Title Page

Allergen Delivery Inhibitors: A Rationale for Targeting Sentinel Innate Immune Signaling of Group 1

House Dust Mite Allergens Through Structure-Based Protease Inhibitor Design

Jihui Zhang, Jie Chen, Gary K Newton, Trevor R Perrior, Clive Robinson

Institute for Infection and Immunity, St George's, University of London, Cranmer Terrace, London

SW17 0RE, United Kingdom (JZ, JC, CR)

State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of

Sciences, Beijing, P.R. China (JZ)

Domainex Ltd, Chesterford Research Park, Little Chesterford, Saffron Walden, CB10 1XL, United

Kingdom (GKN, TRP)

Running Title Page

Running Title: Allergen Delivery Inhibitors

Correspondence:

Professor Clive Robinson, Institute for Infection and Immunity, St George's, University of London,

SW17 0RE, UK

c.robinson@sgul.ac.uk

Number of pages: 68 (including references, tables and figures)(word count = 19,752)

                        26 (main text)(word count = 10,945)

Number of Tables: 3

Number of Figures: 9

Abstract: 250 words

Number of references: 139

ABBREVIATIONS: ADAM, A disintegrin and metalloprotease; ADI, allergen delivery inhibitor; Blo t, *Blomia tropicalis*; CD, cluster of differentiation; CCL, C-C chemokine ligand; CCLR, chemokine ligand receptor; CH_2Ph, benzyl; CXCR, C-X-C chemokine ligand; DC, dendritic cell; DC-SIGN, dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin; DC-SIGNR; dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin receptor; Der f, *Dermatopagoides farinae;* Der p, *Dermatophagoides pteronyssinus*; DPI, dry powder inhaler; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; Eur m, *Euroglyphus maynei*, GM-CSF, granulocyte macrophage-colony stimulating factor; HDM, house dust mite; IL, interleukin; ILC2, group 2 innate lymphoid cell; LPS, lipopolysaccharide; MD-2, myeloid differentiation protein-2; MRGPR, Mas-related G-protein coupled receptor; MyD88, myeloid differentiation primary response 88; NFκB, nuclear factor kappa B; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PAR, protease-activated receptor; PRR, pattern recognition receptor; ROS, reactive oxygen species; TAK-242, ethyl-(6*R*)-6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate; *t*-Btu, *tertiary*-butyl; Th2, type 2 T-helper cell; TJ, tight junction; TLR, Toll-like receptor; TIRAP, TRIF-related adapter molecule; TRAM, Toll-interleukin 1 receptor domain containing adapter protein; TSLP, thymic stromal lymphopoietin

Abstract

Diverse evidence from epidemiological surveys and investigations into the molecular basis of allergenicity have revealed that a small cadre of 'initiator' allergens promote the development of allergic diseases such as asthma, allergic rhinitis and atopic dermatitis. Pre-eminent among these initiators are the group 1 allergens from house dust mites (HDM).   In mites, group 1 allergens function as cysteine peptidase digestive enzymes to which humans are exposed by inhalation of HDM fecal pellets. Their protease nature confers the ability to activate high gain signaling mechanisms which promote innate immune responses leading to the persistence of allergic sensitization. An important feature of this process is that the initiator drives responses both to itself and unrelated allergens lacking these properties through a process of collateral priming. The clinical significance of group 1 HDM allergens in disease, their serodominance as allergens and their IgE-independent bioactivities in innate immunity make these allergens interesting therapeutic targets in the design of new small-molecule interventions in allergic disease. The attraction of this new approach is that it offers a powerful, root-cause level intervention from which beneficial effects can be anticipated by interference in a wide range of effector pathways associated with these complex diseases. This review addresses the general background to HDM allergens and the validation of group 1 as putative targets. We then discuss structure-based drug design of the first-in-class representatives of allergen delivery inhibitors aimed at neutralizing the proteolytic effects of HDM group 1 allergens which are essential to the development and maintenance of allergic diseases.

Introduction

Over the past 40 years pharmacology, immunology and cell biology have contributed enormously to understanding the mechanisms of diseases with allergic aetiologies *viz.* atopic dermatitis, allergic rhinitis and much of asthma. The pharmacologic contribution to this research effort has created a wealth of new molecular and biologic entities that have enjoyed success as experimental tools. However, for new entities acting upon unprecedented targets this drug discovery enterprise has largely failed when assessed against the metric of clinical success.   These endeavors have revealed the inherent weakness in treating the effects rather than causes of disease and have demonstrated convincingly the difficulties faced when making focused interventions against strategic effector mechanisms that are backed-up by multiple redundancies. Indeed, the supremacy of inhaled corticosteroids as standard of care for asthma highlights the benefits of a broad spectrum of action but, despite this, discrete targets in downstream effector pathways continue to attract attention. The more recent focus on interventions based on new biologic entities such as monoclonal antibodies directed against IL-5, the shared IL-4/IL-13 receptor α-subunit or thymic stromal lymphopoietin (TSLP) has demonstrated that success with new approaches is certainly feasible when patients are rigorously stratified for treatment. Nevertheless, the overarching philosophy in these newly-approved or emerging biologic approaches remains directed at downstream effectors and, inevitably, the reminders of the high risk of failure associated with this level of intervention are ever present because not all anti-cytokine development programmes have enjoyed positive outcomes in asthma. This raises strategic questions for the design of new approaches to these diseases, and among the most challenging topics are the identification of tractable new targets and the future that remains for small-molecule design in an era when biologics are in the limelight.

Encouragingly, alternative philosophies have delivered success, as demonstrated by omalizumab, the monoclonal antibody therapy which depletes circulating IgE to eventually reduce the amount of antibody bound to high affinity IgE receptors on mast cells. The development of omalizumab represented a significant change from a focus on symptom amelioration to an upstream intervention potentially capable of disease modification. However, amongst other significant issues, biologic therapies such as omalizumab pose significant reimbursement challenges which are problematic for patient access to treatment in chronic diseases affecting large populations.

From a theoretical perspective an ideal intervention would target a root cause of allergic disease, namely allergens themselves. This is, of course, partly the basis of the long-standing practice of allergen-specific immunotherapy. For small-molecule interventions this root cause approach has been generally viewed as an unattainable goal: there are many allergens in the environment, many of which remain poorly characterized, and exactly why they are allergenic, that is break immune tolerance and promote IgE-directed immune responses has, until relatively recently, been a surprisingly unfashionable topic in allergy. However, progress in the characterization of allergens has shed new light on the molecular basis of allergenicity and this has coincided with a renaissance of interest in innate immunity. This creates the possibility of making interventions at critical checkpoints in these pathways to prevent the development of allergic disease. Particularly, it is now possible to design an approach to target a major asthma trigger at root cause level, with the advantage that this circumvents concerns about the chronic consequences of blocking checkpoints which are of fundamental importance to immunity. Unlike allergen-specific immunotherapy an important advantage of this new line of attack is the prevention of innate immune responses which are the engine of allergy development. The purpose of this review is to examine the biologic mechanisms by which a major house dust mite (HDM) allergen triggers allergic disease and describe the design of an intervention to specifically neutralize this activity. The choice and nature of the target are unprecedented and may be unfamiliar to those outside the immediate field, so as an aid to interested newcomers we also describe the historical background to HDM allergens and allergic disease, their clinical significance and a brief overview of the complete spectrum of denominated HDM allergens.

HDM Allergens and Disease

House dust is a complex mixture of biologically diverse components from various sources with properties that render it inhalable by humans. House dust as a cause of allergic disease was first recognized in 1921 by the American physician Richard Kern who observed that many patients with rhinitis or asthma had positive skin reactions to extracts of dust from their own homes. This idea was elaborated further in the following two decades by Willem Storm van Leeuwen, Professor of Pharmacology and Director of the Pharmacotherapeutic Institute at the University of Leiden, who proposed that the cause of asthma could be the presence of mites in house dust. However, conclusive proof remained elusive until 1967 when Spieksma and Voorhorst established that *Dermatophagoides pteronyssinus* was an important source of indoor allergens – a linkage which we now regard as intuitive, but which at the time was received with fierce scepticism from many allergists. It is now

recognized that HDM such as *D. pteronyssinus* or *D. farinae* form the single most important indoor allergen source associated with asthma in temperate climates and they lead to the development of high titer allergen-specific IgE. The growth in sensitization of human populations is, in part, promoted by indoor, sedentary, affluent lifestyles and the creation of warm, humid, draught-free habitation and working conditions which provide optimal growth conditions for HDM. Consequently, many people are extensively exposed to HDM with the result that allergic diseases triggered by indoor allergens are major healthcare problems with significant socioeconomic impact.

Substantial evidence associates allergic conditions such as asthma, chronic rhinitis, atopic dermatitis and, less frequently, conjunctivitis with exposure to HDM or other indoor allergens (Gelber et al., 1993; Peat et al., 1996; Pichavant et al., 2005; Platts-Mills et al., 1987; Platts-Mills et al., 1997; Platts-Mills, 2009; Smith et al., 1969; Sporik et al., 1990; Squillace et al., 1997). Data from longitudinal investigations suggest that the development of sensitization to HDM occurs before polysensitization (Purello-D'Ambrosio et al., 2001; Silvestri et al., 1999), that is the development of allergy to other triggers, a phenomenon which occurs in ~50-80 % of people (Calderon et al., 2012). At first, this ordering of events in polysensitization seems puzzling until it is recognized that the bioactivity of certain allergens makes them unusually potent stimulants and providers of collateral immune priming on which other allergens depend. In this regard, certain HDM proteins function broadly as bio-initiators as well as simply being allergenic.

HDM are small arthropods, ~250 μm in length, related to spiders and scorpions. Many mite species are found in house dust, but the pyroglyphid family dominates in most areas of the world (eg *D. pteronyssinus, D. farinae and Euroglyphus maynei).* In the tropics, allergy to *Blomia tropicalis* may also be prevalent. Dust mites live on a diet of exfoliated human skin flakes and other biodebris. Existing on this diet means that HDM have digestive enzymes which can process tough structural proteins. Food waste is excreted, together with the digestive enzymes, in pellets which are of a respirable aerodynamic diameter (10-30 μm). As described later, these excreted digestive enzymes are significant because they are allergenic with a bioactivity profile which provides them with the ability to promote sensitization to themselves and unrelated inhalant allergens, regardless of source. Sensitization to HDM allergens occurs through inhalation of their fecal pellets which impact on the airway epithelium where airway surface liquid triggers the release of the pellet contents, resulting in a high concentration of allergen at the site of deposition. Sensitization by inhalation may also be the

case in people who develop atopic dermatitis rather than allergic conditions of the airways, although in established dermatitis any ensuing allergic responses are more likely to result from skin contact.

Allergen Nomenclature

Allergens are classified according to a system devised in 1984 by the Allergen Nomenclature Sub-Committee which was established jointly by the World Health Organization and the International Union of Immunological Societies. The systematic naming of allergens takes the accepted binomial nomenclature of the source and uses the first three letters of the genus combined with the first one or two letters of the species name followed by an Arabic numeral reflecting the order in which the allergen was isolated or its clinical importance (or, in reality, a hybrid of both). This numbering allows the denomination of allergens by group. In the case of <u>*Dermatophagoides pteronyssinus*</u>, the systematic naming takes the form Der p X, where X is the group number, and this is replicated in similar allergens from related species. Thus, HDM cysteine protease allergens from *D. pteronyssinus, D. farinae, Euroglyphus maynei,* and *Blomia tropicalis* are individually known as Der p 1, Der f 1, Eur m 1, and Blo t 1, respectively, collectively as the group 1 HDM allergens, or as the HDM cysteine proteases. Denomination is further resolved to accommodate the allelic variation in proteins which gives rise to isoallergens. Those allergens from the same source species with >67 % sequence identity are denoted using suffixes which may be up to four digits in length according to the complexity of the isoallergenic variations (eg Der p 1.0101, Der p 1.0102 *etc*). As an additional refinement it is sometimes necessary to denote whether allergens are in their native (n) forms or are products of recombinant protein engineering (r).   Recombinant allergens differ most notably in protein glycosylation and is usually due to the need to modify potential glycosylation sites *eg* to prevent hyperglycosylation of proteins expressed in yeast, an expression system which is often favored because it generally provides correct protein folding and thus mimics conformational epitopes and other biological characteristics seen in native allergens. While some glycosylation changes may have neutral or little impact on bioactivities such as the protease behavior of Der p 1 (Zhang et al., 2009), they are more relevant to processing of allergens by antigen presenting cells. Thus, the prefixes n and r, respectively, may be appended (eg nDer p 1, rDer p 1).

More than 30 groups of HDM allergens have now been denominated making HDM probably the single largest source of allergens to which humans are exposed. These allergens do not, however, have equal clinical importance because a clear serodominance is observable for those belonging to

groups 1 and 2. A summary of HDM allergens and their properties is provided in **Table 1**. Molecular

biology, genomics and bioinformatics have played key roles in the elucidation of these groups,

particularly in predicting their functional properties in the host – information which provides

important insights into why those molecules behave as allergens in humans. Further information

about the characteristics of these allergen groups can be obtained by using the GenBank and

UniProt accession codes listed in **Table 1**.

Group 1 HDM Allergens as a Drug Design Target

As clan CA, C1 family cysteine proteases, group 1 HDM allergens have a basic structure, comprising a

substrate binding groove separating two globular domains, which is archetypally defined by the

extensively studied representatives, papain, actinidin and cathepsin K. Proteolytic latency of the

HDM allergens is maintained in the proenzyme form because the prodomain occludes the catalytic

center of the enzyme while covalently tethered to the rest of the molecule and in so doing it masks

some IgE epitopes. This results in proteolytically latent HDM allergens being less immunogenic than

their catalytically competent mature forms (Takai et al., 2005). Differences in prodomain structure

between the HDM allergens and cathepsins have functional implications which place them in an

identifiable sub-family of C1 enzymes (Zhang et al., 2007). The group 1 allergen prodomains from

Dermatophagoides species comprise 80 amino acids arranged as 4 α-helices. Thus, they can be

distinguished from cathepsins K, L and S in which the longer propieces comprise only 3 α-helices and

contain a conserved 'ERFNIN' motif (EX_3RX_2 (I/V)$FX_2NX_3IX_3N$) absent in the HDM allergens (Zhang et

al., 2007). Other cathepsins, notably cathepsin B, have prodomains which lack a further α-helix and

which are shorter than the HDM allergens.   In cathepsins, untethered propeptide units generally

behave as regulators of their cognate proteases, but this seems not the case in Der p 1 (and, by

inference, other group 1 HDM allergens) where maturation of the protease results in rapid

degradation of the propiece and unconstrained enzymatic activity (Zhang et al., 2007). The

corresponding group 1 allergen Blo t 1 of *Blomia tropicalis*, found only in tropical regions, represents

an intermediate case between the more pervasive *Dermatophagoides* group 1 allergens and

cathepsins. At the time of writing, catalytic competence of Blo t 1 is only inferred from alignment of

its putative catalytic residues (C^{119} H^{263} N^{283}) with Der p 1 and formal proof of the enzymatic activity

of unambiguously pure allergen is awaited. It differs from its *Dermatophagoides* counterparts in

sharing only ~30 % sequence identity overall and having a modified ERFNIN motif (ERFQVN) which

may indicate that its prodomain could remain a potential inhibitor after maturation, restricting its

behavior. In that regard it is noteworthy that while Blo t 1 is clearly a major allergen of *B. tropicalis*,

unlike the group 1 HDM allergens it is not obviously serodominant. It is tempting to speculate that the lack of serodominance may be related to inferences that can be drawn about control of its proteolytic capacity.

Of numerous difficulties encountered in studying the biology of allergens, a fundamental challenge is their purification from the complex mixture of bioactive materials with which they are normally associated. While such extraneous materials may have deep relevance to host immune responses and disease development, they can be serious confounding factors when investigating functions and mechanisms at the molecular level. A pertinent example was the claim, which has enjoyed a persistence in some literature, that Der p 1 is a multifunctional protease with both cysteine and serine protease activities (Hewitt et al., 1997). It is now clear that this is incorrect: No evidence for this notion exists within the crystal structure of Der p 1, and studies with native and recombinant Der p 1 in the presence of highly selective inhibitors provide no functional support. The likeliest explanation for the misleading claim is that the native Der p 1 preparation studied by those researchers was contaminated with serine peptidase allergens because native Der p 1 prepared by a strategy to rigorously eliminate such contamination fails to degrade the chymotrypsin substrate *N*-succinyl-AAPF-*p*-nitroanilide. In contrast, both trypsin and Der p 1 degrade *N*-Bz-FVR-*p*-nitroanilide but whereas Der p 1 is inhibited by ADZ 50,000 it is not affected by a generic serine peptidase inhibitor, aminoethylbenzenesulfonyl fluoride, which blocks the action of trypsin (our unpublished observations).

The cleavage specificity of Der p 1 has been evaluated in peptide sequences from suspected target proteins and by library screening campaigns. Substrates in combinatorial library of general formula Abz-1-2-3-4-Y(NO_2)-D-NH_2, with cleavage between residues 3 and 4, revealed the following preferences for Der p 1 – 1: V>A,Q,L,F; 2: A>>Q or K; 3: L, J or A>S; 4: S. Noting that J is an isostere of K, these findings are similar to the results of screening crude HDM extracts in a different combinatorial library where the preferences for the S_1-S_4 pockets were K, A, V and J, respectively. A more recent campaign using phage display has proposed a preference for K/R,V,L/V,V,V for the S_1-S_5 pockets and used a bioinformatics approach to predict potential cellular substrates, some of which have not previously been revealed by hypothesis-driven investigation (Jacquet et al., 2017). While informative about the selectivity or promiscuity of enzyme pockets, this information does not, of course, translate directly into obvious function or provide immediate chemical design solutions for inhibitor discovery. Potential substrates may be clandestine in cellular reality and therefore of

marginal biological significance. Conversely, apparently disfavored substrates may be presented in ways which encourage attack or whose cleavage initiates amplification mechanisms which transform the wider biological significance of the target. For inhibitor design, an appreciation of the binding site topology taken together with substrate preferences is merely a first step in the creation of developable chemical entities as opposed to tool compounds whose value is restricted.

