metalloprotease 10; EGFR, epidermal growth factor receptor; MDA-5, melanoma differentiation-associated protein 5; PAR, protease-activated receptor; RIG-1, retinoic acid-inducible gene I; ROS, reactive oxygen species; TLR3, Toll-like receptor 3.

from the activation of PAR2.⁷⁴ The prothrombinase activity of Der p 1 raises the possibility that, like the airways, the innate response of skin stimulated by HDM may require the canonical activation of PAR1 and PAR4. However, unlike the airways where the accepted view is that TLR4, TSLP, and IL-33 have significant roles in sensitization and allergic inflammation, deficiency in TLR4 or IL-33 but not TSLP seems tolerated in skin.^{10,21,75}

Airway exposure of naive mice to papain, bromelain, or *Aspergillus*-associated protease promotes IL-33 production.^{8,76,77} Such protease-dependent IL-33 secretion is critical for the recruitment and activation of ILC2 cells.⁷⁸ Serine proteases from HDM, *Alternaria* or pollen extracts can induce the release of other DAMPs than IL-33, such as uric acid and ATP by human bronchial epithelial cells.^{13,79}

Der p 1 and HDM extracts downregulate indoleamine 2,3-dioxygenase (IDO), a key molecule to preserve immune tolerance, in both dendritic cells and AECs.^{80,81} Although the underlying mechanism is unknown, it is noteworthy that ligation of TLR4 decreases IDO activity in AECs,⁸⁰ suggesting that the primary signal which breaks tolerance might be the Der p 1-dependent formation

of thrombin and activation of signaling via PAR1 and PAR4 with the consequent activation of TLR4 by endogenous ligands.^{6,62,63}

The proteolytic activity of fungal Pen ch 13 downregulates CD44 expression in human bronchial epithelial cells.⁸² However, whether this change in expression of a receptor involved in epithelial repair resulted from the proteolytic cleavage by Pen ch 13 is unknown.

Der p 1 and Per a 10 (Cockroach) reduce the expression of CD40 on DCs, an important receptor for IL-12/IFN γ production through CD40 and CD40L (expressed on T cells) interaction pathway.^{83,84} However, only Der p 1 was shown to shed CD40 (sCD40 release) directly from DCs surface.⁸³ Furthermore, DC-SIGN (CD209), a C-type lectin receptor primarily expressed on DCs and macrophages, can be cleaved by Der p 1,⁸⁵ down-regulating DC-SIGN-ICAM3 signaling for the differentiation of Th1 cells.⁸⁶ A closely related molecule, DC-SIGNR/L-SIGN (CD299), was also known to be cleaved by Der p 1.⁸⁵

Der p 1 upregulated CD86 expression and mediated the release of pro-Th2 TARC (CCL17) and MDC (CCL22) in DCs from HDM-allergic patients through its protease activity. Purified T cells from HDM-sensitive patients stimulated by autologous active Der p 1-pulsed DCs produced large amounts of IL-4, an effect prevented by anti-CD86 antibodies.^{87,88} Proteolytically active Der p 1

or papain can mediate an-IgE-independent activation of mouse basophils leading to secretion of IL-4 and TSLP.^{3,89} Der p 1, but not papain, can induce similar effects in human basophils. The identity of the papain/Der p 1-activated sensor(s) expressed by basophils remains unknown. PARs are unlikely candidates as they cannot be functionally activated by cysteine proteases, but Mas-related G-protein-coupled receptors (Mrgprs), innate nociceptive sensors,⁹⁰ participate in IgE-independent basophil activation. Of note, human MRGPRX1 and mouse MRGPRC11 can be activated by Der p 1 and Der f 1 through cleavage of the N-terminal activation region, leading to IL-6 secretion.⁹¹

Der p 1 or Per a 10 selectively sheds cell surface receptors such as CD23 (FcεRII, low-affinity IgE Fc receptor) on B cells and CD25 (IL-2 receptor) on both CD4 and CD8 T cells.^{36,92–94} Soluble CD23 (sCD23) positively regulates the IgE production,⁹⁵ whereas shedding of CD25 by protease allergens downregulates Th1 polarization.⁹⁶ Active Der p 1, Der f 1, or papain can directly mediate the degranulation of human eosinophils and the production of superoxide anion.⁹⁷ Active Der p 1 can directly exert proasthmatic-like effects by its capacity to mediate MAPK-dependent rabbit airway smooth muscle responsiveness in vitro.^{98,99} The serine protease activity of fungal Asp f 13 provoked AHR by infiltrating bronchial submucosa in mice.⁹⁸ Without prior allergic sensitization, Asp f 13 modulates in human ASM cells profound changes in cell morphology, Ca²⁺ mobilization, and degradation of the extracellular matrix.

5 | PROTEASE-ACTIVATED RECEPTOR (PAR) ACTIVATION BY PROTEASE ALLERGENS

A key mechanism of the innate immune response initiated by some protease allergens involves the proteolytic activation of G-protein-coupled PARs, best known for their stimulation by the coagulation cascade enzyme, thrombin.^{100–102} Activation of this unique family of receptors is caused by the proteolytic unmasking of a “tethered” receptor-activating ligand. The protease-revealed N-terminal sequence interacts with the extracellular receptor domain 2 to stimulate signal transduction as illustrated in Figure 4A for PAR2 but is also the same for PAR1 and PAR4.