Examination of the structure of Der p 1 determined from crystals of isoallergen Der p 1.0105 reveals the presence of three disulphide bridges (C^{84}-C^{197}, C^{111}-C^{151} and C^{145}-C^{183}) and suggests that the amino acid residues surrounding the substrate binding groove, with its characteristic catalytic triad of C^{114}, H^{250} and N^{270}, are well conserved. The clear implication is that substrate preferences are consistent among isoallergens in a single species of HDM and between homologous allergens in different HDM species. This is supported by enzyme kinetic and inhibitor data (our unpublished observations) and structural comparison of the active sites of Der p 1 and Der f 1 where substitution of R^{231} in Der p 1 by Q^{232} is the most notable difference.

High resolution structural data allow the binding pockets of Der p 1 to be compared with off-target enzymes which have potential to constitute selectivity nuisances in drug design. An empirical observation made in early discovery research was that non-optimized inhibitors of Der p 1 were effective inhibitors of cathepsin B but showed good selectivity over cathepsins S and K. We believe that the selectivity of our initial inhibitors over cathepsins S and K was imparted to some extent by a small P_2 group, whereas cathepsins S and K prefer larger groups in this position. Despite cathepsin B being the least like Der p 1 when judged by amino acid sequence, optimization of selectivity over cathepsin B requires more chemical design effort to exploit the greater openness of the S_3 pocket in Der p 1 which results from substitution by T^{74} in place of the bulkier Y^{75} in cathepsin B. The appreciation of these differences provided encouragement that novel inhibitors of the HDM allergens with appropriate selectivity profiles were achievable.

While not compromising the view that they are a single target for structure-based protease inhibitor design, detailed crystallographic investigations have revealed some minor differences between Der p 1 and Der f 1. Examples include the presence of a binding site for divalent cations which may be occupied in the case of Der p1, but not Der f 1 (Chruszcz et al., 2009; de Halleux et al., 2006; Meno et al., 2005; Meno et al., 2006). Removal of divalent cations does not alter the proteolytic activity of

Der p 1 (Zhang, unpublished data), which distinguishes it from papain in which divalent cations increase catalytic activity (Chruszcz et al., 2009). A further distinction between Der p 1 and other cysteine peptidases is its tendency to form oligomers in solution or in crystalline solid state (Chruszcz et al., 2009; de Halleux et al., 2006), although Der f 1 lacks this behavior (Chruszcz et al., 2009). Whether this oligomerization has biological significance is unknown - the property is uncommon in cysteine peptidases generally, with cathepsin C being one of the few enzymes where it may be physiologically relevant. Although the IgE-binding epitopes of these allergens remain to be fully defined, structural analysis of a monoclonal antibody which binds both Der p 1 or Der f 1 shows that mutagenesis of the conserved allergen binding site diminishes IgE binding, providing insight into a surface region which provides linear and/or conformational features for species cross-reactivity between Der p 1, Der f 1 and Eur m 1 (Chruszcz et al., 2012). This region is distant from the catalytic sites of these allergens, suggesting that IgE-bound allergens are catalytically active, creating the potential for the exercise of multiple bioactivities when interacting with sentinels such as mast cells and basophils. A further feature of this site is its poor conservation in Blo t 1, consistent with the low cross-reactivity of *Blomia* and *Dermatophagoides* allergens in allergic sera (Chruszcz et al., 2012).

Allergy Initiators: Biological Effects of Group 1 HDM Allergens

For any protein to merit interest as a drug design target, a minimum requirement is that plausible evidence must exist to validate its association with a disease-related pathway. For group 1 HDM allergens, a clear association of the allergen with disease through precedented (ie IgE-dependent mechanisms) is historical reality and persuasive contemporary evidence now links proteolytic activity to mechanisms considered core to the pathogenesis of allergy. These are summarized in **Table 2** and examined in depth in the subsequent sections. For these reasons, the group 1 HDM allergens may be considered 'initiator' allergens, a highly exclusive cadre whose actions underpin and facilitate progressive polysensitization to allergens from unrelated sources (although the number of people exhibit monosensitization is also significant). It is clear from **Tables 1** and **2** that the HDM allergen repertoire contains 3 serine proteases which replicate some of the effects of the group 1 cysteine proteases. In the airways, the nature of this redundancy underscores the selection of group 1 HDM allergens as the favored target because the latter enable the redundancy of the serine peptidases through their ability to inactivate airway serpins while evading antiprotease defences themselves. This provides a basis to anticipate that inhibition of a group 1 allergen will boost the defence against

serine peptidase allergens by serpins. As discussed later, the correct proposal of protease target is endorsed by encouraging data for group 1 inhibitors in a variety of mechanistic models.

Mucosal Defenses

Antigen-presenting cell networks of the lung (dendritic cells) and skin (Langerhans cells) are the sentinels which link innate and adaptive immune responses. These cells process signals from activated innate immune mechanisms, such as pattern recognition receptors (PRRs), and present antigen to T cells after their migration to lymph nodes. To minimize nuisance triggering by low grade external threats or the host microbiome, antigen presenting cells and PRRs exhibit a polarized distribution with many of these receptors and cells protected by epithelium. Epithelial cells, airway macrophages and components of airway surface liquid are thus the front line of defence against allergens and other immunological threats. In understanding the initiation of sensitization and the processes which ensure its persistence, a key question is how allergens contact antigen presenting cell networks, either by crossing mucosal barriers themselves or by promoting the ability of antigen presenting cells to sample from the external environment. Of all allergens, the proteases of HDM have been the most studied in this regard, notably in the airways whose simple epithelial structure, intercellular junctions and permeability characteristics are well characterized. The more complex stratified epithelium of skin is less extensively studied in the context of allergen disposition, reflecting that the role of tight junctions (TJs) in regulating skin permeability was itself unclear until the beginning of this millennium.

The first detailed studies of HDM allergen interactions with epithelial cells employed a combination of functional, biochemical and 2-photon molecular excitation microscopy with 3-dimensional isosurface image reconstruction, although earlier examination of permeability effects had been reported several years earlier (Herbert et al., 1990; Herbert et al., 1995; Wan et al., 2000; Wan et al., 2001; Wan et al., 1999). These investigations revealed that TJs in human airway epithelial cells exposed to HDM fecal pellets were cleaved under conditions that mimic daily exposure to the allergens (**Figure 1**) resulting in a non-specific increase in paracellular permeability (ie the barrier becomes leaky to all allergens, regardless of origin, and may also facilitate dendritic, and other, cell migration). The underlying mechanism is a proteolytic attack by cysteine peptidase and serine peptidase HDM allergens on the extracellular domains of occludin and claudins (Wan et al., 2001; Wan et al., 1999), transmembrane proteins which form the contiguous intercellular contacts at the apical pole of the cells and which are

adhesive components of the supramolecular TJ complex. In turn, this extracellular proteolytic cleavage initiates intracellular processing of the TJ plaque protein, ZO-1. While epithelial permeability control is likely to depend on the ensemble function of various TJ adhesion proteins, lung-specific claudin-18 is known to be important in the Th2-high asthma phenotype. In common with claudins of TJs in other tissues, claudin-18 is downregulated by IL-13 (whose release is augmented by peptidase allergens) and its loss impairs epithelial permeability control in model systems *in vitro* and *in vivo* (Sweerus et al., 2017). Thus, the ability of protease allergens to cleave TJs directly and to affect their function through cytokine expression provides a powerful mechanism to compromise epithelial defense. As described later, this is accompanied by protease-driven inflammatory events in which the intracellular generation of reactive signaling molecules play a key role and which provide a link to the activation of Toll-like receptor 4 (**Figure 1, Table 2**).

An analogous mechanism operates in skin where HDM protease allergens cause the epidermis to become leaky and then impede its restitution (Jeong et al., 2008; Nakamura et al., 2006), while at the same time promoting cytokine and chemokine production which is similar to the response in airways (Arlian and Morgan, 2011; Arlian et al., 2008; Ogawa et al., 2008; Oshio et al., 2009)(**Table 2**). The cleavage of TJs in keratinocytes affords the opportunity for Langerhans cells, which express TJ proteins themselves, to form new TJs with keratinocytes enabling an upregulation of antigen sampling while, at least initially, retaining barrier integrity (Kubo et al., 2009). Similar behavior occurs in allergic rhinitis where, unlike non-rhinitic controls, the dendrites of antigen-presenting cells penetrate beyond the apical surface of the nasal epithelium (Takano et al., 2005). However, chronic allergen exposure or other predisposing factors for epithelial leakiness such as loss of function mutations in filaggrin or dysregulation of lipid composition in the stratum corneum of the skin result in augmented immunologic responsiveness to allergens. Indeed, emerging evidence suggests that clinically unaffected skin barrier properties in atopic dermatitis are compromised as decreased levels of claudins-1, -4 and -23 have been found in non-lesional skin and an inverse relationship between claudin-1 and Th2-polarized responses has been observed (Brandner et al., 2015; De Benedetto et al., 2011).

Although the allergen repertoire of HDM consist of several proteases, the particular importance of group 1 allergens such as Der p 1 is indicated by the prevention of transepithelial delivery of allergen when TJ cleavage is blocked (Wan et al., 1999) and when the proteolytic activity of Der p 1 is inhibited, intranasally administered HDM allergens no longer evoke allergic sensitization in mice. Thus the

serine protease HDM allergens, despite cleaving TJs (Wan et al., 2001), have a subordinate role in driving allergic sensitization.

In addition to the physical defenses, epithelial surfaces offer protection against pathogens through biochemical mechanisms (**Table 2**). Antiproteases are one facet of biochemical defense relevant to interactions with protease allergens, especially in the airways where epithelial surface liquid is rich in α_1-antitrypsin capable of inhibiting the effects of serine protease allergens. However, Der p 1 inactivates this serpin and is itself resistant to most mammalian antipeptidases except α_2-macroglobulin, whose molecular weight restricts its presentation at the airway surface (Brown et al., 2003; Kalsheker et al., 1996). Other targets of Der p 1 in airway surface liquid are surfactant proteins –A and –D, members of the collectin family of C-type lectin receptors. The function of these proteins in innate immunity is opsonization of pathogens for phagocytosis, but as their names imply they also control surfactant production and may offer some protection against allergy development (Deb et al., 2007).

Cell Signaling and HDM Protease Allergens

Additional to the events that promote the physical interaction of allergens with antigen presenting cells, HDM protease allergens initiate a sophisticated series of events within the sphere of innate immunity. Cells that are in the front line of interactions with allergens (*viz.* macrophages, epithelial cells, keratinocytes, dendritic cells and mast cells) express an extensive palette of innate immune receptors which are central to the events which initiate and perpetuate allergy. Cytokine production is one of their outputs with a prominent role in progressively driving disease symptoms and exacerbations. The main goal of clinical management in allergy is prevention of exacerbations, so interruption of this chain of events at key checkpoints should offer significant patient benefit.

Group 1 HDM Allergens are Prothrombinases

Unexpectedly, one of the proteolytic features of Der p 1 is its ability to behave as a prothrombinase, forming thrombin from prothrombin independently from classical coagulation pathways (Zhang et al., 2016)(**Table 2**). In airway epithelial cells, this effect of Der p 1 is augmented by the subsequent activation of an endogenous prothrombinase which plays a major role in controlling the generation of reactive signaling molecules (Zhang et al., 2018). These recent discoveries cast an exciting new

perspective on how group 1 HDM allergens upregulate cytokine expression through the operation of a signaling cycle which interlinks components having a pleiotropic role in allergy specifically, or innate immunity generally (**Figure 2A**). At the time of writing, the exact relationships between many key events in this cycle remain to be defined, but what is known suggests that group 1 HDM allergens are, by means of their proteolytic activity, the ignition keys of fundamental innate responses. Consequently, novel molecular entities which inhibit the proteolytic activity of group 1 HDM allergens affect a broad spectrum of events relevant to allergy initiation and maintenance (Zhang et al., 2018).

Through its interstitial prothrombinase activity, Der p 1 enables the canonical activation of protease-activated receptors (PAR)-1 and PAR-4 by thrombin in a manner consistent with the formation of a ternary complex of thrombin with receptor hetero-oligomers (Chen et al., 2017)(**Table 2**). This is the beginning of a regenerative cycle (**Figure 2A**) from which key outputs are reactive oxygen and nitrogen species. Although natural mixtures of HDM allergens contain both serine and cysteine proteases, with the former as candidates for the activation of PAR-2, potent and selective inhibitors of Der p 1 attenuate the production of virtually all the reactive species suggesting that it is the major component within the repertoire of the allergen mixture responsible for their generation. That PAR-2 cleavage makes only a minor contribution is indicated by the small effects of either its pharmacologic antagonism or silencing by siRNA. Probe studies with a DNA-binding triphenylphosphonium analog of dihydroethidium indicate that at least some of this generation occurs in mitochondria through a two electron reduction of oxygen, and this establishes the basis for a mechanism for the upregulation of cytokine production through well-characterized mechanisms, notably those dependent on the transcription factor NFκB (Chen et al., 2017; Zhang et al., 2016). Reactive oxidant species (ROS) activate transcription factors and induce histone modifications favoring the induction of pro-allergic cytokines, while through direct transformation of proteins they activate mitogen-activated protein kinase and the signal transducer and activation of transcription pathways which are implicated in allergy and asthma (Comhair and Erzurum, 2010; van Rijt et al., 2017). Other studies have provided direct evidence of DNA damage resulting from HDM exposure in mice and human lung cells, mirroring underlying events observed in asthma (Chan et al., 2016).

Group 1 HDM Allergens, Pannexon Gating and ATP Release

Downstream from the Der p 1-dependent activation of PAR1/4 lies the opening of pannexons which *inter alia* result in the extracellular release of ATP (**Figure 2A**)(**Table 2**). These pannexons are assembled from pannexin-1 and the release of ATP operates as an innate alarm mechanism by signaling through P_2X_7, P_2Y_2 and, to a lesser extent, P_2Y_4 receptors leading to the activation of ADAM 10 and eventuating in the production of ROS. The recruitment of ADAM 10 into this cycle is notable in that its other actions, namely promoting IgE synthesis through its CD23 sheddase activity on B-lymphocytes (also a direct effect of Der p 1), recruiting dendritic cells, eosinophils, neutrophils and T-cells through CCL20, CCL2, CCL5, CXCL8 and CXCL16 release, signify a pleiotropic role in allergy while its sheddase action on E-cadherin of adherens junctions suggest an augmenting role in dysregulation of epithelial barrier permeability (Gough et al., 2004; Inoshima et al., 2011; Mathews et al., 2011; Post et al., 2015; Schulz et al., 1995; Weskamp et al., 2006) and the promotion of epithelial-mesenchymal transition (EMT). However, in airway epithelial cells responding to HDM allergens it also appears that ADAM 10 initiates further conversion of prothrombin to thrombin, indirectly, directly or both, but via events that are significantly downstream from the prothrombinase activity of Der p 1. The ligand shedding activity of ADAM 10 is Ca^{2+}-dependent (Nagano et al., 2004; Reiss and Saftig, 2009), so an association with the opening of pannexons, which are permeable to both mono- and divalent cations, may be critical in its activation. Recent studies from our laboratory indicate that ADAM 8 may provide some support for ADAM 10 in this cycle, suggesting an analogous relationship to that involved in CD23 shedding where ADAM 10 is the constitutive sheddase whose action can be duplicated, if necessary, by ADAM 8. The endogenous production of thrombin activated by ADAM 10 and ADAM 8 is required for the generation of reactive signaling species, which led us to consider what the further downstream consequences of these reactions might be.

Group 1 HDM Allergens and TLR4 Ligation

Revealingly, we found that effective blockade of ROS formation occurred by siRNA silencing of Toll-like receptor (TLR) 4 or pharmacologic inhibition of its interaction with the adapter proteins TIRAP and TRAM by TAK-242 (Zhang et al., 2018). Attenuation of responses by a Der p 1 inhibitor shows that this activation of TLR4 requires Der p 1 and is independent of ligation by bacterial lipopolysaccharides (LPS), the archetypal agonists of this receptor (Zhang et al., 2018). Although LPS is present in house dust and HDM allergen mixtures, LPS is only weakly effective in triggering ROS production, at least in healthy airways, because of a deficiency in the MD-2 co-receptor and the polarized basolateral distribution of TLR4 which protects it from nuisance triggering by exogenous

signals. However, TLR4 is activated by a range of putative endogenous ligands which include fibrinogen and its cleavage products (Cho et al., In Press; Erridge, 2010; Hodgkinson et al., 2008; Millien et al., 2013; Yu et al., 2010).   Airway epithelial cells secrete fibrinogen from their basolateral aspect in a vectorial, microtubule-dependent manner and are one of few extrahepatic sites where all three component chains of the protein are expressed (Guadiz et al., 1997). This leads to the possibility that fibrinogen cleavage products are the agencies by which Der p 1-dependent TLR4 activation is achieved, although ensemble participation of other TLR4 ligands should not be excluded. The involvement of fibrinogen is indicated by attenuation of responses in airway epithelial cells in which expression of the FGA gene, which encodes the α-chain of the protein, has been silenced by siRNA (**Figure 2B**).   The Der p 1-dependent activation of TLR4 is, therefore, a consequence of the chain of signaling events initiated by its activation of PAR-1 and PAR-4, and the TLR4-dependent action of selective P_2Y_2R or P_2X_7R agonists suggests that the coupling of TLR4 ligation to ROS production occurs downstream from the gating of pannexons (Zhang et al., 2018).

Discovery of a linkage between Der p 1 and TLR4 is of considerable interest because TLR4 expressed on airway epithelial cells is an indispensable requirement for the development of allergic sensitization to HDM allergens (Hammad et al., 2009). Ligation of TLR4 leads to an activation of cells by IL-1α and the release of GM-CSF and IL-33 (Willart et al., 2012), the latter being an bioactivation target of the Der p 1 enzyme also (Cayrol et al., 2018)(**Table 2**). The importance of TLR4 and this triad of cytokines is demonstrated by depletion of the receptor or cytokine neutralization preventing allergic responses (Hammad et al., 2009; Willart et al., 2012). Until the discovery of how Der p 1 leads to TLR4 activation the mechanisms offered to account for the indispensability of TLR4 were (i) the presence in PAR-2 of a potential Toll/interleukin-1 receptor domain which might bind to myeloid differentiation primary response 88 (MyD88) in the TLR4 signaling complex which regulates early NFκB-dependent gene transcription (Barrett et al., 2009), and (ii) the similarity between group 2 HDM allergens and myeloid differentiation protein-2 (MD-2) which functions as a co-receptor protein in the response to LPS (Eisenbarth et al., 2002; Trompette et al., 2009). A structural feature linking the group 2 HDM allergens with MD-2 is the presence of a large hydrophobic pocket capable of binding lipophilic ligands such as LPS. The attraction of this MD-2 mimicry by group 2 HDM allergens is that it might provide a means to compensate for a relative deficiency of MD-2 in airway epithelial cells and offers a mechanistic connection to LPS which is implicated in allergy development under certain conditions. However, MD-2 mimicry suffers limitations as a central initiator mechanism, not least in failing to accommodate the cellular distribution of TLR4 in healthy airways epithelium. Unlike group 1 HDM allergens, those of group 2 neither initiate ROS production nor

have a direct effect on epithelial barrier properties when protease contamination has been rigorously excluded. Indeed, studies on the transepithelial disposition of Der p 2 show that significant permeation occurs only in the presence of proteolytically active group 1 allergens and can be prevented by a novel Der p 1 inhibitor of high potency. Moreover, inhibition of Der p 1 proteolytic activity prevents allergic sensitization in mice (Gough et al., 1999; Gough et al., 2001; Robinson et al., 2012; Zhang et al., 2009), an effect which is hard to reconcile with the indispensability of TLR4 being solely reliant on MD-2 mimicry. Our data provide an alternate rationale where the decisive event is thrombin formation by the direct prothrombinase activity of group 1 HDM allergens which, in turn, facilitates the formation of endogenous TLR4 ligands in the airway epithelium through activation of an additional endogenous prothrombinase (Zhang et al., 2016; Zhang et al., 2018). The identities of these endogenous TLR4 activators remain unclear, but gene silencing of any one of the three component chains of fibrinogen abrogates ROS production by Der p 1 in human airway epithelial cells, although other ligands appear to participate too (**Figure 2C** and our unpublished observations). Significantly, selective inhibitors of Der p 1 provide effective inhibition of all these events (Zhang et al., 2018).   It will be evident from the foregoing that the airway epithelium is the cellular host of a sophisticated signaling nexus which combines the physical delivery of allergen to antigen presenting cells, and thereby T-lymphocytes, with the creation of signaling environment which transduces the progression from innate to acquired immunity with an allergic polarization.

Signaling Convergence Between Group 1 HDM Allergens and Viral RNA Sensors

A fascinating aspect of this HDM allergen-dependent route to TLR4 ligation and the production of reactive intermediates which regulate gene expression is that the signaling mechanisms converge with cellular responses initiated by ligation of the viral RNA sensors TLR3 and TLR7 (**Figure 2A, Table 2**)(Zhang et al., 2016; Zhang et al., 2018). The point of convergence lies upstream from the myosin motor-dependent gating of pannexons and ATP release (Zhang et al., 2018). In allergic asthma, disease exacerbations arise from interactions between allergens and respiratory viruses (principally rhinovirus, respiratory syncytial virus and influenza) so the identification of a nexus linking these stimuli provides new insight into how these exacerbations are precipitated. Interestingly, given the regenerative cycle which underlies this production of ROS, activation of PAR-1 contributes to the pathogenicity of influenza A, and PAR-1 and TLR3 are both upregulated by infections caused by respiratory viruses (Antoniak et al., 2013; Groskreutz et al., 2006).

Thrombin and ATP: Innate Effector-Perpetuators in Allergy

It has been known for some time that concentrations of thrombin in airway surface liquid are elevated in asthma to levels which are capable of driving cell proliferation, and they are also raised following respiratory virus infection (Terada et al., 2004). Whereas some thrombin may result from tissue repair mechanisms activated by inflammation, the more recent data suggest that it also functions as an innate strategic initiator and an effector-perpetuator of allergy through its direct generation by inhaled Der p 1 (Zhang et al., 2016). Similarly, ATP is present in elevated concentrations in bronchoalveolar lavage fluid in asthma (Muller et al., 2011), consistent with the allergen-dependent gating of pannexons. In addition to triggering ROS generation, it initiates the release of IL-33 which is pivotal in the orchestration of responses mediated by ILC2 cells and promotes a Th2 bias in dendritic cells (Idzko et al., 2007; Muller et al., 2011). The actions of ATP extend downstream from these events and additionally activate mast cells, eosinophils and cause dyspnoea (Basoglu et al., 2005; Schulman et al., 1999). In keeping with the foregoing, novel Der p 1 inhibitors which abrogate thrombin generation and pannexon-dependent ATP release also attenuate eosinophil recruitment, inhibit the release of IL-33 and TSLP and impair acute allergic bronchoconstriction (Newton et al., 2014 and our unpublished observations). Their ability to reduce IL-33 production in the airways removes a critical component of innate immune signalling which directs the development and persistence of allergic sensitization and some of its key pathophysiologic features.

Activation of PAR-2 and MRGPRX1

The discovery of PARs naturally prompted speculation that they could be receptors for protease allergens, especially those, like the group 3 and group 6 HDM allergens, with substrate preferences like canonical activators of these receptors. That group 1 HDM allergens would interact with one or more of these receptors was assumed to occur through an example of biased agonism. The ability of HDM allergen extracts and purified allergens to stimulate cytokine (eg IL-6, IL-8, CCL-11, GM-CSF) release from airway epithelial cells or keratinocytes in a manner which paralleled the effects of PAR agonist peptides reinforced this view, especially highlighting PAR-2 as an important molecular recognition system for protease allergens with a central mechanism in disease (Kato et al., 2009; King et al., 1998).   However, subsequent investigations suggest that an association of PAR-2 with asthma and other allergic conditions is complex. Illustratively, in mouse models of HDM sensitization it is dispensable (Adam et al., 2006; Asokananthan et al., 2002; Post et al., 2014) and in some disease models PAR-2 activation dampens rather than escalates inflammation (De Campo and

Henry, 2005; Ebeling et al., 2005), and in the case of group 1 HDM allergens there are conflicting accounts of whether PAR-2 cleavage is an activation signal at all.   Furthermore, the discovery that Der p 1 is a prothrombinase which initiates the PAR-1- and PAR-4-dependent ligation of TLR4 introduces a significant new dimension to how protease-mediated signaling promotes and maintains allergy.

An aspect of chronic asthma which may link Der p 1 and PAR-2 is airway remodelling and EMT in which transforming growth factor-β (TGFβ) is a key element (Hackett, 2012). Group 1 HDM allergens activate latent TGFβ and promote EMT characterized by a PAR-2 and EGFR-dependent reduction of E-cadherin expression by epithelial cells (Frisella et al., 2011; Heijink et al., 2010a; Heijink et al., 2010b; Nakamura et al., 2006), which in turn is a stimulus for the upregulation of TARC and TSLP (Heijink et al., 2007). As described earlier, Der p 1 activates PAR-4 which itself is implicated as an initiator of EMT and the ensuing cycle which leads to ROS formation involves ADAM 10, a key sheddase of E-cadherin (Ando et al., 2007; Chen et al., 2017; Inoshima et al., 2011).

Attention has also turned to the activation of other receptors in conjunction with PARs. Data have been obtained to show that Der p 1 activation of the orphan receptor MRGPRX1 from the mas-related G-protein coupled receptor family contributes to PAR-2-dependent cytokine release from cultured human airway epithelial cells (Reddy and Lerner, 2017), although the relevance to allergy remains untested. However, a possible link between Der p 1 and MRGPRs is intriguing because of their expression in sensory neurons and mast cells. The latter degranulate in response to the proteolytic action of Der p 1 through an uncharacterized mechanism independent from IgE cross-linkage (Machado et al., 1996). In the context of MRGPRs (and also PARs) transducing the nociception of pain and itch, the ability of a protease to activate such physiologic responses is well-established, not least through the use of spicules from pods of the cowhage plant (*Mucuna pruriens*), which contain the cysteine protease mucunain, as an experimental stimulus in human volunteers (Broadbent, 1953; Reddy et al., 2008; Shelley and Arthur, 1955; Wolff and Goodell, 1952). Others will be aware of its efficacy at inducing a burning pain by prank exposure to 'itching powder', a favorite product of novelty shops. One unexpected feature which became quickly evident in early studies with cowhage is that its ability to induce pruritis is heavily reliant on histamine release (Broadbent, 1953), suggesting that the concept of pseudo-allergic, protease-dependent mast cell activation should be revisited in the context of contemporary receptor biology and innate immunity.

Group 1 HDM Allergens and Deficits in Antioxidant Defense

One of the long-standing enigmas of allergy is why environmentally pervasive inhalant allergens evoke sensitization in only a sub-set of the exposed population. The generation of ROS by airway epithelial cells as an innate response to group 1 HDM allergens suggests a scheme to account for some of this difference in susceptibility. It is well established that a deficit in enzymatic and non-enzymatic anti-oxidant defences is common in asthma (Comhair and Erzurum, 2010; Sackesen et al., 2008). Some of these are genetically determined (Fryer et al., 2000; Mapp et al., 2002; Spiteri et al., 2000), while others may arise consequentially from the development of disease. These deficits are exacerbated by the pathogenetic upregulation of oxidant production, such as the induction of NADPH oxidase subunits by cytokines. HDM allergen extract increases the selective expression of DUOX-1 in human airway epithelial cells and this is correlated with enhanced release of hydrogen peroxide and IL-33 (Hristova et al., 2016). In asthma, allergen exposure increases DUOX-1 expression by the nasal epithelium and neutrophil-derived ROS production by Der f 1 is greater than in controls without asthma (Fukunaga et al., 2011). The enhancement of ROS production by certain allergens may itself be a further factor in promoting the allergenicity of other exogenous proteins through carbonyl adduction to form reactive aldehydes which can direct a Th2 proliferative bias in T-lymphocytes (Moghaddam et al., 2011).

A key role for oxidant/antioxidant balance in shaping the development of allergy is suggested by compelling evidence from disease models. In mice, the cysteine protease papain promotes sensitization through mechanisms involving oxidative stress (Tang et al., 2010), and partial inhibition of protease activity blunts the capacity of HDM allergen extract to induce allergic inflammation (Utsch et al., 2015). Correspondingly, mice deficient in the master antioxidant regulator nuclear factor (erythroid-derived 2)-like 2 (Nrf2), or which are unable to upregulate it, develop enhanced responses to ovalbumin or HDM allergens (Li et al., 2013; Rangasamy et al., 2005; Utsch et al., 2015; Williams et al., 2008). Similarly, glutathione depletion exacerbates airway hyperreactivity and inflammation (Nadeem et al., 2014). Consistent with this theme, an Nrf2 activator protected against IL-33 release and allergic responses (Uchida et al., 2017) while over-expression of Nrf2 enhanced the expression of ZO-1, occludin and E-cadherin in airway epithelium (Comhair et al., 2001).

Antigen-Presenting Cell Recruitment

Accumulation of antigen-presenting cells at mucosal surfaces and their departure for interaction with T-lymphocytes is a strategic conduit linking innate and acquired immune responses to allergens. This is demonstrated by the observations that HDM allergen exposure recruits antigen-presenting cells to lung (DCs) and skin (Langerhans cells) through the agency of chemokine release (CCL-2, CCL-5, CCL-20 and CXCL-10) by epithelial cells (Pichavant et al., 2005), and that humanized SCID mice reconstituted with monocyte-derived DCs from patients with atopic asthma are predisposed to develop immune responses with a Th2 polarization (Hammad et al., 2002).   Transcriptomic analysis in HDM allergen-exposed airway epithelial cells or clinical airway specimens suggests that chemokines directed against antigen presenting cells constitute part of an upregulated core gene repertoire (Vroling et al., 2008a; Vroling et al., 2008b). Both CCL-2 and CCL-20 have been proposed as pivotal in the increased steady-state numbers of DCs in the bronchial mucosa of patients with asthma, with a notable increase in Der p 1-dependent CCL-20 release in airway samples in atopic asthma (Pichavant et al., 2005).   A role for CCL-20 in response to HDM allergens is replicated in a mouse model where its release is TLR4-dependent (Hammad et al., 2009). CCL-20 is a ligand for CCR-6 which is expressed on Langerhans cell-like precursors of conventional DCs that are associated with inflammatory events. In mice deficient in CCR6, allergic pulmonary responses are attenuated due to impaired migration of DCs (Lukacs et al., 2001). In contrast to HDM allergen responses, signaling via the CCL-20/CCR-6 axis does not occur with ovalbumin (Robays et al., 2007) which requires the collateral priming effects of the proteolytic activity of Der p 1 or another adjuvant for the development of robust IgE responses (Fattouh et al., 2005; Gough et al., 1999; Gough et al., 2001; Zhang et al., 2009).   Release of CCL-20 from human airway epithelial cells by HDM allergen extracts also occurs through a β-glucan-dependent route. This mechanism is protease- and TLR4-independent but involves Syk activation suggesting that it might be mediated through the C-type lectin receptor, dectin-1 (Nathan et al., 2009). Other work has suggested a role for β-glucan-dependent, TLR2-mediated signaling by HDM allergen extracts in the nasal mucosa (Ryu et al., 2013). Like chitins (β-(1,4)-poly-N-acetyl-D-glucosamine polymers) which also ligate dectin-1 (Lee et al., 2011), β-glucans are extraneous factors in HDM culture extracts and components, from multiple sources, of environmental house dust, so this activation of chemokine production by multiple mechanisms is anticipated as a multi-layered reinforcement of allergic polarization. While these observations suggest the importance of CCL-20/CCR-6 signaling in the allergic recruitment of DCs, the pattern of chemokine and receptor activation seems likely to be more complex given that the high phenotypic plasticity of antigen-presenting cells provides them with a versatile repertoire of delegated effector roles. For this reason, selective intervention at targets within this particular

checkpoint in the initiation and maintenance of allergy may be of variable benefit compared to other options. Illustrating the difficulty in validating CCL-20/CCR-6 as a discrete, primary target pairing, CCL-2, CCL-5 and CXCL-10 chiefly act via ligation of CCR1, CCR2, CCR5 and CXCR3 found on DCs of monocyte derivation, and of these combinations the interaction of CCL2-CCL2R is particularly interesting in allergy development because CCR2^{+}Ly6c^{hi} monocytes are precursors of inflammatory CD11b^{+} DCs found in allergy (Hammad et al., 2009; Robays et al., 2007).

While antigen-presenting cell recruitment and activation may be driven primarily through signaling events triggered by HDM allergens in the airway epithelium, the allergens must also have direct interactions with antigen-presenting cells and these have been the subject of some scrutiny in the context of protease-dependent activation and a programming of Th2 polarization.   One approach has been to use *in silico* prospecting for potential substrates of Der p 1 among the cell surface proteins found on dendritic cells, resulting in identification of C-type lectin receptor dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) and its homologue DC-SIGNR as potential substrates of Der p 1 (**Table 2**). In support of this, recombinant DC-SIGN and DC-SIGNR are cleaved by Der p 1 *in vitro* (Furmonaviciene et al., 2007). DC-SIGN expression is reduced in DCs exposed to Der p 1, although it is hard to ascertain how much of this disappearance is due to proteolysis or to protease-independent endocytosis (Furmonaviciene et al., 2007; Kauffman et al., 2006).

The challenge of devising a successful antigen presentation checkpoint intervention is further demonstrated by another potential target of Der p 1 in DCs (**Table 2**). Der p 1 causes a down-regulation of indoleamine 2,3-dioxygenase in HDM-sensitive individuals (Maneechotesuwan et al., 2009).   Indoleamine 2,3-dioxygenase catalyzes the conversion of tryptophan to kynurenine and it causes immunosuppression through a combination of tryptophan depletion (which activates a sequence of events through induction of general control nonrepressed 2 kinase) and the direct cellular effects of kynurenine and other tryptophan metabolites (Johnson et al., 2009). Consequently, down-regulation of the enzyme by Der p 1 is a potentially interesting mechanism leading to the breaking of immune tolerance and the development and maintenance of sensitization. Indoleamine 2,3-dioxygenase activity is particularly important in antigen-presenting cells, though there is notable heterogeneity in its significance in different DC subsets, especially between conventional DCs and plasmacytoid DCs (Harden and Egilmez, 2012). The success of attempts to induce tolerance to an allergen by manipulation of indoleamine 2,3-dioxygenase activity

may therefore depend on which subset(s) of DCs are most relevant. It should also be noted that chronically stimulating indoleamine 2,3-dioxygenase activity may not be generally beneficial because of the inherent risks associated with a concomitant attenuation of routine immune surveillance for pathogens and tumors (Harden and Egilmez, 2012; Muller et al., 2008). For these, and other reasons, we preferred a different approach focussed on the allergen rather than innate host checkpoints.

Allergen Delivery Inhibitors (ADIs)

The strong causative association of serodominant group 1 HDM allergens with major allergic diseases makes them a compelling target in the search for unprecedented interventions intended to modify the basic immunologic events that are responsible for the development of disease. As outlined in the earlier sections, and summarized by the actions listed in **Table 2**, understanding how their proteolytic activity drives these sentinel events provides encouragement that inhibitors of this proteolytic activity offer the prospect of disease modification. The chemical design challenge associated with this unprecedented mechanism was significant and the identification of developable candidates necessitated the rigorous pursuit of a clear vision of the required attributes, some of which would be deemed an unusual focus in most early stage discovery research. Given the similarity of group 1 HDM allergens as proteases, this enabled us to use Der p 1 as the archetypal template for inhibitor design.   We call this new class of drugs 'Allergen Delivery Inhibitors' to reflect the effect that they interfere with the ability of HDM allergens to engage with antigen-presenting cells. As will now be evident, this process of 'delivery' is a combination of biophysical events in intercellular junctions and the creation of a complex molecular signaling pathway which leads from innate immunity to the acquisition and persistence of allergic sensitization.