PARs are widely expressed in the airways by lung epithelial cells, airway smooth muscle and both tissue resident and recruited inflammatory cells, as well as by esophageal epithelium.⁴⁹ The multiple signaling pathways activated following PAR engagement have been reviewed in depth previously.^{102–105}

To date, few serine protease allergens have been shown to target PARs directly, with PAR2 as the main example. The allergens and crude extracts with documented PAR2-regulating proteases are HDM, cockroach, and mold allergens.^{106–108} Initially, it was reported that the major HDM allergen Der p 1 could activate PAR2,¹⁰⁹ but subsequent studies showed that Der p 1 cleaved PAR2 at a non-activating site¹⁰⁸ (Figure 4B). In contrast, trypsin-like Der p 3^{108,110} and collagenase-like Der p 9¹¹⁰ activate PAR2 in AECs and induce

the release of cytokines and chemokines including GM-CSF and eotaxin. Similarly, the *D. farinae* allergen-derived enzyme, Der f 3 (80% homologous to Der p 3) induces IL-6, IL-8, and GM-CSF release from AECs through its protease activity and PAR2 activation.¹⁸ Tyr p 3 from the storage mite *Tyrophagus putrescentiae* has serine protease-dependent effects on AECs that are prevented by both a PAR2 and a serine protease inhibitor.¹⁵ These observations illustrate the clear activity of serine protease allergens on PAR2, although the specificity of Der f 3 to induce cytokines associated with IL-17 responses is hard to explain based alone on PAR2-mediated activation of AECs.^{111,112}

Alternaria alkaline serine protease activates PAR2 directly and induces allergic airway inflammation in mice.¹⁰⁶ Additionally, various fungal allergen extracts, including *Alternaria*^{113,114} and *Aspergillus*¹¹⁵ extracts, have been shown to activate AEC, eosinophils, and induce cytokines through PAR2 but the exact proteases mediating these effects have not been identified.¹¹⁶ Fungal Pen c 13 can cleave PAR1 and PAR2 at their activation sites and desensitize AECs to increased intracellular calcium induced by PAR1 and PAR2 agonists.¹¹⁷ In addition, incubation of human AECs with Pen c 13 induces cytokine production that is inhibited by antibodies that prevent either PAR1 or PAR2 cleavage.

Cockroach extracts activate AECs in a PAR2-dependent fashion^{118,119} and mediate PAR2-dependent sensitization of mice.^{120–122} Less is known regarding the cockroach allergens that can activate PAR2. Per a 10 induces PAR2-mediated effects in purified cells^{123,124} and can elicit allergic sensitization in mice.¹²³ Similarly, using a trypsin-specific activity based probe, three serine proteases from German cockroach (*Blattella germanica*) were shown to be highly homologous to Per a 10 and to activate PAR2.¹⁰⁷ Finally, German cockroach extracts mediated the PAR2-dependent inflammatory responses of human eosinophils.¹²⁵

PAR2 is not the only PAR activated by allergens; Pen c 13 can also activate PAR1.¹¹⁷ Among HDM allergens, Der f 3 has been shown to activate PAR1 in addition to PAR2,¹⁸ while Der p 3 can activate PAR4 and lead to PAR4-mediated activation of store-operated Ca²⁺ channels in mast cells.¹⁷ Interestingly, allergens indirectly activate PARs. Der p 1 can activate thrombin that then has PAR1-mediated effects on AECs,⁶² and Cry j 1, the major pollen allergen of *Cryptomeria japonica* (Japanese cedar), can potentially indirectly cause PAR activation by upregulating a cellular serine protease like thrombin.¹²⁶

Even though we have learned more about the role of PARs in asthma and allergy from in vitro and murine studies, there are limited data linking PARs and asthma or allergies in humans in vivo. Notwithstanding, PAR2 expression is increased in the airways of patients with asthma,^{127,128} and in peripheral monocytes of patients with severe asthma.¹²⁹ Furthermore, in a murine model of eosinophilic esophagitis, SPINK5 or SPINK7 deficiency unleashes serine protease activity, triggering PAR2-dependent TSLP release and subsequent type 2 immunity.^{48,49} Given that SPINK5 deficiency underlies Netherton syndrome,¹³⁰ which is marked by allergic inflammation, the impact of this deficiency may be evidence for a human link to PAR2-mediated inflammatory disease.