Our approach to Der p 1 inhibitor design was informed by the fact that the targets are inhalant allergens and preventing their effects at the site of initial impaction in the lungs would be advantageous. With a non-human inhaled target, the interaction with an inhibitor has no requirement for cell permeation which, in combination with properties designed to minimize systemic exposure, means a significant opportunity to mitigate safety risks in chronic therapy. There are several device options for the delivery of drugs by inhalation *viz*. nebuliser, metered dose inhaler and dry powder inhaler (DPI). We elected to pursue compounds that would be compatible with DPI delivery, with the expectation that this would bring confidence for usage in other devices. Delivery

by DPI imposes a burden on chemical design and pharmaceutics because it demands non-hygroscopic, stable, crystalline compounds which can produce particles of consistent size that will distribute uniformly to the site of action in the lung, in addition to embodying the pharmacologic profile for target selectivity, potency, safety and duration of action.

Opening explorations of Der p 1 inhibitor design were based around the introduction of inhibitory warheads into a modified peptide substrate sequence based on the scaffold peptide VAJS.   Contrary to the development of compounds for oral delivery, it is usually advantageous for inhaled asthma drugs to have poor oral bioavailability to minimize systemic exposure and thus achieve a good safety profile. Compounds which violate Lipinski 'rules' therefore provide interesting freedoms for inhaled drug design which would not usually be tolerated in other settings.   A peptide template, with a high proportion of hydrogen bond donors and acceptors in a flexible scaffold, was therefore considered a useful departure point for such an application. An exemplar derived from this scaffold is aldehyde **1 (Figure 3)**, but despite encouraging potency it was compromised by expectations of instability and non-specific reactivity.   Surprisingly, some warheads effective in the design of cysteine peptidase inhibitors directed at other targets were found to be poor inhibitors of Der p 1, such as vinylogous esters and sulfones elaborated from the scaffold of **1**. Initial progress in the search for potent Der p 1 inhibitors was made with the identification of a series of acyloxymethylketones exemplified by **2**, **3**, and **4 (Figure 3)**. Docking of **3** in Der p 1.0105 (PDB: A2S8) suggested that the norleucine side chain makes hydrophobic interactions with the P_1 pocket while the carbonyl initially forms a reversible covalent bond with C^{114} and the NH a hydrogen bond with Y^{249} (Newton et al., 2014). Consistent with the mechanism of inhibition for acyloxymethyl ketones, C^{114} subsequently migrates to displace the acyl group forming an irreversible covalent bond with the inhibitor. The amino and carbonyl groups of alanine hydrogen bond with D^{154} and the preference for compact groups at P_2 arises because of I^{156}. The side chain of valine makes hydrophobic interaction with the P_3 pocket and the benzoyl capping group forms hydrophobic π-stacking associations with tyrosine residues.   While docking analysis provided useful information to guide subsequent chemical design, the acyloxymethyl ketones *per se* lack developability, primarily through concerns about their irreversible inhibition of the target. The challenge was therefore to identify functional groups which would enable the creation compounds whose target inhibition was reversible whilst maintaining the benefits of slow off-rate. To this end we investigated a series of inhibitors which were capable of forming a reversible covalent bond with C^{114}. An advance towards this goal was a series of amino ketones which demonstrated the achievability of incorporating a reversible inhibitor motif into a scaffold which offered good prospects for optimization. However, despite initially encouraging

progress, it proved impossible to increase potency beyond that of compound **5 (Figure 4)**. This activity was deemed insufficient when rated against a compound developability profile whose potency requirements had been defined from empirical estimates of target exposure in humans and the need to be mindful of device dosage practicalities (Newton et al., 2014).

Reassurance that an uplift in potency could be achieved in a reversible inhibitor was provided by **6 (Figure 4)**, albeit using a functionality of limited developability. A pursuit of alternative groups led to the identification of a series of pyruvamides such as **7 (Figure 4)** which became the focus for detailed exploration (Newton et al., 2014). The inhibitory potency of **7** against the Der p 1 target is compatible with the declared profile for development, but it is not optimized with respect to properties which influence the persistence at the site of action or its selectivity over obvious off-target nuisances. As a design tool for optimization, a computational model was created based on the structure of Der p 1.0105 and on the structures of peptidic inhibitors bound to cathepsin K. Compound **7** was built and energy-minimized in the substrate binding groove of Der p 1 with the electrophilic carbonyl of the pyruvamide moiety orientated to interact with C^{34} and the peptide backbone aligned to follow a similar trajectory to that of the other peptidic inhibitors. As shown in **Figure 5**, this revealed interactions between the amide carbonyl of the pyruvamide and the backbone NH of C^{114}, the NH of the P_1 subunit and the carbonyl of Y^{249} and the formation of a donor:acceptor pair with the backbone carbonyl and NH of D^{154}. This tool enabled the prioritization of chemical design decisions which were rigorously explored by iterative rounds of synthesis and screening, details of which are provided elsewhere (Newton et al., 2014). Counter-screening against Der f 1 provided confidence that compounds designed using Der p 1 as template were similarly active in other HDM species.

Pursuant to producing compounds which were efficacious against real-life allergen exposure and not just a single purified allergen, a range of *in vivo* studies were undertaken to establish confidence in developability (Newton et al., 2014). An example for compound **8** is shown in **(Figure 6)**. Eosinophils in bronchoalveolar lavage fluid was chosen as a readout in these studies because elevations in blood eosinophils and eosinophil recruitment to the airways are hallmark features of allergic asthma in many patients and correlate with disease severity. Importantly, these animals were sensitized and challenged with the complete palette of house dust mite allergens in a naturally-derived mixture and not simply with the purified target allergen. As illustrated, a single inhaled dose of the compound produced a striking reduction in eosinophil recruitment after acute allergen challenge in an IgE-

dependent model. This is an interesting result because our expectation is that ADIs will exhibit greatest benefits against underlying IgE-independent mechanisms which drive the acquisition and persistence of disease.   Thus, even in a model where a strong IgE response exists and allergen challenge comprised to full palette of HDM allergens, targeting only the Der p 1 component produced a result suggesting useful efficacy (Newton et al., 2014).

To further explore the chemical features necessary for a persistence of effect, we evaluated a subset of potent compounds which were stable in contact with airway cells. To neutralize additional variables created in IgE-dependent challenge models, we electively conducted investigations using animals which were HDM allergen-naïve because aerosol challenge with Derp 1 or HDM allergen mixtures results in eosinophil recruitment to the airways in the absence of HDM sensitization (Newton et al., 2014). The kinetics of this IgE-independent innate response are indistinguishable from sensitized animals, but the effect is smaller in magnitude. Compounds selected for these studies spanned a range of log $D_{7.4}$ values and polar surface areas **(Figure 7)**. Better efficacy was associated with lipophilicity, but good activity also existed in quaternary ammonium compounds (eg **8**, **14**) (Newton et al., 2014). As anticipated, the latter benefited from having low oral absorption, thus restricting systemic exposure in a manner which offers some advantages over alternate approaches where deliberately promoting the biotransformation of absorbed drugs can generate unexpected metabolic liabilities.

As the electrophilic pyruvamide motif has potential for non-specific interaction with suitable nucleophiles, it was imperative to achieve selectivity over potential off-targets, even though many of these are intracellular and thus intrinsically resilient to reaction compared to the facile extracellular interaction with Der p 1. As already described, the S_3 pocket of Der p 1 is more capacious than in cathepsins, and our screening campaign confirmed that that steric bulk in this position increased selectivity while retaining potency on target. Inspection of the structural model suggested that the S_2 pocket of Der p 1 was shallow compared to cathepsins B, K or S and so with compact substituents at this position it was possible to obtain a useful balance of potency and selectivity (Newton et al., 2014). As an aside, Blo t 1 exhibits a similar feature at its putative S_2 pocket, suggesting that compounds designed against a template from a pervasive HDM species may have characteristics which translate to allergens from mites whose evolution has diverged to suit niche environments. Once confidence in the contributions of the P_2 and P_3 substituents had been obtained, we explored greater variation in the P_4 and P' groups which pleasingly revealed the feasibility of synthesizing

compounds such as **16-21** of high inhibitory potency and good selectivity consistent with developability **(Figure 8)** (Newton et al., 2014).

We examined exemplars of optimized compounds *in vivo* using a variety of readouts to establish confidence in efficacy and duration of protection. These studies confirmed the structural features required for compliance with the exacting candidate drug template and provided test data from which clinical candidates could be selected (Newton et al., 2014). A wider examination of the effects of ADIs in experimental models reveals the potential for a fascinating spectrum of effects in the clinic and a profile which is interestingly differentiated from current standard of care and other entities in development for asthma (**Figure 9** and **Table 3**). The overall impression is of an intervention which has the breadth normally associated with corticosteroids. However, ADIs can affect events which are not directly regulated by steroids and, notably, their broad range of effects is achieved without incurring penalties from non-specific immunosuppression. Pre-eminent in the readouts which show positive effects of ADIs are those strongly associated with events identified by experimental biology as being indispensable in allergy. Some of the signaling entities which drive these indispensable events are now the target of biologic therapies in development. The ability to attenuate the same signals simultaneously by means of an inhaled small-molecule provides a superior option with the potential for usage across a broader spectrum of patients.

Concluding Remarks

From the earliest scientific investigations of allergy, the causative link between allergens and the diseases they produce has attracted attention as a means of disease treatment. The outstanding example of this is the pioneering work of Freeman and Noon led to the development of allergen immunotherapy which attempts to tolerize the immune system to a particular allergen (Freeman and Noon, 1911). However, targets for small molecule interventions at the root cause level have been non-obvious until recently, so the pharmacotherapy of allergy adopted a different approach which exploited progress in the identification of bioactive mediators and the elucidation of effector mechanisms. The renaissance of innate immunity and an increasing understanding the molecular basis of allergenicity now creates the first opportunity for pharmacotherapy to target the causative agents of many allergic diseases and thereby prevent the activation of sentinel mechanisms which initiate and sustain disease. In this review we have described progress towards that ambitious objective for what is arguably the most significant of all indoor allergens. The power of the

intervention exploits the growing awareness that the allergome is not a molecular democracy: An exclusive cadre of initiator allergens play decisive roles in driving disease. This vulnerability creates a pharmacologic opportunity to exert a broad spectrum of benefits by careful selection of target and chemical design, as exemplified by the pioneer work with ADIs. Naturally, it will be of considerable interest to follow the progression into the clinic of the new drugs from this unique programme.

Acknowledgements

Author Contributions

Wrote, or contributed to the writing of, this manuscript: Zhang, Chen, Newton, Perrior, Robinson

References

Adam E, Hansen KK, Astudillo Fernandez O, Coulon L, Bex F, Duhant X, Jaumotte E, Hollenberg MD and Jacquet A (2006) The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. *J Biol Chem* **281**: 6910-6923.

Ando S, Otani H, Yagi Y, Kawai K, Araki H, Fukuhara S and Inagaki C (2007) Proteinase-activated receptor 4 stimulation-induced epithelial-mesenchymal transition in alveolar epithelial cells. *Respiratory Research* **8**: 31.

Antoniak S, Owens AP, 3rd, Baunacke M, Williams JC, Lee RD, Weithauser A, Sheridan PA, Malz R, Luyendyk JP, Esserman DA, Trejo J, Kirchhofer D, Blaxall BC, Pawlinski R, Beck MA, Rauch U and Mackman N (2013) PAR-1 contributes to the innate immune response during viral infection. *J Clin Invest* **123**: 1310-1322.

Arlian LG and Morgan MS (2011) Immunomodulation of skin cytokine secretion by house dust mite extracts. *Int Arch Allergy Immunol* **156**: 171-178.

Arlian LG, Morgan MS and Peterson KT (2008) House dust and storage mite extracts influence skin keratinocyte and fibroblast function. *Int Arch Allergy Immunol* **145**: 33-42.

Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ and Stewart GA (2002) House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol* **169**: 4572-4578.

Baker SF, Yin Y, Runswick SK, Stewart GA, Thompson PJ, Garrod DR, Robinson C (2003) Peptidase allergen Der p 1 initiates apoptosis of epithelial cells independently of tight junction proteolysis. *Mol Membr Biol* **20**: 71-81.

Barrett NA, Maekawa A, Rahman OM, Austen KF and Kanaoka Y (2009) Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J Immunol* **182**: 1119-1128.

Basoglu OK, Pelleg A, Essilfie-Quaye S, Brindicci C, Barnes PJ and Kharitonov SA (2005) Effects of aerosolized adenosine 5'-triphosphate vs adenosine 5'-monophosphate on dyspnea and airway caliber in healthy nonsmokers and patients with asthma. *Chest* **128**: 1905-1909.

Brandner JM, Zorn-Kruppa M, Yoshida T, Moll I, Beck LA and De Benedetto A (2015) Epidermal tight junctions in health and disease. *Tissue Barriers* **3**: e974451.

Broadbent JL (1953) Observations on itching produced by cowhage, and on the part played by histamine as a mediator of the itch sensation. *Brit J Pharmacol Chemother* **8**: 263-270.

Brown A, Farmer K, MacDonald L, Kalsheker N, Pritchard D, Haslett C, Lamb J and Sallenave JM (2003) House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Respir Cell Mol Biol* **29**: 381-389.

Calderon MA, Cox L, Casale TB, Moingeon P and Demoly P (2012) Multiple-allergen and single-allergen immunotherapy strategies in polysensitized patients: looking at the published evidence. *J Allergy Clin Immunol* **129**: 929-934.

Cayrol C, Duval A, Schmitt P, Roga S, Camus M, Stella A, Burlet-Schiltz O, Gonzalez-de-Pedero A, Girard J-P (2018) Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nature Immunol* **19**: 375-385.

Chan TK, Loh XY, Peh HY, Tan WN, Tan WS, Li N, Tay IJ, Wong WS and Engelward BP (2016) House dust mite-induced asthma causes oxidative damage and DNA double-strand breaks in the lungs. *J Allergy Clin Immunol* **138**: 84-96 e81.

Chen J, Zhang J, Tachie-Menson T, Shukla N, Garrod DR and Robinson C (2017) Allergen-dependent oxidant formation requires purinoceptor activation of ADAM 10 and prothrombin. *J Allergy Clin Immunol* **139**: 2023-2026 e2029.

Cho HJ, Lee HJ, Kim SC, Kim K, Kim YS, Kim CH, Lee JG, Yoon JH, Choi JY (2012) Protease-activated receptor-2-dependent fluid secretion from airway submucosal glands by house dust mite extract. *J Allergy Clin Immunol* **129**; 529-535.e5 https://doi.org/10.1016/j.jaci.2011.11.024

Cho M, Lee JE, Lim H, Shin HW, Khalmuratova R, Choi G, Kim HS, Choi WS, Park YJ, Shim I, Kim BS, Kang CY, Kim JO, Tanaka S, Kubo M and Chung Y (In Press) Fibrinogen cleavage products and Toll-like receptor 4 promote the generation of programmed cell death 1 ligand 2-positive dendritic cells in allergic asthma. *J Allergy Clin Immunol* . https://doi.org/10.1016/j.jaci.2017.09.019

Chruszcz M, Chapman MD, Vailes LD, Stura EA, Saint-Remy JM, Minor W and Pomes A (2009) Crystal structures of mite allergens Der f 1 and Der p 1 reveal differences in surface-exposed residues that may influence antibody binding. *J Mol Biol* **386**(2): 520-530.

Chruszcz M, Pomes A, Glesner J, Vailes LD, Osinski T, Porebski PJ, Majorek KA, Heymann PW, Platts-Mills TA, Minor W and Chapman MD (2012) Molecular determinants for antibody binding on group 1 house dust mite allergens. *J Biol Chem* **287**: 7388-7398.

Comhair SA, Bhathena PR, Farver C, Thunnissen FB and Erzurum SC (2001) Extracellular glutathione peroxidase induction in asthmatic lungs: evidence for redox regulation of expression in human airway epithelial cells. *FASEB J* **15**: 70-78.

Comhair SAA and Erzurum SC (2010) Redox Control of Asthma: Molecular Mechanisms and Therapeutic Opportunities. *Antioxid Redox Sign* **12**(1): 93-124.

Daan de Boer J, van't Veer C, Stroo I, van der Meer AJ, de Vos AF, van der Zee JS, Roelofs JJTH, van der Poll T (2014) Protease-activated receptor-2 deficient mice have reduced house dust mite-evoked allergic lung inflammation. *Innate Immunity* **20**: 618-625. DOI: 10.1177/1753425913503387

De Benedetto A, Slifka MK, Rafaels NM, Kuo IH, Georas SN, Boguniewicz M, Hata T, Schneider LC, Hanifin JM, Gallo RL, Johnson DC, Barnes KC, Leung DY and Beck LA (2011) Reductions in claudin-1 may enhance susceptibility to herpes simplex virus 1 infections in atopic dermatitis. *J Allergy Clin Immunol* **128**: 242-246 e245.

De Campo BA and Henry PJ (2005) Stimulation of protease-activated receptor-2 inhibits airway eosinophilia, hyperresponsiveness and bronchoconstriction in a murine model of allergic inflammation. *Brit J Pharmacol* **144**: 1100-1108.

de Halleux S, Stura E, VanderElst L, Carlier V, Jacquemin M and Saint-Remy JM (2006) Three-dimensional structure and IgE-binding properties of mature fully active Der p 1, a clinically relevant major allergen. *J Allergy Clin Immunol* **117**: 571-576.

Deb R, Shakib F, Reid K and Clark H (2007) Major house dust mite allergens Dermatophagoides pteronyssinus 1 and Dermatophagoides farinae 1 degrade and inactivate lung surfactant proteins A and D. *J Biol Chem* **282**: 36808-36819.

Ebeling C, Forsythe P, Ng J, Gordon JR, Hollenberg M and Vliagoftis H (2005) Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. *J Allergy Clin Immunol* **115**: 623-630.

Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA and Bottomly K (2002) Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* **196**: 1645-1651.

Erridge C (2010) Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J Leukocyte Biol* **87**: 989-999.

Fattouh R, Pouladi MA, Alvarez D, Johnson JR, Walker TD, Goncharova S, Inman MD and Jordana M (2005) House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am J Respir Crit Care Med* **172**: 314-321.

Freeman J and Noon L (1911) Further observation on the treatment of hay-fever by hypodermic inoculation of pollen vaccine. *Lancet* **2**: 1572-1573.

Frisella PD, Silverberg J, Joks R and Frieri M (2011) Transforming growth factor beta: a role in the upper airway and rhinosinusitis-Dermatophagoides pteronyssinus-induced apoptosis with pulmonary alveolar cells. *Am J Rhinology & Allergy* **25**: 231-235.

Fryer AA, Bianco A, Hepple M, Jones PW, Strange RC and Spiteri MA (2000) Polymorphism at the glutathione S-transferase GSTP1 locus. A new marker for bronchial hyperresponsiveness and asthma. *Am J Respir Crit Care Med* **161**: 1437-1442.

Fukunaga M, Gon Y, Nunomura S, Inoue T, Yoshioka M, Hashimoto S and Ra C (2011) Protease-mediated house dust mite allergen-induced reactive oxygen species production by neutrophils. *Int Arch Allergy Immunol* **155 Suppl 1**: 104-109.

Furmonaviciene R, Ghaemmaghami AM, Boyd SE, Jones NS, Bailey K, Willis AC, Sewell HF, Mitchell DA and Shakib F (2007) The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. *Clin Exp Allergy* **37**: 231-242.

Gelber LE, Seltzer LH, Bouzoukis JK, Pollart SM, Chapman MD and Platts-Mills TA (1993) Sensitization and exposure to indoor allergens as risk factors for asthma among patients presenting to hospital. *Am Rev Respir Dis* **147**: 573-578.

Gough L, Campbell E, Bayley D, Van Heeke G, Shakib F (2003) Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model *Clin Exp Allergy* **33**: 1159-1163.

Gough L, Schulz O, Sewell HF and Shakib F (1999) The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* **190**: 1897-1902.

Gough L, Sewell HF and Shakib F (2001) The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen. *Clin Exp Allergy* **31**: 1594-1598.

Gough PJ, Garton KJ, Wille PT, Rychlewski M, Dempsey PJ and Raines EW (2004) A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. *J Immunol* **172**: 3678-3685.

Groskreutz DJ, Monick MM, Powers LS, Yarovinsky TO, Look DC and Hunninghake GW (2006) Respiratory Syncytial Virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. *J Immunol* **176**: 1733-1740.

Guadiz G, Sporn LA, Goss RA, Lawrence SO, Marder VJ and Simpson-Haidaris PJ (1997) Polarized secretion of fibrinogen by lung epithelial cells. *Am J Respir Cell Mol Biol* **17**: 60-69.

Hackett TL (2012) Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. *Curr Opinion Allergy Clin Immunol* **12**: 53-59.

Hammad H, Chieppa M, Perros F, Willart MA, Germain RN and Lambrecht BN (2009) House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nature Med* **15**: 410-416.

Hammad H, Lambrecht BN, Pochard P, Gosset P, Marquillies P, Tonnel AB and Pestel J (2002) Monocyte-derived dendritic cells induce a house dust mite-specific Th2 allergic inflammation in the lung of humanized SCID mice: involvement of CCR7. *J Immunol* **169**: 1524-1534.

Harden JL and Egilmez NK (2012) Indoleamine 2,3-dioxygenase and dendritic cell tolerogenicity. *Immunol Invest* **41**: 738-764.

Heijink IH, Kies PM, Kauffman HF, Postma DS, van Oosterhout AJ and Vellenga E (2007) Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *J Immunol* **178**: 7678-7685.

Heijink IH, Postma DS, Noordhoek JA, Broekema M and Kapus A (2010a) House dust mite-promoted epithelial-to-mesenchymal transition in human bronchial epithelium. *Am J Respir Cell Mol Biol* **42**: 69-79.

Heijink IH, van Oosterhout A and Kapus A (2010b) Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. *Eur Respir J* **36**: 1016-1026.

Herbert CA, Holgate ST, Robinson C, Thompson PJ and Stewart GA (1990) Effect of mite allergen on permeability of bronchial mucosa. *Lancet* **336**(8723): 1132.

Herbert CA, King CM, Ring PC, Holgate ST, Stewart GA, Thompson PJ and Robinson C (1995) Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *Am J Respir Cell Mol Biol* **12**: 369-378.

Hewitt CR, Horton H, Jones RM and Pritchard DI (1997) Heterogeneous proteolytic specificity and activity of the house dust mite proteinase allergen Der p I. *Clin Exp Allergy* **27**: 201-207.

Hodgkinson CP, Patel K and Ye S (2008) Functional Toll-like receptor 4 mutations modulate the response to fibrinogen. *Thromb Haemostasis* **100**: 301-307.

Hristova M, Habibovic A, Veith C, Janssen-Heininger YM, Dixon AE, Geiszt M and van der Vliet A (2016) Airway epithelial dual oxidase 1 mediates allergen-induced IL-33 secretion and activation of type 2 immune responses. *J Allergy Clin Immunol* **137**: 1545-1556 e1511.

Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC, Jr. and Lambrecht BN (2007) Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nature Med* **13**: 913-919.

Inoshima I, Inoshima N, Wilke GA, Powers ME, Frank KM, Wang Y and Bubeck Wardenburg J (2011) A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. *Nature Med* **17**: 1310-1314.

Jacquet A, Campisi V, Szpakowska M, Dumez ME, Galleni M and Chevigne A (2017) Profiling the Extended Cleavage Specificity of the House Dust Mite Protease Allergens Der p 1, Der p 3 and Der p 6 for the Prediction of New Cell Surface Protein Substrates. *Int J Mol Sci* **18**.

Jeong SK, Kim HJ, Youm JK, Ahn SK, Choi EH, Sohn MH, Kim KE, Hong JH, Shin DM and Lee SH (2008) Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery. *J Invest Dermatol* **128**: 1930-1939.

Johnson BA, 3rd, Baban B and Mellor AL (2009) Targeting the immunoregulatory indoleamine 2,3 dioxygenase pathway in immunotherapy. *Immunotherapy* **1**: 645-661.

Kalsheker NA, Deam S, Chambers L, Sreedharan S, Brocklehurst K and Lomas DA (1996) The house dust mite allergen Der p1 catalytically inactivates alpha 1-antitrypsin by specific reactive centre loop cleavage: a mechanism that promotes airway inflammation and asthma. *Biochem Biophys Res Commun* **221**: 59-61.

Kato T, Takai T, Fujimura T, Matsuoka H, Ogawa T, Murayama K, Ishii A, Ikeda S, Okumura K and Ogawa H (2009) Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes. *Allergy* **64**: 1366-1374.

Kauffman HF, Tamm M, Timmerman JA and Borger P (2006) House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. *Clin Mol Allergy* **4**: 5.

King C, Brennan S, Thompson PJ and Stewart GA (1998) Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* **161**: 3645-3651.

Kubo A, Nagao K, Yokouchi M, Sasaki H and Amagai M (2009) External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J Exp Med* **206**: 2937-2946.

Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang MJ, He CH, Takyar S and Elias JA (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Ann Rev Physiol* **73**: 479-501.

Li N, Wang M, Barajas B, Sioutas C, Williams MA and Nel AE (2013) Nrf2 deficiency in dendritic cells enhances the adjuvant effect of ambient ultrafine particles on allergic sensitization. *J Innate Immunity* **5**: 543-554.

Lukacs NW, Prosser DM, Wiekowski M, Lira SA and Cook DN (2001) Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J Exp Med* **194**: 551-555.

Machado DC, Horton D, Harrop R, Peachell PT and Helm BA (1996) Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *Eur J Immunol* **26**: 2972-2980.

Maneechotesuwan K, Wamanuttajinda V, Kasetsinsombat K, Huabprasert S, Yaikwawong M, Barnes PJ and Wongkajornsilp A (2009) Der p 1 suppresses indoleamine 2, 3-dioxygenase in dendritic cells from house dust mite-sensitive patients with asthma. *J Allergy Clin Immunol* **123**: 239-248.

Mapp CE, Fryer AA, De Marzo N, Pozzato V, Padoan M, Boschetto P, Strange RC, Hemmingsen A and Spiteri MA (2002) Glutathione S-transferase GSTP1 is a susceptibility gene for occupational asthma induced by isocyanates. *J Allergy Clin Immunol* **109**: 867-872.

Maruo K, Akaike T, Inada Y, Ohkubo I, Ono T, Maeda H (1993) Effect of microbial and mite proteases on low and high molecular weight kininogens. Generation of kinin and inactivation of thiol protease inhibitory activity. *J Biol Chem* **268**: 17711-17715.

Maruo K, Akaike T, Matsumura Y, Kohmoto S, Inada Y, Ono T, Arao T, Maeda H (1991) Triggering of the vascular permeability reaction by activation of the Hageman factor-prekallikrein system by house dust mite proteinase. *Biochim Biophys Acta* **1074**: 62-68.

Maruo K, Akaike T, Ono T, Okamoto T, Maeda H (1997) Generation of anaphylatoxins through proteolytic processing of C3 and C5 by house dust mite protease. *J Allergy Clin Immunol* **100**: 253-260.

Mathews JA, Ford J, Norton S, Kang D, Dellinger A, Gibb DR, Ford AQ, Massay H, Kepley CL, Scherle P, Keegan AD and Conrad DH (2011) A potential new target for asthma therapy: a disintegrin and metalloprotease 10 (ADAM10) involvement in murine experimental asthma. *Allergy* **66**: 1193-1200.

Meno K, Thorsted PB, Ipsen H, Kristensen O, Larsen JN, Spangfort MD, Gajhede M and Lund K (2005) The crystal structure of recombinant proDer p 1, a major house dust mite proteolytic allergen. *J Immunol* **175**: 3835-3845.

Meno K, Thorsted PB, Ipsen H, Kristensen O, Larsen JN, Spangfort MD, Gajhede M and Lund K (2006) The metal binding site of the major house dust mite allergen Der p 1. *J Allergy Clin Immunol* **118**: 971.

Millien VO, Lu W, Shaw J, Yuan X, Mak G, Roberts L, Song LZ, Knight JM, Creighton CJ, Luong A, Kheradmand F and Corry DB (2013) Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* **341**: 792-796.

Moghaddam AE, Gartlan KH, Kong L and Sattentau QJ (2011) Reactive carbonyls are a major Th2-inducing damage-associated molecular pattern generated by oxidative stress. *J Immunol* **187**: 1626-1633.

Muller AJ, Sharma MD, Chandler PR, Duhadaway JB, Everhart ME, Johnson BA, 3rd, Kahler DJ, Pihkala J, Soler AP, Munn DH, Prendergast GC and Mellor AL (2008) Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. *Proc Natl Acad Sci USA* **105**: 17073-17078.

Muller T, Vieira RP, Grimm M, Durk T, Cicko S, Zeiser R, Jakob T, Martin SF, Blumenthal B, Sorichter S, Ferrari D, Di Virgillio F and Idzko M (2011) A potential role for P_2X_7R in allergic airway inflammation in mice and humans. *Am J Respir Cell Mol Biol* **44**: 456-464.

Nadeem A, Siddiqui N, Alharbi NO, Alharbi MM and Imam F (2014) Acute glutathione depletion leads to enhancement of airway reactivity and inflammation via p38MAPK-iNOS pathway in allergic mice. *Int Immunopharmacol* **22**: 222-229.

Nagano O, Murakami D, Hartmann D, De Strooper B, Saftig P, Iwatsubo T, Nakajima M, Shinohara M and Saya H (2004) Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular $Ca^{(2+)}$ influx and PKC activation. *J Cell Biol* **165**: 893-902.

Nakamura T, Hirasawa Y, Takai T, Mitsuishi K, Okuda M, Kato T, Okumura K, Ikeda S and Ogawa H (2006) Reduction of skin barrier function by proteolytic activity of a recombinant house dust mite major allergen Der f 1. *J Invest Dermatol* **126**: 2719-2723.

Nathan AT, Peterson EA, Chakir J and Wills-Karp M (2009) Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *J Allergy Clin Immunology* **123**: 612-618.

Newton GK, Perrior TR, Jenkins K, Major MR, Key RE, Stewart MR, Firth-Clark S, Lloyd SM, Zhang J, Francis-Newton NJ, Richardson JP, Chen J, Lai P, Garrod DR and Robinson C (2014) The discovery of potent, selective, and reversible inhibitors of the house dust mite peptidase allergen Der p 1: an innovative approach to the treatment of allergic asthma. *J Med Chem* **57**: 9447-9462. doi: 10.1021/jm501102h and see online supplementary material https://pubs.acs.org/doi/suppl/10.1021/jm501102h/suppl_file/jm501102h_si_001.pdf

Ogawa T, Takai T, Kato T, Kikuchi Y, Niyonsaba F, Ikeda S, Okumura K and Ogawa H (2008) Upregulation of the release of granulocyte-macrophage colony-stimulating factor from keratinocytes stimulated with cysteine protease activity of recombinant major mite allergens, Der f 1 and Der p 1. *Int Arch Allergy Immunol* **146**: 27-35.

Oshio T, Sasaki Y, Funakoshi-Tago M, Aizu-Yokota E, Sonoda Y, Matsuoka H and Kasahara T (2009) Dermatophagoides farinae extract induces severe atopic dermatitis in NC/Nga mice, which is effectively suppressed by the administration of tacrolimus ointment. *Int Immunopharmacol* **9**: 403-411.

Peat JK, Tovey E, Toelle BG, Haby MM, Gray EJ, Mahmic A and Woolcock AJ (1996) House dust mite allergens. A major risk factor for childhood asthma in Australia. *Am J Respir Crit Care Med* **153**: 141-146.

Pichavant M, Charbonnier AS, Taront S, Brichet A, Wallaert B, Pestel J, Tonnel AB and Gosset P (2005) Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. *J Allergy Clin Immunol* **115**: 771-778.

Platts-Mills TA, Hayden ML, Chapman MD and Wilkins SR (1987) Seasonal variation in dust mite and grass-pollen allergens in dust from the houses of patients with asthma. *J Allergy Clin Immunol* **79**: 781-791.

Platts-Mills TA, Vervloet D, Thomas WR, Aalberse RC and Chapman MD (1997) Indoor allergens and asthma: report of the Third International Workshop. *J Allergy Clin Immunol* **100** (6 Pt 1): S2-24.

Platts-Mills TAE (2009) Indoor Allergens, in *Middleton's Allergy Principles and Practice* pp 539-555, Mosby Elsevier.

Post S, Heijink IH, Petersen AH, de Bruin HG, van Oosterhout AJ and Nawijn MC (2014) Protease-activated receptor-2 activation contributes to house dust mite-induced IgE responses in mice. *PloS one* **9**: e91206.

Post S, Rozeveld D, Jonker MR, Bischoff R, van Oosterhout AJ and Heijink IH (2015) ADAM10 mediates the house dust mite-induced release of chemokine ligand CCL20 by airway epithelium. *Allergy* **70**: 1545-1552.

Purello-D'Ambrosio F, Gangemi S, Merendino RA, Isola S, Puccinelli P, Parmiani S and Ricciardi L (2001) Prevention of new sensitizations in monosensitized subjects submitted to specific immunotherapy or not. A retrospective study. *Clin Exp Allergy* **31**: 1295-1302.

Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, Yamamoto M, Kensler TW, Tuder RM, Georas SN and Biswal S (2005) Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med* **202**: 47-59.

Reddy VB, Iuga AO, Shimada SG, LaMotte RH and Lerner EA (2008) Cowhage-evoked itch is mediated by a novel cysteine protease: a ligand of protease-activated receptors. *J Neurosci* **28**: 4331-4335.

Reddy VB and Lerner EA (2017) Activation of mas-related G-protein-coupled receptors by the house dust mite cysteine protease Der p1 provides a new mechanism linking allergy and inflammation. *J Biol Chem* **292**: 17399-17406.

Reiss K and Saftig P (2009) The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. *Sem Cell Dev Biology* **20**: 126-137.

Robays LJ, Maes T, Lebecque S, Lira SA, Kuziel WA, Brusselle GG, Joos GF and Vermaelen KV (2007) Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation. *J Immunol* **178:** 5305-5311.

Robinson C, Zhang J, Richardson J, Chen J, Francis N, Garrod D, Perrior T, Newton G, Jenkins K, Beevers R, Major M and Stewart M (2012) Cysteine peptidase inhibitor ADZ 51,457 attenuates the recruitment of dendritic cells and eosinophils to the airways of mice challenged with house dust mite allergens. *Am J Respir Crit Care Med* **185**: A2854.

Ryu JH, Yoo JY, Kim MJ, Hwang SG, Ahn KC, Ryu JC, Choi MK, Joo JH, Kim CH, Lee SN, Lee WJ, Kim J, Shin DM, Kweon MN, Bae YS and Yoon JH (2013) Distinct TLR-mediated pathways regulate house dust mite-induced allergic disease in the upper and lower airways. *J Allergy Clin Immunol* **131**: 549-561.

Sackesen C, Ercan H, Dizdar E, Soyer O, Gumus P, Tosun BN, Buyuktuncer Z, Karabulut E, Besler T and Kalayci O (2008) A comprehensive evaluation of the enzymatic and nonenzymatic antioxidant systems in childhood asthma. *J Allergy Clin Immunol* **122**: 78-85.

Schulman ES, Glaum MC, Post T, Wang Y, Raible DG, Mohanty J, Butterfield JH and Pelleg A (1999) ATP modulates anti-IgE-induced release of histamine from human lung mast cells. *Am J Respir Cell Mol Biol* **20**: 530-537.

Schulz O, Laing P, Sewell HF and Shakib F (1995) Der p I, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *Eur J Immunol* **25**: 3191-3194.

Shelley WB and Arthur RP (1955) Studies on cowhage (*Mucuna pruriens*) and its pruritogenic proteinase, mucunain. *AMA Arch Dermatol* **72**: 399-406.