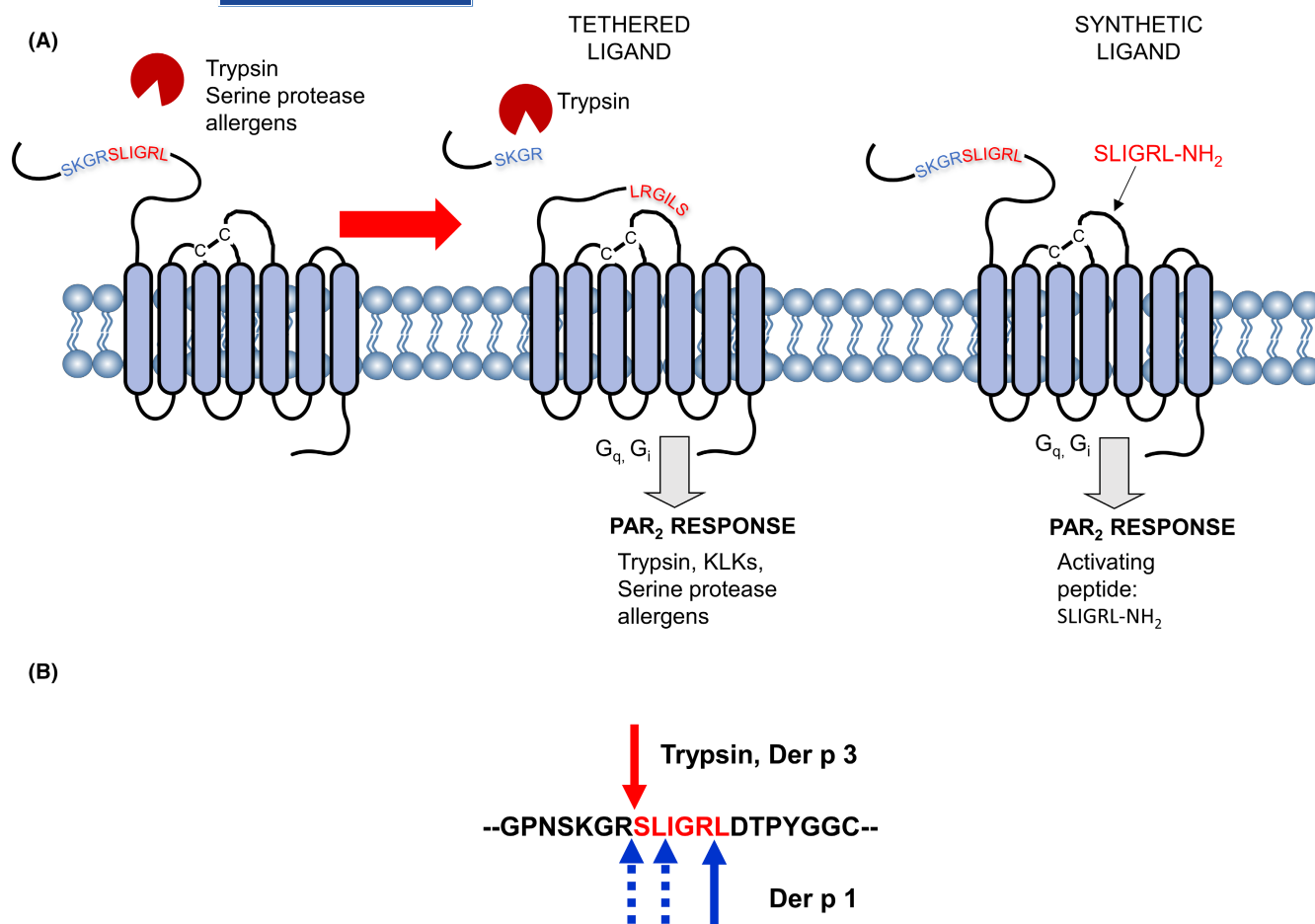


FIGURE 4 Activation of Protease-activated receptor 2 (PAR2) by trypsin/KLKs or trypsin-like protease allergens. (A) PAR2 cleavage by trypsin/KLK or serine protease allergens unmasks PAR2's tethered ligand which interacts with the extracellular loop 2 domain to initiate receptor signaling via the G-proteins, Gq, G_{12/13} and Gi. Similar effects are triggered by a synthetic tethered ligand-mimicking peptide. (B) Differential cleavage of the N-terminus of rat PAR2 by trypsin and Der p 3, which unmasks the 'canonical' tethered receptor-activating ligand versus Der p 1, that cleaves primarily at a distinct site to 'disarm' the receptor for trypsin activation. The solid and dotted blue arrows indicate major and minor cleavage sites, respectively, for Der p 1. KLK, kallikrein.

6 | THE RIPIL-33 PATHWAY, IL-33 MATURATION, AND PROTEASE ALLERGENS

It is well established that the alarmin IL-33, a member of the IL-1 cytokine family, has a critical role in the context of allergic inflammation. IL-33 secreted into the extracellular space binds ST2-expressing ILC2s, mast cells, Th2 cells, eosinophils, basophils, and DCs. Activation of IL-33/ST2 axis thereby contributes to the development and amplification of an allergic inflammatory response. The importance of IL-33 and its receptor in allergic responses is substantiated by genetic linkage studies. The genes *IL-33* (9p24) and *ST2/IL1RL1* (2q12) are top hits in genome-wide association studies of asthma, blood eosinophil counts in the general population, and virus-induced exacerbations in severe childhood-onset asthma.¹³¹⁻¹³⁴ Indeed, early clinical trials with anti-IL-33 and anti-ST2 monoclonal antibodies have revealed improvements in asthma and lowering of blood eosinophil levels.^{135,136}

IL-33 is normally localized in the nucleus of epithelial and endothelial cells¹³⁷ as an immature form of 270 amino acids (full-length IL-33, $_{FL}$ IL-33) tightly bound to histone proteins associated with chromatin, through an N-terminal chromatin binding domain.¹³⁸ The nuclear sequestration of IL-33 is essential for fine tuning its cytokine activity. Another control mechanism for regulating IL-33 activity is modification by proteases.

A variety of type 2 stimuli including allergens, parasites, and viral component (including TLR3 ligands) trigger IL-33 proteolytic maturation and activation¹³⁹ and subsequent epithelial cell release in a large molecular weight complex with histones.¹⁴⁰ Exposure of epithelial cells to a broad range of aeroallergens (including *Alternaria alternata*, HDM, *Aspergillus fumigatus*, cat dander, and cockroach extracts), but not birch pollen, Bermuda grass, peanut, whole wheat or cow milk extracts, triggers the assembly of the ripoptosome complex containing activated caspase-8, which subsequently activates caspase 3 and 7 leading to the $_{FL}$ IL-33 intracellular cleavage at position D175 and D178, resulting in biospecies that preserve their chromatin binding