Shi J, Luo Q, Chen F, Chen D, Xu G, Li H, (2010) Induction of IL-6 and IL-8 by house dust mite allergen Der p 1 in cultured human nasal epithelial cells is associated with PAR/PI3K/NFκB signaling. *ORL J Otorhinolaryngol Relat Spec* **72:** 256-265. doi: 10.1159/000312687

Silvestri M, Rossi GA, Cozzani S, Pulvirenti G and Fasce L (1999) Age-dependent tendency to become sensitized to other classes of aeroallergens in atopic asthmatic children. *Ann Allergy, Asthma & Immunol* **83**: 335-340.

Smith JM, Disney ME, Williams JD and Goels ZA (1969) Clinical significance of skin reactions to mite extracts in children with asthma. *Br Med J* **1** (659): 723-726.

Spiteri MA, Bianco A, Strange RC and Fryer AA (2000) Polymorphisms at the glutathione S-transferase, GSTP1 locus: a novel mechanism for susceptibility and development of atopic airway inflammation. *Allergy* **55 Suppl 61**: 15-20.

Sporik R, Holgate ST, Platts-Mills TA and Cogswell JJ (1990) Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study. *N Engl J Med* **323**: 502-507.

Squillace SP, Sporik RB, Rakes G, Couture N, Lawrence A, Merriam S, Zhang J and Platts-Mills AE (1997) Sensitization to dust mites as a dominant risk factor for asthma among adolescents living in central Virginia. Multiple regression analysis of a population-based study. *Am J Respir Crit Care Med* **156**: 1760-1764.

Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S (2001) Interaction of mite allergens Der p 3 and Der p 9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* **167:** 1014-1021.

Sweerus K, Lachowicz-Scroggins M, Gordon E, LaFemina M, Huang X, Parikh M, Kanegai C, Fahy JV and Frank JA (2017) Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma. *J Allergy Clinical Immunol* **139**: 72-81 e71.

Takahashi K, Aoki T, Kohmoto S, Nishimura H, Kodera Y, Matsushima A, Inada Y (1990) Activation of kallikrein-kinin system in human plasma with purified serine protease from *Dermatophagoides farinae*. *Int Arch Allergy Appl Immunol* **91:** 80-85.

Takai T, Kato T, Ota M, Yasueda H, Kuhara T, Okumura K and Ogawa H (2005) Recombinant Der p 1 and Der f 1 with in vitro enzymatic activity to cleave human CD23, CD25 and alpha1-antitrypsin, and in vivo IgE-eliciting activity in mice. *Int Arch Allergy Immunol* **137**: 194-200.

Takano K, Kojima T, Go M, Murata M, Ichimiya S, Himi T and Sawada N (2005) HLA-DR- and CD11c-positive dendritic cells penetrate beyond well-developed epithelial tight junctions in human nasal mucosa of allergic rhinitis. *J Histochem Cytochem* **53**: 611-619.

Tang H, Cao W, Kasturi SP, Ravindran R, Nakaya HI, Kundu K, Murthy N, Kepler TB, Malissen B and Pulendran B (2010) The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. *Nature Immunol* **11**: 608-617.

Terada M, Kelly EA and Jarjour NN (2004) Increased thrombin activity after allergen challenge: a potential link to airway remodeling? *Am J Respir Crit Care Med* **169**: 373-377.

Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP and Karp CL (2009) Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* **457**: 585-588.

Uchida M, Anderson EL, Squillace DL, Patil N, Maniak PJ, Iijima K, Kita H and O'Grady SM (2017) Oxidative stress serves as a key checkpoint for IL-33 release by airway epithelium. *Allergy* **72**: 1521-1531.

Utsch L, Folisi C, Akkerdaas JH, Logiantara A, van de Pol MA, van der Zee JS, Krop EJ, Lutter R, van Ree R and van Rijt LS (2015) Allergic sensitization is associated with inadequate antioxidant responses in mice and men. *Allergy* **70**: 1246-1258.

van Rijt LS, Utsch L, Lutter R and van Ree R (2017) Oxidative Stress: Promoter of Allergic Sensitization to Protease Allergens? *Int J Mol Sci* **18** (6).

Vroling AB, Jonker MJ, Breit TM, Fokkens WJ and van Drunen CM (2008a) Comparison of expression profiles induced by dust mite in airway epithelia reveals a common pathway. *Allergy* **63**: 461-467.

Vroling AB, Jonker MJ, Luiten S, Breit TM, Fokkens WJ and van Drunen CM (2008b) Primary nasal epithelium exposed to house dust mite extract shows activated expression in allergic individuals. *Am J Respir Cell Mol Biol* **38**: 293-299.

Wan H, Winton HL, Soeller C, Gruenert DC, Thompson PJ, Cannell MB, Stewart GA, Garrod DR and Robinson C (2000) Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clin Exp Allergy* **30**: 685-698.

Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ, Cannell MB, Stewart GA, Garrod DR and Robinson C (2001) The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. *Clin Exp Allergy* **31**: 279-294.

Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB and Robinson C (1999) Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* **104**: 123-133.

Weskamp G, Ford JW, Sturgill J, Martin S, Docherty AJ, Swendeman S, Broadway N, Hartmann D, Saftig P, Umland S, Sehara-Fujisawa A, Black RA, Ludwig A, Becherer JD, Conrad DH and Blobel CP (2006) ADAM10 is a principal 'sheddase' of the low-affinity immunoglobulin E receptor CD23. *Nature Immunol* **7**: 1293-1298.

Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN and Hammad H (2012) Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med* **209**: 1505-1517.

Williams MA, Rangasamy T, Bauer SM, Killedar S, Karp M, Kensler TW, Yamamoto M, Breysse P, Biswal S and Georas SN (2008) Disruption of the transcription factor Nrf2 promotes pro-oxidative dendritic cells that stimulate Th2-like immunoresponsiveness upon activation by ambient particulate matter. *J Immunol* **181**: 4545-4559.

Winton HL, Wan H, Cannell MB, Thompson PJ, Garrod DR, Stewart GA, Robinson C (1998) Class specific inhibition of house dust mite proteinases which cleave cell adhesion, induce cell death and which increase the permeability of lung epithelium. *Br J Pharmacol* **124**: 1048-1059.

Wolff HG and Goodell H (1952) Pain Sensations and Reactions. Bailliere, Tindall & Cox, London.

Yu L, Wang L and Chen S (2010) Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med* **14**: 2592-2603.

Zhang J, Chen J, Allen-Philbey K, Perera Baruhupolage C, Tachie-Menson T, Mangat SC, Garrod DR and Robinson C (2016) Innate generation of thrombin and intracellular oxidants in airway epithelium by allergen Der p 1. *J Allergy Clin Immunol* **138**: 1224-1227.

Zhang J, Chen J, Mangat SC, Perera Baruhupolage C, Garrod DR and Robinson C (2018) Pathways of airway oxidant formation by house dust mite allergens and viral RNA converge through myosin motors, pannexons and Toll-like receptor 4. *Immunity, Inflammation and Disease* **6:** 276-296. doi: 10.1002/iid3.216

Zhang J, Garrod DR and Robinson C (2009) Novel Der p 1 inhibitors attenuate house dust mite sensitization in mice. *Am J Crit Care Med* **179**: A4249.

Zhang J, Hamilton JM, Garrod DR and Robinson C (2007) Interactions between mature Der p 1 and its free prodomain indicate membership of a new family of C1 peptidases. *Allergy* **62**: 1302-1309.

Footnotes

Legends For Figures

Figure 1. **A,B** 3-Dimensional isosurface reconstruction of fluorescent antibody labelling of TJs (green) and desomosomes (red) in human airway epithelial cells. Normal cells are shown in **A**, note the contiguous rings of TJs compared to the punctate staining of desmosomes. **B** Two hours after exposure to HDM allergen, note the loss of TJ staining, whereas desmosomes remain intact. **C,D** Human airway epithelial cells labelled with NucBlue and MitoSOX red in the absence of HDM allergen stimulation (**C**) or following exposure to mixed HDM allergens showing generation of intracellular ROS (**D**). **E** Progress curves showing formation of rhodamine in calu-3 cells loaded with dihydrorhodamine under control conditions (circles), after stimulation by mixed HDM allergens (triangles) or after allergen stimulation in the presence of an allergen delivery inhibitor (squares). **F** Initial rates of ROS formation derived from rhodamine formation in **E**. **G** Silencing of TLR4 expression by siRNA attenuates intracellular ROS generation evoked by mixed HDM allergens in calu-3 cells. Data for transfected control cells (con) and nontransfected cells are shown for completeness. **H** Concentration-dependent inhibition of intracellular ROS formation by TAK-242 (an inhibitor of the association between TLR4 and the signaling adapter proteins TIRAP and TRAM) in calu-3 cells stimulated by mixed HDM allergens (circles) or Der p 1 (triangles). The bar chart depicts the rate of ROS formation (dihydrorhodamine oxidation) in unstimulated cells or in the presence of allergen activation. **I** Gene silencing of the production of the α-chain of fibrinogen inhibits ROS generation by mixed HDM allergens. For **E-H** all data are shown as mean ± s.e. with n= 4-8.   *P<0.001 *v* vehicle (veh); **P<0.001 *v* HDM; †P<0.05 *v* non-transfected HDM control. HDM 0.1, 1 refer to a natural mixture of allergens containing 0.1 μg mL^{-1} or 1 μg mL^{-1}, respectively. Further methodological details concerning the studies in panels **C-I** are available (Zhang et al., 2016; 2018) and these form the basis of previously unpublished or re-composited data shown here.

Figure 2. A The prothrombinase activity of Group 1 HDM allergens stimulates intracellular ROS

formation in airway epithelial cells through an ATP and ADAM 10-dependent regenerating cycle

initiated by the canonical cleavage of PAR-1 and PAR-4. The formation of endogenous ligands for

TLR4 play a key permissive role in this process because silencing of receptor expression or

prevention of TLR4 interactions with intracellular adapter proteins blunts ROS formation. This

signaling cycle receives convergent stimulatory inputs from viral RNA sensors (TLR3/MDA-5/RIG-

I/TLR7), upstream from the gating of pannexons by myosin motors. ROS provide transcriptional

regulation of gene expression through multiple mechanisms. Direct formation of thrombin by Group

1 HDM allergens (prothrombinases) appears to be functionally compartmentalized from the ADAM

10-dependent generation of prothrombin because PAR-1, PAR-4 and pannexin-1 are required for the

former. While the underlying reasons for this dichotomy are not established, it may reflect poor

accessibility of apically-generated ligands to TLR4 in a polarized epithelium. Figure revised and

updated with added mechanistic details from the scheme originally published by Zhang et al., 2018.

B Gene silencing production of the α-chain of fibrinogen in calu-3 airway epithelial cells inhibits ROS

generation activated by ligation of P_2X_7 (BzATP) or P_2Y_2 (MRS2768) receptors. *P<0.001 *vs* vehicle

(veh) controls; **P<0.001 *vs* stimulated cells (nontransfected and transfection control, con). **C** Gene

silencing of ADAM 10 or ADAM 8 attenuates ROS production by calu-3 cells stimulated by mixed

HDM allergens containing Der p 1 at 1 μg mL^{-1}). *P<0.001 *vs* veh controls; †P<0.001 *vs* stimulated

cells; ‡P<0.001 *vs* veh controls. Data in **B** and **C** are shown as mean ± s.e. (n = 8). The new data

shown in **B,C** used methods which have been published elsewhere (Zhang et al., 2016; 2018).

Figure 3. **A** Inhibitors of Der p 1 identified in early discovery research. Activity for the reversible

inhibitor aldehyde **1** is expressed as the inhibition constant, whereas data for the acyloxymethyl

ketones with irreversible action are expressed as second order inhibitor rate constants.

Measurements were performed as described in Newton et al., 2014 and its online supporting

information https://pubs.acs.org/doi/suppl/10.1021/jm501102h and

https://pubs.acs.org/doi/suppl/10.1021/jm501102h/suppl_file/jm501102h_si_001.pdf, using

(3S,6S,9S,12S,15S,18S)-1-(2-aminophenyl)-9-butyl-18-carbamoyl-15-(4-hydroxy-3-nitrobenzyl)-12-

(hydroxymethyl)-3-isopropyl-6-methyl-1,4,7,10,13,16-hexaoxo-2,5,8,11,14,17-hexaazaicosan-20-oic

acid as substrate. **B, C** Docking of compound **3** in the substrate binding groove of Der p 1.0105

shown, respectively, as surface representation and simplified stick view revealing hydrogen bonding

interactions with Derp 1. **D** General mechanism for irreversible inhibition of Der p 1 using

acyloxymethyl ketone **3** as exemplar.

Figure 4. **A** Transition from irreversible to reversible binding mode inhibitors of Der p 1 by

modification of the cysteine binding motif and identification of pyruvamide **7**. Inhibitor

characteristics were determined as disclosed in Newton et al., 2014 and its online supporting

information https://pubs.acs.org/doi/suppl/10.1021/jm501102h;

https://pubs.acs.org/doi/suppl/10.1021/jm501102h/suppl_file/jm501102h_si_001.pdf, using

(3S,6S,9S,12S,15S,18S)-1-(2-aminophenyl)-9-butyl-18-carbamoyl-15-(4-hydroxy-3-nitrobenzyl)-12-

(hydroxymethyl)-3-isopropyl-6-methyl-1,4,7,10,13,16-hexaoxo-2,5,8,11,14,17-hexaazaicosan-20-oic

acid as substrate.   **B** General mechanism for reversible inhibition of Der p 1 using amino ketone **5** as

exemplar.

Figure 5. Docking model of compound **7** bound to the active site of Der p 1.0105. Procedural details

are presented in Newton et al., 2014 https://pubs.acs.org/doi/suppl/10.1021/jm501102h.

Figure 6. **A**. Quaternary ammonium compound **8**. **B** Effect of a single aerosolised dose of **8** (18

nmol/kg) on the recruitment of eosinophils to the airways following aerosol challenge with mixed

HDM allergens in sensitized Brown Norway rats. The compound was administered 2 h prior to HDM

allergen by means of a Penn-Century aerosoliser. Data are displayed as means ± s.e. mean in groups

of 10 animals. [*]P<0.05 *v* vehicle (veh) challenge; [**]P<0.05 *v* HDM without test substance pre-

treatment. Full details of treatment protocols are provided in Newton et al., 2014 and its online

supporting information https://pubs.acs.org/doi/suppl/10.1021/jm501102h and

https://pubs.acs.org/doi/suppl/10.1021/jm501102h/suppl_file/jm501102h_si_001.pdf.

Figure 7. **A** Relationship between inhibitor polar surface area and log $D_{7.4}$ for a subset of

compounds used for the examination of *in vivo* efficacy. Symbols depict ionization state at pH7.4:

neutral (purple circles), positively charged basic center (orange squares) and positively-charged

quaternary ammonium (blue diamonds) **B** Percentage reduction in bronchoalveolar eosinophil

counts 48 h after challenge of non-sensitized rats with a natural mixture of HDM allergens following

a single aerosolized dose of test substance administered 2 h prior to allergen challenge. The

percentage reduction was calculated relative to vehicle-pretreated animals which underwent similar

HDM allergen challenge. Test substances were administered at a drug:target ratio of 50:1 with the

exception of compound **11** tested at 15:1. Data are mean ± s.e. mean with 10 animals per treatment

group. [*]P<0.001 compared to HDM challenge control. Column coloration depicts ionization state of

test substance as in **A**. **C** Structural information and potency data for compounds used in this study.

Full experimental details for chemical syntheses and biological studies are provided in Newton et al.,

2014 and its online supporting information https://pubs.acs.org/doi/suppl/10.1021/jm501102h and

https://pubs.acs.org/doi/suppl/10.1021/jm501102h/suppl_file/jm501102h_si_001.pdf

Figure 8. Further optimization of the P_4 (R) and P' groups produces compounds showing high target

potency and intrinsic selectivity over cathepsin B. Chemical syntheses and enzymatic activity assays

are described in Newton et al., 2014 and its online supporting information

https://pubs.acs.org/doi/suppl/10.1021/jm501102h and

https://pubs.acs.org/doi/suppl/10.1021/jm501102h/suppl_file/jm501102h_si_001.pdf.

Figure 9. Predicted effects profiles for ADIs based on preclinical discovery research models

(published and on-file data). **A** Effects of HDM exposure in the airways with a focus on innate

immune responses. **B** Example effects of HDM exposure in the presence of ADI drug with affected

pathways greyed out.

Table 1. Group denominations of HDM allergens, with exemplars, and their bioactivity profiles

based on bioinformatics predictions and/or empirical observations. The list omits storage mite

species exemplars of the sub-class Acari, although in many cases their allergens show the same

grouping and characteristics as the HDM allergens. Exemplar GenBank and UniProt accession codes

of the listed allergens are provided for supporting literature references and bioinformatics

substantiation (eg by hyperlink to simple modular architecture research tool 'SMART'), but codes for

isoallergenic variants are omitted for brevity.

HDM Allergen Group exemplars	MW (kDa)	Known or predicted bioactivity	GenBank/EMBL	UniProt
Group 1 eg Der p 1 Der f 1 Eur m 1 Der m 1 Blo t 1	25 (39 Blo t 1)	Cysteine protease	U11695 AB03496 AF047610 - AF277840	P08176 Q58A71 P25780 P16312 Q95PJ4
Group 2 eg Der p 2 Der f 2 Eur m 2 Blo t 2	14	NPC2 family; MD-2–related protein, lipid binding, binds LPS	AF276239 D10447 AF047613 AY288141	P49278 Q00855 Q9TZZ2 Q1M2P1
Group 3 eg Der p 3 Der f3 Eur m3 Blo t 3	25	Trypsin	U11719 D63858 AF047615 AY291323	P39675 P49275 O97370 A1KXI1

HDM Allergen Group exemplars	MW (kDa)	Known or predicted bioactivity	GenBank/EMBL	UniProt
Group 4 eg Der p 4 Der f 4 Eur m 4 Blo t 4	60	Amylase	AF144060 KM016832 AF144061 AY291324	Q9Y197 A0A089FLV3 Q9Y196 A1KXI2
Group 5 eg Der p 5 Blo t 5	14	Function unknown; ligand-binding protein?	S76337 U59102	P14004 O96870
Group 6 eg Der p 6 Der f 6 Blo t 6	25	Chymotrypsin	- AF125187 AY291325	P49277 P49276 A1KXI3
Group 7 eg Der p 7 Der f 7 Blo t 7	26-31	Bactericidal permeability-increasing like protein of unknown function; belongs to the juvenile hormone binding family of proteins found in insects; may have lipid binding properties	U37044 S80655 MF740745	P49273 Q26456 -
Group 8 eg Der p 8 Der f 8 Blo t 8	27	Glutathione *S*-transferase	S75286 KC305499 GQ398117	P46419 L7V2G7 C8CGT7
Group 9 eg Der p 9	29	Collagenase-like serine protease	AY211952	Q7Z163
Group 10 eg Der p 10	36	Tropomyosin	Y14906	O18416

HDM Allergen Group exemplars	MW (kDa)	Known or predicted bioactivity	GenBank/EMBL	UniProt
Der f 10 Blo t 10			D17682 EU106615	Q23939 A7XZI4
Group 11 eg Der p 11 Der f 11 Blo t 11	103	Paramyosin	AY189697 AF352244 AF525465	Q6Y2F9 Q967Z0 Q8MUF6
Group 12 eg Blo t 12	14	Possible chitinase; shows homology with Der f 15 due to chitin-binding domain	U27479	Q17282
Group 13 eg Der p 13 Der f 13 Blo t 13	15	Fatty acid–binding protein	HM560018 AY283293 U58106	E0A8N8 Q1M2P5 Q17284
Group 14 eg Der p 14 Der f 14 Eur m14	177	Vitellogenin or lipophorin	AF373221 D17686 AF149827	Q8N0N0 Q94507 Q9U785
Group 15 eg Der p 15 Der f 15	98, 109[1]	GH18 superfamily chitinase; shows homology with mite group 18.	DQ078741 AF178772	Q4JK69 Q9U6R7
Group 16 eg Der f 16	53	Gelsolin/villin	AF465625	Q8MVU3
Group 17 eg Der f 17	30	Calcium-binding protein	-	-

---

[1] Glycosylated forms of 63 kDa protein predicted by DNA sequence

HDM Allergen Group exemplars	MW (kDa)	Known or predicted bioactivity	GenBank/EMBL	UniProt
Group 18 eg Der p 18 Der f 18	60	GH18 superfamily chitinase; homologous with group 15	DQ078739 AY093656	Q4JK71 Q86R84
Group 19 eg Blo t 19	7	Antimicrobial peptide homology	KF771884	W5RZ24
Group 20 eg Der p 20 Der f 20	40	Arginine kinase	EU684970 KM009994.1	B2ZSY4 -
Group 21 eg Der p 21 Der f 21 Blo t 21	14	Function unknown; shows homology with group 5 allergens	DQ354124 KF732965.1 DQ788679	Q2L7C5 B2GM84 A7IZF1
Group 22 eg Der f 22	17	Shows homology with group 2 mite allergen; belongs to MD-2-related lipid recognition (ML) domain family; implicated in lipid binding	DQ643992	A5X5X4
Group 23 eg Der p 23 Der f 23	14-19	Unknown function; shows homology with peritrophin-A domain and contains a chitin-binding domain	EU414751.1 KU166910	L7N6F8 -
Group 24 eg Der p 24 Der f 24	13	Ubiquinol-cytochrome c reductase binding protein-like protein	KP893174 KC669700	A0A0K2GUJ4 M9RZ95
Group 25 eg Der f 25	34	Triosephosphate isomerase	KC305500.1	L7UZA7

HDM Allergen Group exemplars	MW (kDa)	Known or predicted bioactivity	GenBank/EMBL	UniProt
Group 26 eg Der f 26	18	Myosin alkali light chain	KM009996[2]	A0A088SAG5[3]
Group 27 eg Der f 27	48	Serpin – trypsin inhibitor	AIO08851	-
Group 28 eg Der f 28	70	Heat shock protein	KC305502	L7V065
Group 29 eg Der f 29	16	Peptidyl-prolyl *cis-trans* isomerase (cyclophilin)	AY283280.1	A1KXG2
Group 30 eg Der f 30	16	Ferritin	KC305503	L7UZ91
Group 31 eg Der f 31	15	Cofilin, actin binding protein	KM010014	A0A088SAY1
Group 32 eg Der f 32	35	Secreted inorganic pyrophosphatase	KM009993	A0A088SCP3
Group 33 eg Der f 33	52	Alpha-tubulin	KM010005	A0A088SV41
Group 34 eg Der f 34	16	Enamine/imine deaminase	LC120618	A0A1J1DL12
Group 35 eg Der f 35	14	MD-2 related protein homologous with group 2	LC175222	A0A1W7HBY9
Group 36 eg Der p 36 Der f 36	42	Function unknown; contains a C-terminal C2 domain (pfam00168) associated with signal transduction	KY465507 KY465506	A0A291KZD3 A0A291KZC2
Group 37 eg Der p 37	30	Peritrophic-like protein domain	MG520330[4]	-

[2] Note that GenBank/EBI and UniProt incorrectly designate this as a group 30 allergen. Definitively, it is a group 26 allergen as designated by the Allergen Nomenclature Sub-Committee of the World Health Organisation and International Union of Immunological Societies. Please refer to http://www.allergen.org/viewallergen.php?aid=815 for further information.

[3] See footnote 2.

[4] Accession not yet published.

Table 2. Allergy-related **biologic** actions and effects evoked by HDM protease allergens.

Sphere of Action	Action or Effect	Allergen or extract	References
Mucosal defence	Cleavage of tight junctions/reduced epithelial barrier function/allergen delivery	HDM fecal pellet extract, Der p 1, Der f 1, Der p 3, Der p 6	Herbert *et al.,* 1990, 1995; Winton *et al.,* 1998; Wan *et al.,* 1999, 2000, 2001; Nakamura *et al.,* 2006; ADI programme - data on file
	Disruption of epithelial adherens junctions	Der p 1 (directly and indirectly via ADAM 10)	Wan *et al.,* 1999; ADI programme - data on file
	Inactivation of airway antiproteases	Der p 1	Kalsheker *et al.,* 1996; Brown *et al.,* 2003
	Epithelial-mesenchymal transition	HDM extract	Heijink *et al.,* 2010a; Frisella *et al.,* 2011
	Inactivation of surfactant proteins	Der p 1, Der f 1	Deb *et al.,* 2007
	IgE-independent mast cell stimulation	Der p 1, HDM extract	Machado *et al.,* 1996
	Activation of NLRP3 inflammasome and apoptosis	HDM extract, Der p 1	Winton *et al.,* 1998; Baker *et al.,* 2003; Frisella *et al.,* 2011
Cell signaling	Prothrombinase activity	HDM fecal pellet extract, Der p 1	Zhang *et al.,* 2016, 2018
	PAR-1 and PAR-4 activation	Der p 1 (indirectly as prothrombinase)	Zhang *et al.,* 2016; ADI programme - data on file
	PAR-2 activation	HDM fecal pellet extract, Der p 1, Der p 3, Der p 9	Asokananthan *et al.,* 2002; Jeong *et al.,* 2008; Kato *et al.,* 2009; Post *et al.,* 2014;

Sphere of Action	Action or Effect	Allergen or extract	References
			Zhang *et al.,* 2016; Reddy & Lerner, 2017; Sun *et al.,* 2001; Daan de Boer *et al.,* 2014; Cho *et al.,* 2012
	PAR-1 inactivation	Der p 1	Asokananthan *et al.,* 2002
	PAR-2 inactivation	Der p 1	Adam *et al.,* 2006; Kato *et al.,* 2009
	PAR-1 and PAR-2 upregulated expression	Der p 1	Shi *et al.,* 2010
	Mas-related G-protein-coupled receptor activation	Der p 1	Reddy & Lerner, 2017
	EGFR activation in epithelial cells	Der p 1 (indirectly as prothrombinase)	Zhang *et al.,* 2016, 2018; ADI programme - data on file
	Pannexon gating in epithelial cells	Der p 1 (indirectly as prothrombinase)	Zhang *et al.,* 2016, 2018; ADI programme - data on file
	ATP release from epithelial cells	Der p 1 (indirectly as prothrombinase)	Zhang *et al.,* 2016, 2018; ADI programme - data on file
	ADAM 17 activation in epithelial cells	Der p 1 (indirectly as prothrombinase)	Chen *et al.,* 2017; Zhang *et al.,* 2018
	ADAM 10 activation in epithelial cells	Der p 1 (indirectly as prothrombinase)	Chen *et al.,* 2017; ADI programme - data on file
	ADAM 8 activation in epithelial cells	Der p 1 (indirectly as prothrombinase)	ADI programme - data on file

| Sphere of Action	Action or Effect	Allergen or extract	References
	Cleavage of low affinity IgE receptor (CD23)	Der p 1 (directly and indirectly via ADAM 10)	Schulz *et al.,* 1995; ADI programme - data on file
	Activation of endogenous interstitial prothrombinase	HDM extract (due to Der p 1)	Zhang *et al.,* 2016; ADI programme - data on file
Regulation of gene expression and exploitation of antioxidant deficits	ROS generation	Intracellular ROS by HDM fecal pellet extract, Der p 1 in epithelial cells. Der f 1 in neutrophils	Fukunaga *et al.,* 2011; Zhang *et al.,* 2016, 2018; Chen *et al.,* 2017
Immune activation and allergic polarization	Breaking of immune tolerance	HDM extract, Der p 1	Gough *et al.,* 1999, 2001, 2003; ADI programme - data on file
	Suppression of indoleamine 2,3-dioxygenase	Der p 1	Maneechotesuwan *et al.,* 2009
	TLR4 ligation	HDM extract, Der p 1 (indirectly as prothrombinase)	Zhang *et al.,* 2018; ADI programme - data on file
	Bioactivating cleavage of IL-33	Der p 1	Cayrol *et al.,* 2018
	Cytokine/chemokine expression and release (eg IL-33, TSLP, CCL-2, CCL-20, GM-CSF, IL-8, IL-13 etc)	HDM extract, Der p 1, Der p 3, Der p 6, Der p 9	King *et al.,* 1998; Pichavant *et al.,* 2005; Adam *et al.,* 2006; Kaufmann *et al.,* 2006; Ogawa *et al.,* 2008; Arlian *et al.,* 2011; Kato *et al.,* 2009; Shi *et al.,* 2010; ADI programme -data on file

Sphere of Action	Action or Effect	Allergen or extract	References
	Cleavage of IL-2R (CD25)	Der p 1	Schulz *et al.,* 1998
Antigen presenting cell recruitment and activation	Dendritic antigen presenting cell recruitment	HDM extract, Der p 1	Pichavant *et al.,* 2005; Robinson *et al.,* 2012; ADI Programme – data on file
	Cleavage of DC-SIGN/DC-SIGNR	Der p 1	Furmonaviciene *et al.,* 2007
Interaction with viral RNA sensors	Transductional convergence with signaling from TLR3 and TLR7	HDM fecal pellet extract, Der p 1	Zhang *et al.,* 2018; ADI Programme – data on file
Effector mechanisms	Leukocyte recruitment	Der p 1, HDM extract	Robinson *et al.,* 2012; Newton *et al.,* 2014; ADI programme-data on file
	Kinin generation from low and high molecular weight kininogens	Der f 3	Takahashi *et al.,* 1990; Maruo *et al.,* 1991; Maruo *et al.,* 1993
	Decreased cysteine protease inhibitory activity of kininogens	Der f 3	Maruo *et al.,* 1993
	Anaphylatoxin generation	Der f 3	Maruo *et al.,* 1997
	IgE-independent 'pseudoallergic' bronchoconstriction	Der p 1, HDM extract	ADI programme - data on file

**Table 3.** Potential strategic positioning of ADIs in allergic asthma versus alternative approaches.

Intervention	Examples	Remarks
Allergen Delivery Inhibitors	Group 1 HDM protease allergen inhibitors	• Small molecule • Inhaled delivery to target organ • Attractive profile (nonhuman target, extracellular action) with disease modification • Root cause-directed • Potential to prevent exacerbations • Mechanistic differentiation • Potential addition to standard of care at all levels of disease severity • Potentially prescribable at non-specialist level • Low cost of goods compared to biologics • Exploitable as combination therapy and/or other conditions
Alternative small molecules in discovery/development	Downstream signal transduction and effector mechanisms – various targets	• Uncertain potential to surpass or add significantly to inhaled steroids • Multiply redundant effector pathways are confounders of efficacy • Potentially prescribable at non-specialist level • Low cost of goods compared to biologics
Biologics – approved or in development	Anti-cytokine mAbs Anti-receptor mAbs	• High cost of goods

| Intervention	Examples	Remarks
	Anti-IgE mAbs Anti-IgE vaccine (pAb)	• Mainly applicable to severe disease only • Inconvenient to use • Multiply redundant pathways are confounders of efficacy • High safety barriers (esp. IgE vaccine) • Specialist use only • Patchy targeting of innate pathways
Immunotherapy	Allergen-specific immunotherapy Immune deviation	• Moderately high cost of goods • Can be inconvenient to use • Specialists must be involved in GP use • Chronic safety of immune deviation is unproven • Poor targeting of key innate pathways

Figure 1

Figure 2

Figure 3

A

1   Reversible
$K_i = 14$ nM

2   Irreversible
$K_{obs}/[I] = 1.6 \times 10^7$ M^{-1} s^{-1}

3   Irreversible
$K_{obs}/[I] = 6.8 \times 10^7$ M^{-1} s^{-1}

4   Irreversible
$K_{obs}/[I] = 2.1 \times 10^5$ M^{-1} s^{-1}

B

C

D

Figure 4

A

4   Irreversible
$K_{obs}/[I] = 2.1 \times 10^5\ M^{-1}\ s^{-1}$

Change of mechanism

5   Reversible
$IC_{50} = 65\ nM$

No further potential for improvement?