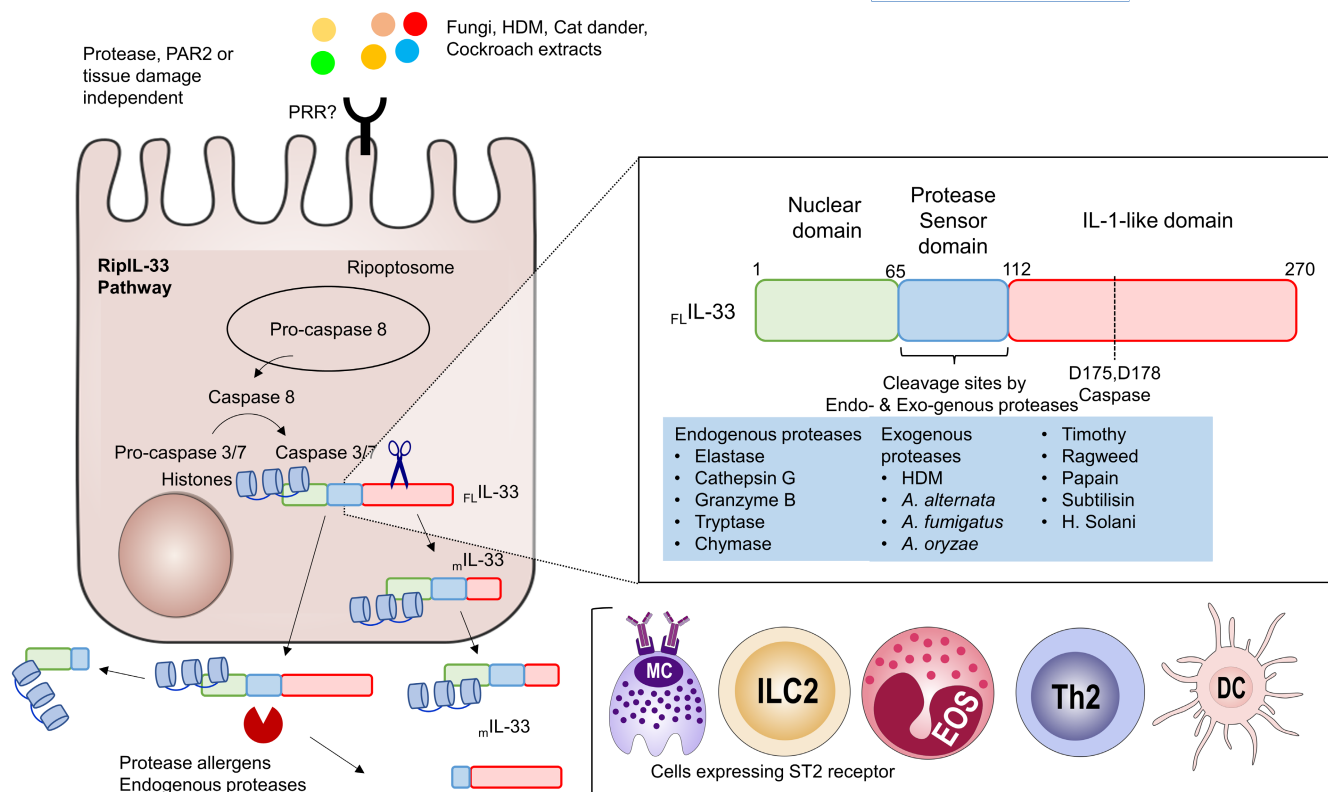


FIGURE 5 IL-33 maturation by intracellular RipIL-33 pathway and by the cleavage of IL-33 protease sensor domain exposed to protease allergens or endogenous proteases. Allergens from different sources trigger the caspase 8–ripiptosome signaling pathway to activate and release mature IL-33 (m IL-33). This RipIL-33 intracellular mechanism is not dependent on PAR2, protease activity of allergens or epithelial damage. In contrast, allergen-induced caspase 8 maturation is required for the downstream cleavage of caspase 3/7. Active caspase 3/7 cleaves the histone-bound full-length IL-33 (FL IL-33) within the IL-1-like domain. The resulting m IL-33 biospecies bound to histone is released and activate the cognate receptor ST2. Both m IL-33 forms had higher bioactivity than FL IL-33. In response to allergens, epithelial cells can release as well histone-bound FL IL-33. Environmental protease allergens from diverse sources (HDM, fungi, pollen, papain) as well as endogenous proteases released from innate effector cells during inflammation (Elastase, Cathepsin G, Granzyme B, Tryptase, chymase) can activate IL-33 maturation through cleavages of the protease sensor domain. Such m IL-33 forms have increased ST2 binding activity compared with FL IL-33.

domain¹⁴¹ (Figure 5). Of note, exogenous allergen-associated proteolytic activity or cell damage was not required for allergen-induced intracellular IL-33 maturation. These caspase-cleaved forms of IL-33 are released before cell death and have increased ST2 bioactivity compared with FL IL-33.¹⁴¹ The ripoptosome-mediated cleavage, activation, and release of IL-33, referred to as RipIL-33, is a canonical pathway by which a broad range of aeroallergens (mentioned above) trigger type 2 immunity and is now considered an upstream allergen sensor.^{141–143} However, the capacity of individual allergens or specific components of allergen extracts to activate the RipIL-33 pathway remains to be investigated. In addition to this intracellular pathway, extracellular proteases from allergenic sources (e.g., Der p 1, papain, *Alternaria alternata*, *Aspergillus fumigatus/oryzae*, ragweed, and Timothy pollens) cleave FL IL-33 between amino acids 94–110, a region referred to as the IL-33 protease sensing domain, resulting in further activation of FL IL-33¹⁴⁴ (Figure 5). In addition, proteases derived from inflammatory cells (e.g., elastase from neutrophils and chymase from mast cells) have potential to activate IL-33, and likely contribute to the temporal and spatial bioactivity of IL-33.

While rodent and human IL-33 can be processed into shorter mature forms by exogenous allergen proteases (e.g. *A. alternata*), differences between the species have been reported as the murine protein can be degraded into additional inactive fragments when exposed to high concentrations of proteases,¹³⁶ suggesting difference in the regulation of human and mouse IL-33 by exogenous protease allergens. Interesting, a Der p 1-specific inhibitor (ADI: Allergen Delivery Inhibitor) suppresses IL-33 accumulation in the airways of sensitized rats subsequently challenged with an aerosolized HDM extract substantiating post-translational modification of IL-33 by exogenous proteases.

7 | NOCICEPTOR ACTIVATION BY PROTEASE ALLERGENS

While IL-33 is a potent pro-atopy alarmin, it operates in coordination with other key signals. Recent findings have drawn attention to the cooperation between IL-33 and sensory neuron signaling.

Sensory neurons originate in the dorsal root ganglia (DRG) and project throughout our body where they are enriched in barrier tissues to detect the presence of harmful stimuli. These stimuli not only include temperature and pressure, but also irritants, leading to pain or itch responses that promote protective avoidance behaviors. Trypsin and mast cell tryptase have previously been shown to activate PAR2-expressing neurons leading to neurogenic inflammation.¹⁴⁵ Could this be a fundamental pathway guiding the detection of environmental protease allergens? In 2019, Serhan and colleagues showed that the cysteine protease activity of *D. farinae* extracts directly activated TRPV1⁺ dorsal root ganglia neurons in vitro.¹⁴⁶ Extending this observation to the in vivo setting, they showed that *D. farinae* led to sensory neuronal release of the neuropeptide Substance P, which then activated mast cells via MRGPRB2 and led to their degranulation.¹⁴⁶ Thus, sensory neurons detected cysteine protease allergen activity and then relayed that activation to mast cells to initiate local innate inflammatory responses. The mechanisms for the sensing of cysteine protease allergens by nociceptors remain unclear. Whereas the PAR2 activation by Der p 1 is controversial,^{108,109} other cell surface receptors could be targeted, such as MRGPRX1.⁹¹ Cutaneous exposure to the cysteine protease papain promotes robust DC activation and migration in vivo, but not in vitro.²² This result suggested that the sensory nervous system may similarly have a role in relaying a signal of protease allergen activation to local DCs. In 2020, Perner and colleagues showed that the cysteine protease allergens papain and from HDM extract, as well as the serine protease allergens from *Alternaria alternata* extract were capable of activating TRPV1⁺ sensory neurons directly, leading to Substance P release and the sensation of itch.²² However, in addition to activating local mast cells, Perner and colleagues showed that Substance P can directly induce the migration of Th2-skewing CD301b⁺ DCs to the draining lymph node through their expression of MRGPRA1. DC migration was independent of mast cells, but was dependent on the presence of TRPV1⁺ neurons and their release of Substance P.²² Importantly, Substance P induced migration was necessary, but not sufficient for Th2 differentiation, indicating the requirement for additional signals in initiating adaptive immune responses to protease allergens (Figure 6).

8 | FACTORS AFFECTING THE POTENTIAL BIOLOGICAL SIGNIFICANCE OF PROTEASE ALLERGENS

The bioactivity of proteases is regulated by intrinsic and extrinsic factors including, interaction with exogenous protease inhibitors, the prevailing redox environment, and their propensity to autolysis. Additional factors could affect the clinical relevance of protease allergens such as their environmental distribution, human exposure levels, and changes in the state of these allergens when in contact with the human immune system.

Serine proteases are vulnerable to inhibition by endogenous antiproteases, although specific information about protease allergens

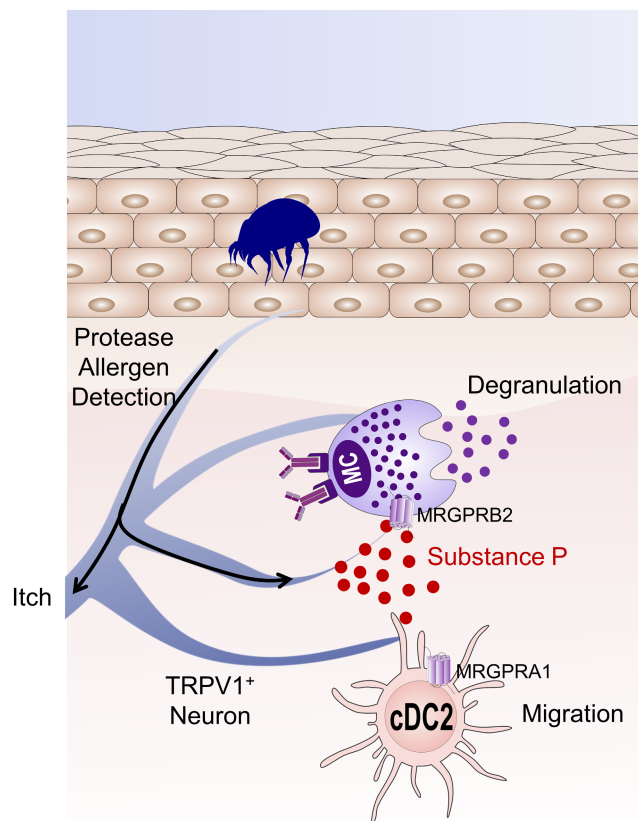


FIGURE 6 Sensing of protease allergens by TRPV1⁺ nociceptors. Cysteine and serine protease allergens from papaya, HDM, and *Alternaria alternata* are detected directly by TRPV1⁺ sensory neurons in the skin leading to itch and the local release of Substance P. Substance P acts directly on mast cells through their expression of MRGPRB2, leading to mast cell degranulation. Substance P also acts on cDC2 through their expression of MRGPRA1, leading to their migration to the draining lymph node where they initiate Th2 differentiation. cDC2, type 2 conventional DC; MRGPR, Mas-related G protein-coupled receptors; TRPV1, Transient receptor potential vanilloid-type 1.

and serpins in this regard is limited at present. The relevant antiproteases are themselves regulated by numerous factors. For example, ROS (which, as described earlier, can be generated by protease allergens) inhibits α_1 -antitrypsin.¹⁴⁷⁻¹⁵⁰ Given that α_1 -antitrypsin is also a putative degradation target of Der p 1, this creates a potentially complex and dynamic system which might govern not only how protease activity interacts with human immunity but could determine which proteases are most influential. This remains an open area for investigation.

As cysteine proteases, group 1 HDM allergens have attracted speculation about whether protease activity does contribute to allergic mechanisms. This is because facile, nonrandom oxidation of the catalytic cysteine residue decreases enzyme activity. Tryptic and chymotryptic serine proteases are also susceptible to oxidative inactivation,^{151,152} but whether the repertoire of serine protease allergen is similarly susceptible to oxidative inactivation remains to be investigated. For cysteine proteases, such as Der p 1, important oxidations of its active site sulphydryl group are facile and reversible.

However, the kinetics underlying some products, notably the formation of sulphinic or sulphonic acids, make these essentially irreversible reactions.⁵⁸ Susceptibility to facile oxidation affects the preparation of any cysteine protease, and this has potential implications for experimental studies. Consistent with extensive experience arising from pioneering work with papain, early studies showed that Der p 1 could be rendered catalytically active after purification. In this state, it promoted allergic sensitization, whereas inactive material did not.

Except in highly oxidized states, purified group 1 HDM allergens can be quickly reactivated by reducing agents, and a similar reactivation occurs in spent medium from HDM cultures.^{34,153,154} In these and numerous other experimental studies described in the literature, this reactivation is performed simply to ensure that allergen preparations have proteolytic activity. However, in a drug discovery setting, such as the design of inhibitors of group 1 HDM allergens, it has been an elective procedural step to ensure uniform behavior across a screening campaign^{7,25,155} where the absence of consistency would be detrimental to sound decision-making in chemical design. Although procedural for a specific purpose, its basis has several plausible biological equivalents in nature. These may be important determinants of protease activation in vivo and thus the significance of protease activity in allergy. In the human respiratory tract, airway surface liquid (ASL) contains small biothiols such as cysteine or glutathione which are known to reactivate group 1 HDM allergens in vitro and probably any other cysteine protease allergen.^{6,7,62,156} The concentration of glutathione in ASL exceeds its levels in plasma and can be increased to millimolar levels in disease.^{157–162} Interestingly, glutathione levels in ASL are reported to be elevated in asthma as judged by measurements in bronchoalveolar lavage.¹⁶² Free thiols on proteins dissolved in ASL or presented on cell membranes provide further potential reductants; among these is α_1 -antitrypsin which is both thiol-rich and cleavable by Der p 1.^{58,60,163–165} Collectively, these observations provide reasons to believe that activatable cysteine protease allergens would be catalytically functional in vivo and they raise an interesting possibility that some cleavage targets might themselves provide the mechanism of activation as part of substrate acquisition by the enzyme's binding pockets. *in vitro* studies show that epithelial cells in culture activate Der p 1 or spent medium from HDM cultures to protease activity levels approaching the behaviour of pre-reduced reagents, consistent with all possibilities already outlined.¹⁶⁶ In passing, a cautionary note concerns the pitfalls of studies investigating allergen activation in vivo, or the difficulties in making retrospective analyses of earlier literature where pre-activated and non-activated allergens have been used in rodent models. The inherent problem is created by the high dilution of ASL by the delivered material. This is most extreme for nasal delivery where ASL is both low in volume and its reductant concentration is ~60% lower than in the lungs.¹⁶⁷ A similar concern applies to aerosol delivery to the lungs where, despite the higher concentration of biothiols, the diluting effect of aerosols contributes a significant experimental variable.¹⁵⁵ Consequently, the resulting concentration of endogenous reductants may fall below a

threshold required to achieve rapid activation of the allergen. This is important when evaluating data from pioneer literature where data for activated and non-activated allergen preparations have been reported, but where the experimental purpose was not to determine the possibility of *in vivo* activation per se.

The effects of protease allergens on the allergic response are largely dependent on the concentration of their active forms from real environments. Although measurements of specific protease allergen content as Der p 1 have been reported in the literature over many years,¹⁶⁸ information about the level of their enzymatic activity is poorly characterized by the lack of specific assays of meaningful sensitivity. Such activity measurements would greatly help in the characterization of the disposition and residence time of the protease allergens in the environment, temperature, and humidity of the microenvironment. We are unaware of data which have addressed these matters systematically in a realistic setting, but it is noteworthy that Der p 1 remains activatable in HDM fecal pellets stored at room temperature for several months and remains able to cleave interepithelial tight junctions.³⁴

One approach to evaluate the contribution of protease activity to allergy is through the use of inhibitors. Excepting group 1 HDM allergens, this is currently restricted to generic inhibitors which lack the potency and selectivity to permit the unambiguous interrogation of even simple experimental models. A surprising feature is the extent to which the acute response to HDM extracts that can be inhibited in sensitized animals with Der p 1-specific inhibitors, suggesting that, at least in experimental models, IgE-independent events driven by group 1 allergens make substantial and decisive contributions to acute allergic responses. However, these findings should not be interpreted as indicating an absence of roles for allergens with other bioactivities, whether they be protease or not, in the complex cascade of events that link events at the airway surface to physiological, immunological, and pathophysiological responses.¹⁵⁵

9 | CONCLUSIONS AND PERSPECTIVES

The present review provides evidence for the prominent role of protease allergens in the initiation-regulation of the allergic response.^{169,170} Proteolytic activity disturbs the integrity of the epithelial barrier and triggers a set of pleiotropic signaling effects favoring Th2 polarization. Although the protease-dependent innate immune mechanisms remain to be fully characterized, TLR4/PAR signaling pathways are activated by protease allergens from various sources to trigger epithelial inflammation in the airway, skin and likely esophagus. IL-33 plays an important role in initiating allergic inflammation in the lung where proteolytically active allergens play a critical role in IL-33 maturation, augmenting its biological activity.¹⁷¹ However, the situation may be more complex in the skin where the role of IL-33 in the development of allergic inflammation is controversial.¹⁷² Recently, intracellular maturation of IL-33 was shown to be concomitant to exposure to protease and non-protease containing allergens, occurring via the caspase-8/riposome and leading

BOX 2 Future research perspectives.

- Standardization of allergen extracts manufacturing and protease allergen isolation to ensure comparative studies.
- Degradome analysis of allergen extracts for the complete identification of proteases.
- IgE binding and cross-linking assays to classify the isolated proteases as allergens.
- Thorough characterization of protease allergens on innate immune activation.
- Degradomics approaches for the identification of protein substrates from immune, epithelial, or neuronal cells.
- Transcriptomic analysis for the elucidation of signaling pathways mediated by protease allergens.
- Elucidation of the structure of identified protease allergens for the design of potent inhibitors and preclinical/clinical evaluation of antiprotease protocols to treat allergic inflammation.
- Effect of protease allergens on host-microbe communication.
- Maturation of cellular protein targets by protease allergens (endogenous proteases, alarmin).
- Impact of protease activity on the stability of allergens in allergenic extracts.
- Interaction of protease and non-protease allergen signaling pathways, such as convergence in IL-33 maturation by the ripoptosome.

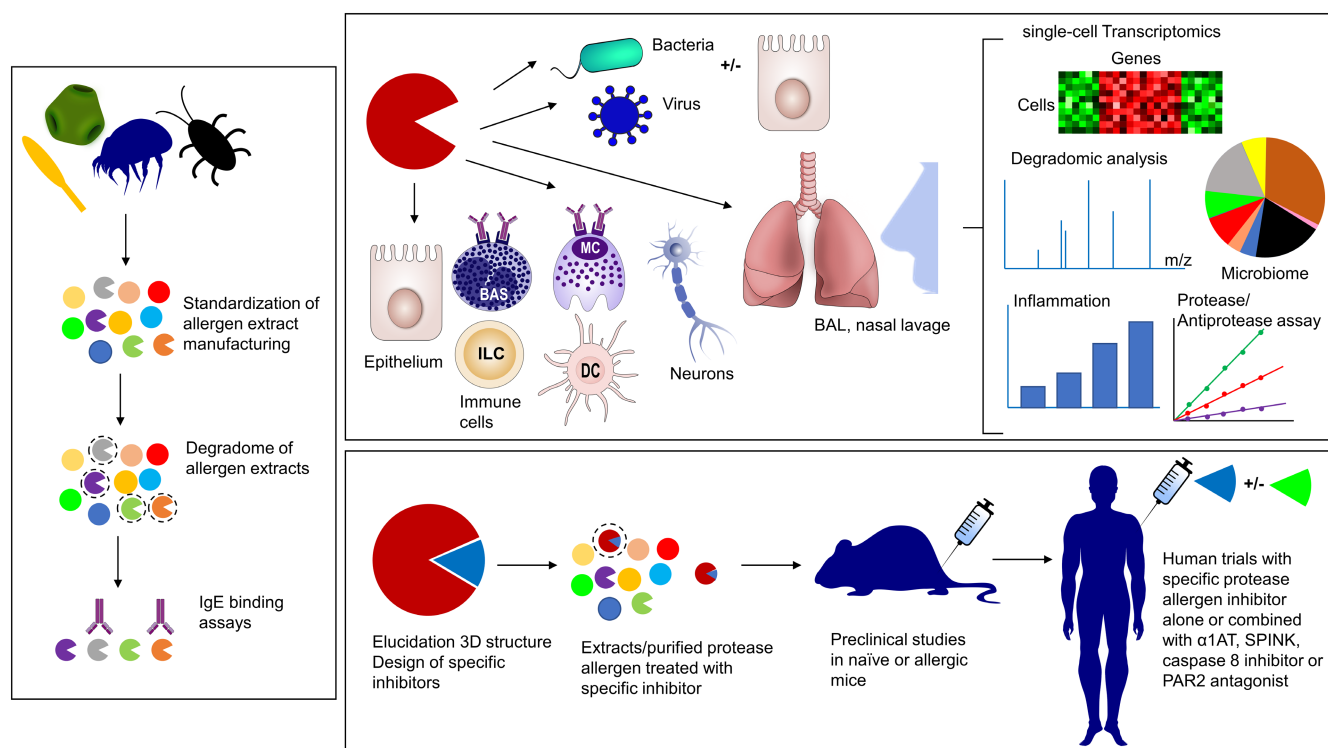


FIGURE 7 Multidisciplinary approaches to tackle important research questions about protease allergens. Degradome analysis combined with IgE binding assays is needed to elucidate the protease allergen repertoire of allergenic extracts. Signaling pathways activated by protease allergens and their protein substrate repertoire can be fully elucidated by single-cell transcriptomics, degradomics and using different human cell samples (epithelial, immune or neuronal cells). The effects of protease allergens on endosymbiotic or pathogenic bacteria, viruses should be investigated to measure their impact on microbiome and microbial virulence. Proteolytic degradation of the airway antiprotease network can be measured from nasal, bronchoalveolar lavage samples. Specific inhibitors can be designed based on the structure of protease allergens, paving the way for innovative therapeutic intervention. Their preclinical and clinical evaluation, alone or in combination with α 1AT/SPINK replacement therapy, caspase 8 inhibitor targeting RipIL-33 pathway or PAR2 antagonist, can pave the way to more effective management of allergic diseases. AT, antitrypsin; SPINK, serine protease inhibitor Kazal.

to the generation and release of bioactive mature IL-33 through a process referred to as "RipIL-33."¹⁷² There, TRPV1⁺ nociceptors have been shown to sense protease allergens upstream from the cDC2-mediated activation and migration. While the understanding of underlying mechanisms has evolved significantly, it is instructive to recall that the expansive repertoire of crucial bioactivities

now known to be associated with protease allergens echo aspects of the "Danger Model" proposed by Matzinger¹⁷³ in which danger/ alarm signals were predicted to direct the development of immune responses. The pleiotropic actions of protease allergens lead to their supremacy in the hierarchy of allergenic proteins as initiators, facilitating both mono- and poly-sensitization to non-protease allergens

from the same or other sources, respectively. Observational birth cohort studies provide a further perspective on the concept of an initiator allergen in identifying them as the entity(ies) responsible for the induction of the first IgE antibody response to a particular allergenic source. Der p 1 has been characterized as such by birth cohort studies, but it is clear that other potential initiators from established allergenic sources (e.g., HDM Der p 2 and Der p 23, pollen Phl p 1 or Bet v 1 and cat Fel d 1 allergens) lack protease activity *per se*.^{169,170,174} While this picture might easily arise from their association with other allergenic or non-allergenic proteases, these allergens are independently able to trigger pro-Th2 innate immune events. In this context, an important allergenic activity consists in the propensity of allergens to transport different lipid cargo and their lipid ligands could, in turn, mediate pro-Th2 TLR2/TLR4 signaling. Interestingly, Der p 2, Bet v 1, or Fel d 1 belong to families of lipid-binding proteins and Der p 2 mediates TLR4- but also TLR2-dependent proinflammatory responses. The complexity of the allergen sensing serves as a reminder that our understanding of the innate immune programming of allergy and its interaction with genetic predispositions remains rudimentary.¹⁷⁵⁻¹⁷⁷

Despite all we have learned, our mechanistic understandings of how protease allergens induce the allergic response remains unresolved. Moreover, most of the data on protease allergens were focused on Der p 1 or proteolytically active HDM extracts, and it is presently unclear as to what extent these principles can be simply applied to other protease allergens. Adding to this uncertainty, many results have been reported using commercial or in-house allergen extracts. Producer-dependent differences in extract manufacturing can inevitably lead to variabilities in allergen composition and the preservation of crucial bioactivities, rendering data interpretation particularly challenging.¹⁷⁸⁻¹⁸⁰ Moreover, allergen extracts frequently contain allergens with lipid-binding properties and contaminating microbial compounds, both capable of generating similar allergic effects, but likely through different mechanisms clouding our ability to study any specific pathway. There is thus an urgent need for standardized allergen extract manufacturing which preserves protease activities to facilitate the comparison of data on the pro-allergic role of proteolytically active extracts, but also on the degradome of allergenic sources. Highly purified proteases from allergenic sources are needed to determine whether they can be classified as allergens (proteins eliciting IgE response) and to attribute their specific role in the allergic response unambiguously.

Several important questions about protease allergens remain unanswered and must be dealt with through multiple areas of research (Box 2 and Figure 7).

The proteolytic degradation or activation of non-protease allergens co-existing with protease allergens are, to our knowledge, fully ignored. Cleavages of these could critically affect the stability, allergenicity and the antigen presentation of these non-protease allergens. Multi-OMIC approaches utilizing, in particular, single-cell transcriptomics and mass spectrometry-based proteomics would enable an extensive characterization of the signaling pathways activated by protease allergens and their protein substrate repertoire. The

cleavage specificity of proteases could be determined from peptide library of FRET substrates by high-throughput screening, a method successfully used for Der p 1.¹⁸¹ Alongside well-characterized and commonly used epithelial cell lines, protease-treated primary cell cultures could more closely mimic the exposure of epithelium to the natural allergens. However, heterogeneity in cellular composition complicates data interpretation.¹⁸² Of note, besides IL-33 other alarmins belonging to the IL-1 family similarly contain a protease sensor domain which could be cleaved by protease allergens, as recently evidenced for IL-1 α and IL-36.¹⁸³ The mechanism of allergen sensing by RipIL-33 deserves attention. The elucidation of the mechanism by which nociceptive neurons sense cysteine protease allergens directly (papain, HDM) is essential to our understanding of the neuron-immune interactions in allergic diseases. In this regard, the unanticipated effect of Der p 1 inhibitors in attenuating acute allergic bronchoconstriction is noteworthy.⁶ Neuronal cell lines or induced-pluripotent stem cell (iPSCs)-derived neurons are urgently needed to replace the primary neurons which are challenging to isolate and culture. As skin, gut or airway epithelia are continuously interacting with endosymbiotic bacteria and pathogens,¹⁸⁴ it remains to be determined whether protease allergens, through cleavage of surface bacterial antigens, could modulate the microbiota and the virulence of pathogenic bacteria. The potentiation of infectivity of influenza or rhinovirus by HDM protease allergens^{71,185} should incite further investigation of the effects of allergenic proteases on the pathogenicity of respiratory viruses including SARS-CoV-2 for which proteolytic cleavage of the COVID-19 spike protein is essential for the virus to infect angiotensin converting enzyme-2 (ACE2)-expressing lung epithelial cells.¹⁸⁶

Structure-based design of specific inhibitors for protease allergens represents an innovative therapeutic intervention for the treatment of allergic diseases. Such an approach was adopted to design allergen delivery inhibitors (ADIs) against group 1 HDM cysteine protease allergens.⁶ Furthermore, epicutaneous application of inhibited papain was able to prevent Th2 inflammation in mice.¹⁸⁷

Preclinical studies showed that pharmaceutically developable Der p 1-specific ADI compounds prevented allergic sensitization; inhibited dendritic cell, B-lymphocyte and eosinophil recruitment to the airways; and attenuated bronchoconstriction.^{6,155} These compounds have the credentials for dry powder delivery to the airways and provide durable protection against HDM challenge.¹⁵⁵ Human trials will be needed to confirm the translation of these promising animal results to clinically significant efficacy in humans.

AUTHOR CONTRIBUTIONS

All the authors contributed to and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

C.L.S. is a paid consultant (expert witness) for Bayer and Merck. C.R. and J.Z. are inventors on patents granted in multiple territories concerning allergens, protease inhibitor design and composition of matter, and medical uses thereof. St George's, University of London is beneficial owner of this patent estate. C.R. is co-founder and holds equity in Aldezon Ltd. M.E.R. is a consultant for Pulm One, Spoon Guru, ClostraBio, Serpin Pharm, Allakos, Celldex, Nextstone One, Bristol Myers Squibb, Astra Zeneca, Ellodi Pharma, GlaxoSmith Kline, Regeneron/Sanofi, Revolo Biotherapeutics, and Guidepoint and has an equity interest in the first seven listed, and royalties from reslizumab (Teva Pharmaceuticals), PEESV2 (Mapi Research Trust) and UpToDate. M.E.R. is an inventor of patents owned by Cincinnati Children's Hospital. The remaining authors declare that they have no conflicts of interest.

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