6   Reversible
$IC_{50} = 4\ nM$

7   Reversible
$IC_{50} = 17\ nM$

B

Cys^{114}—S⁻

SCys

Figure 5

Figure 6

A

8
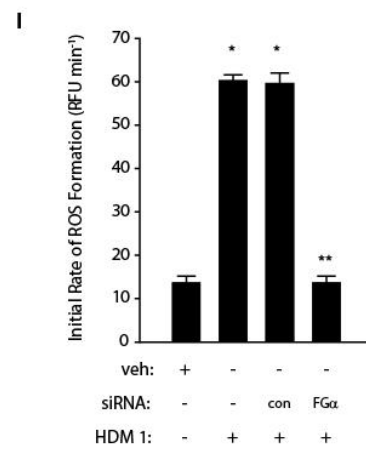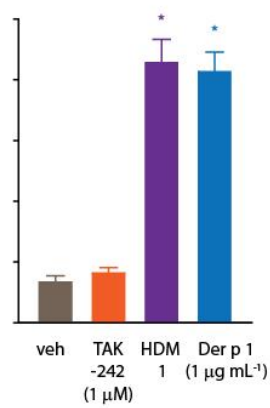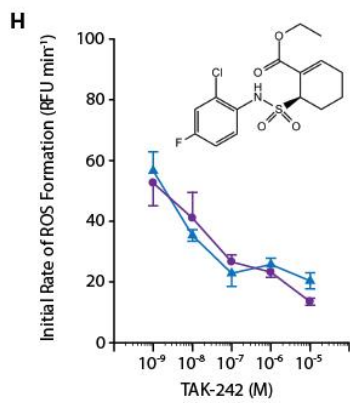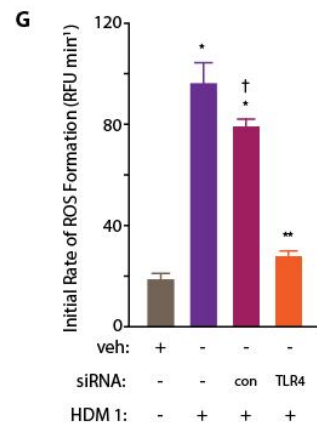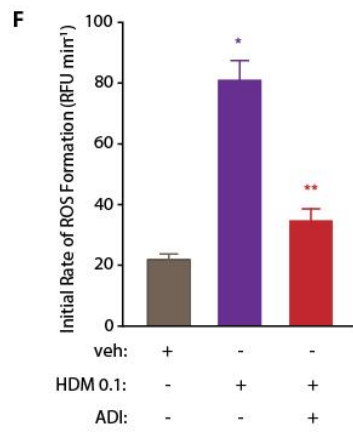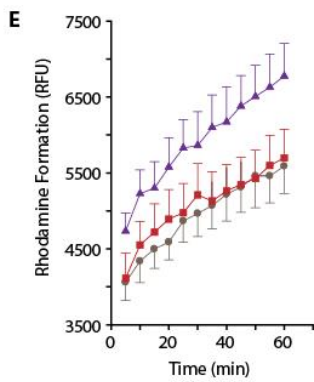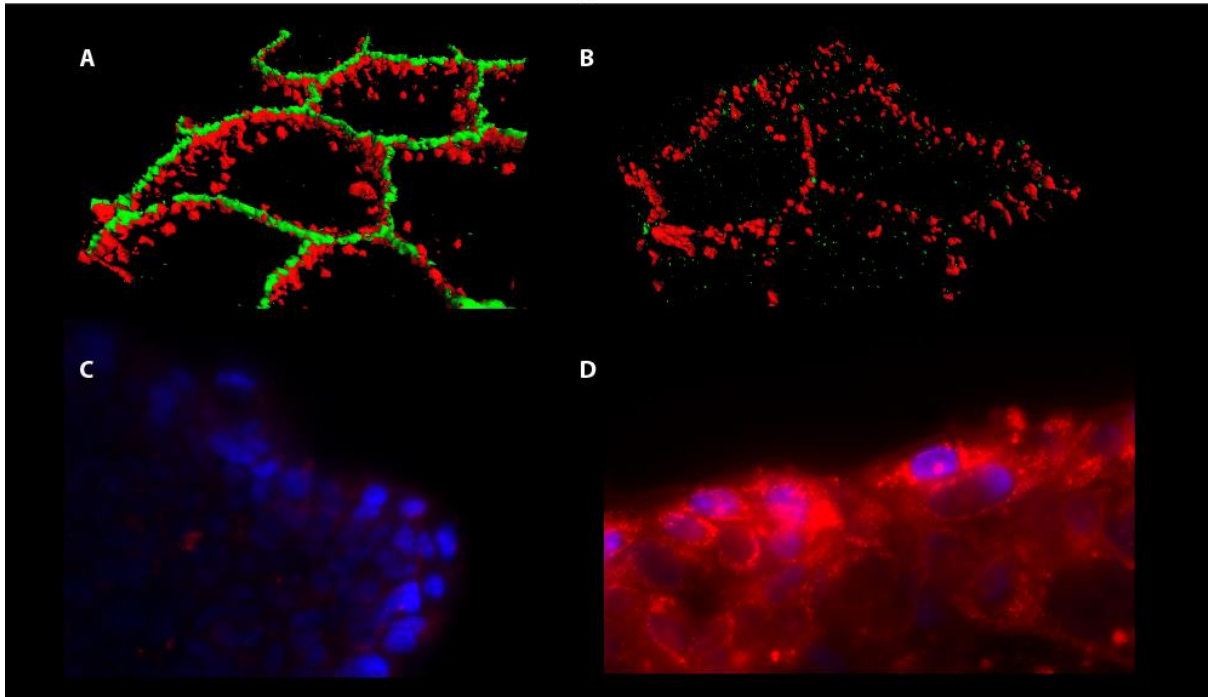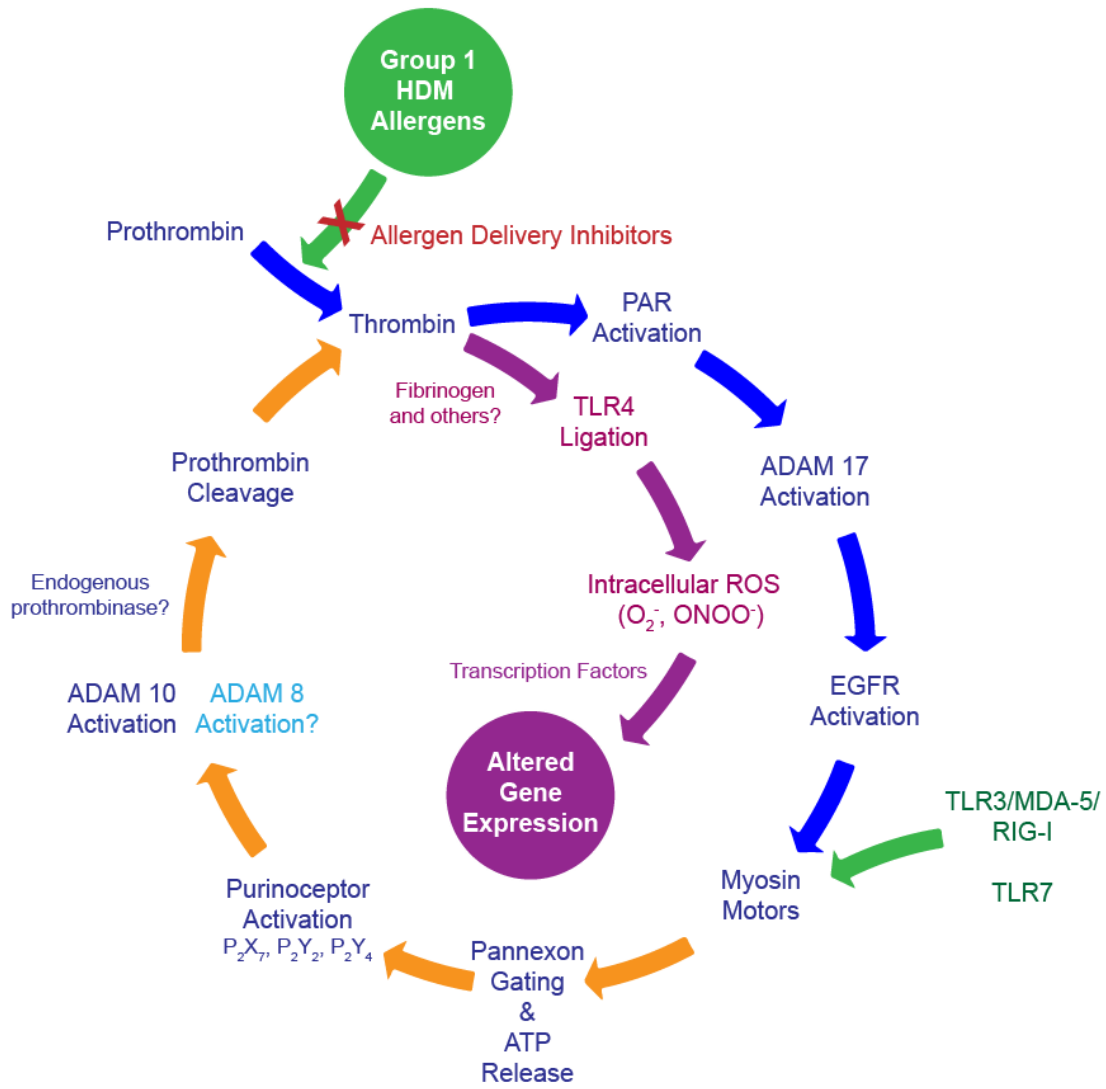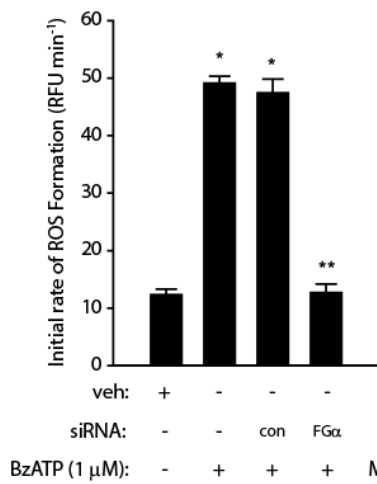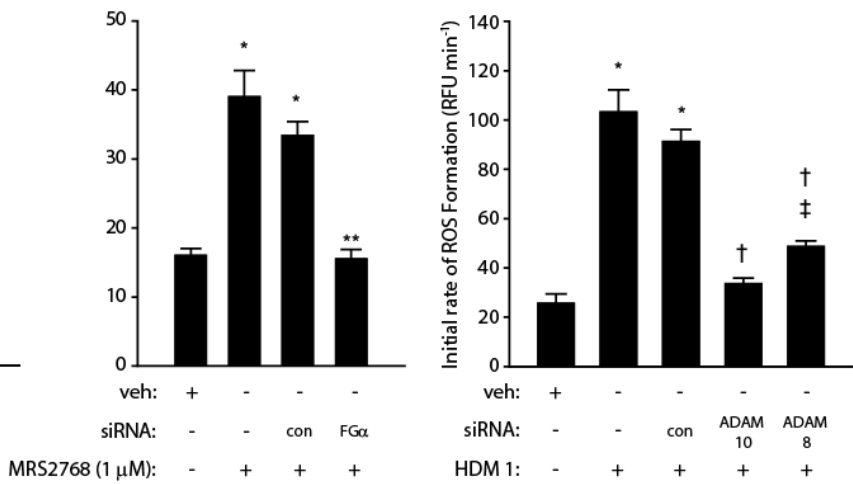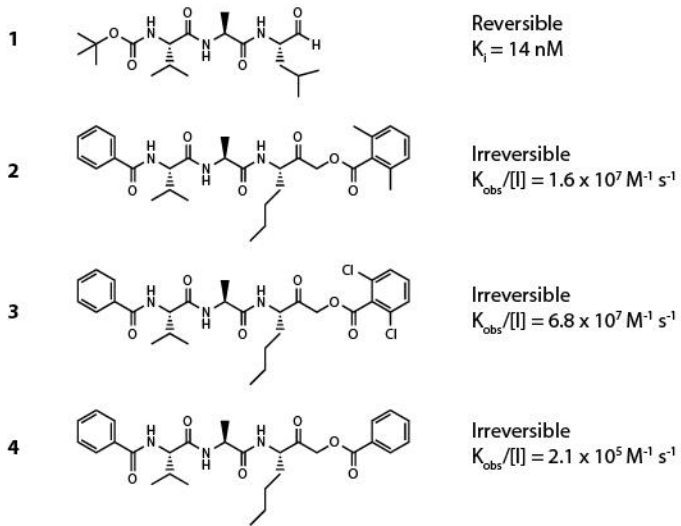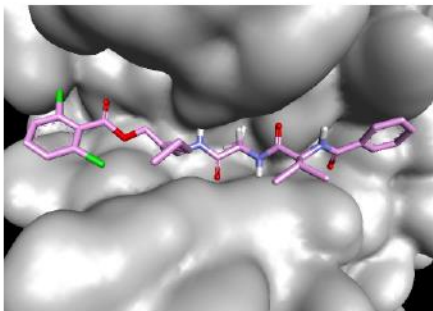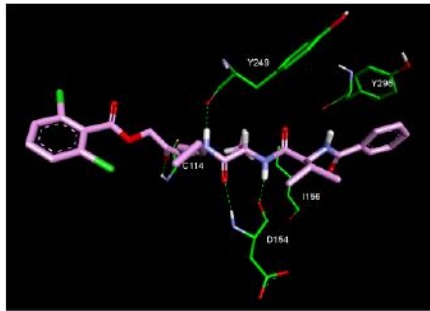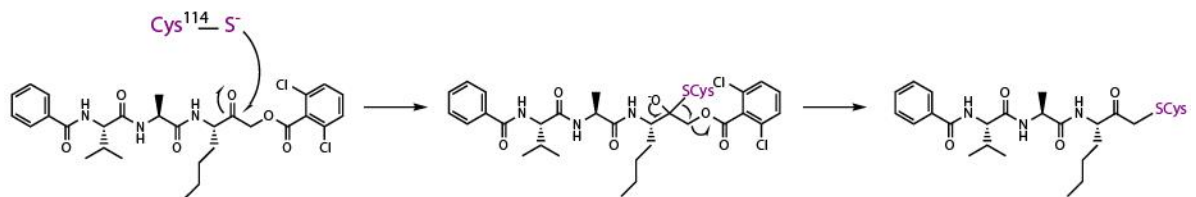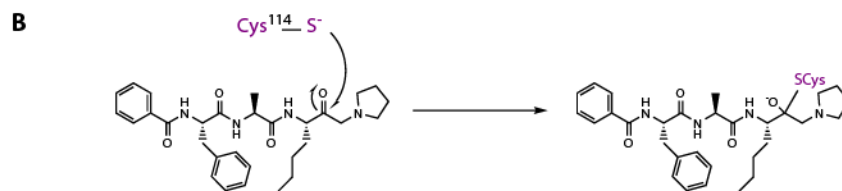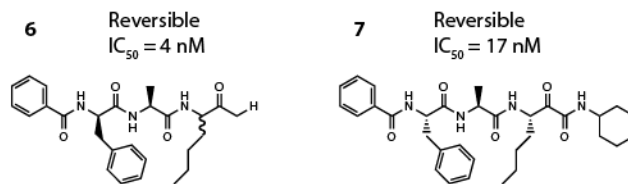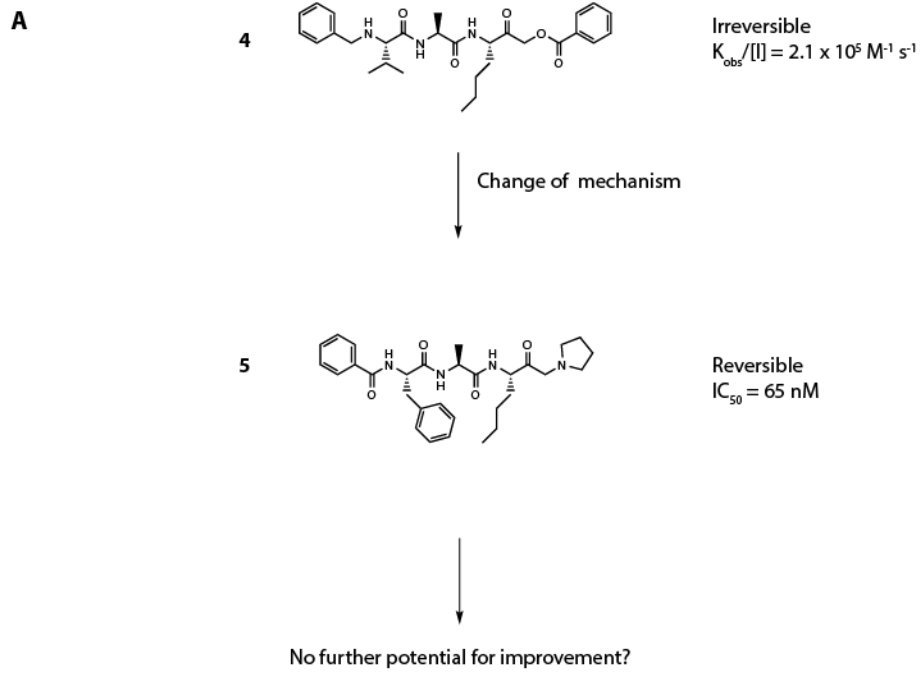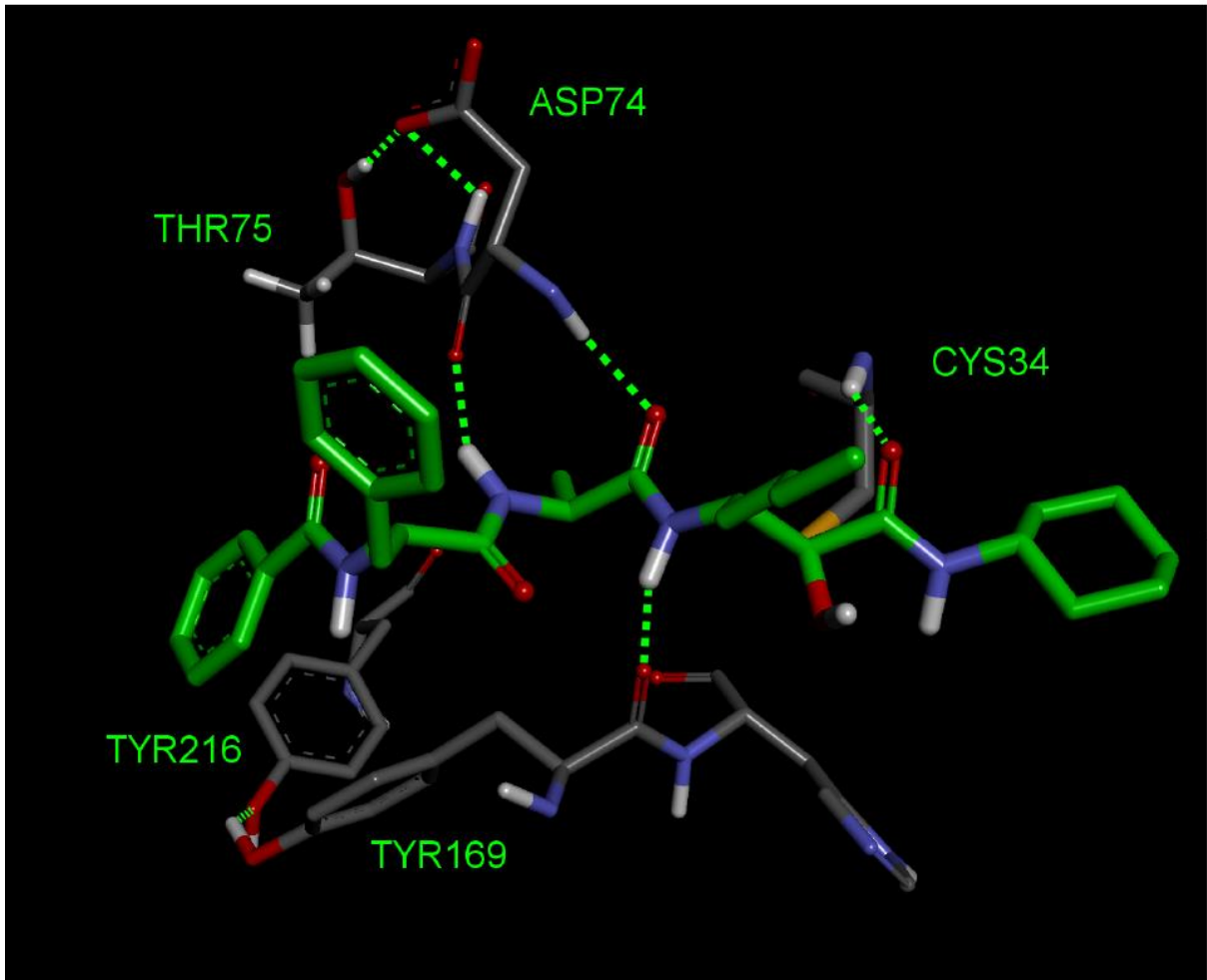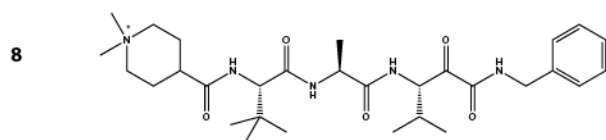
Der p 1 IC_{50} = 6 nM

Cathepsin B IC_{50} = 274 nM

Log $D_{7.4}$ = -0.9

B
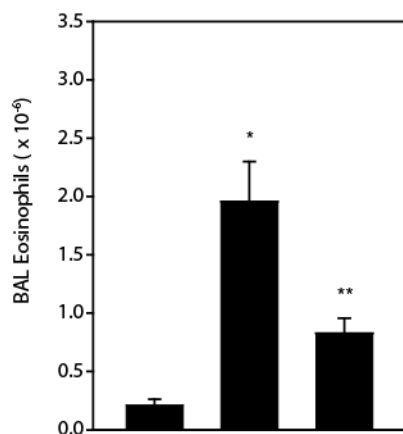
Sensitisation:	+	+	+
Pre-treatment:	-	veh	cpd 8
Challenge:	veh	HDM	HDM
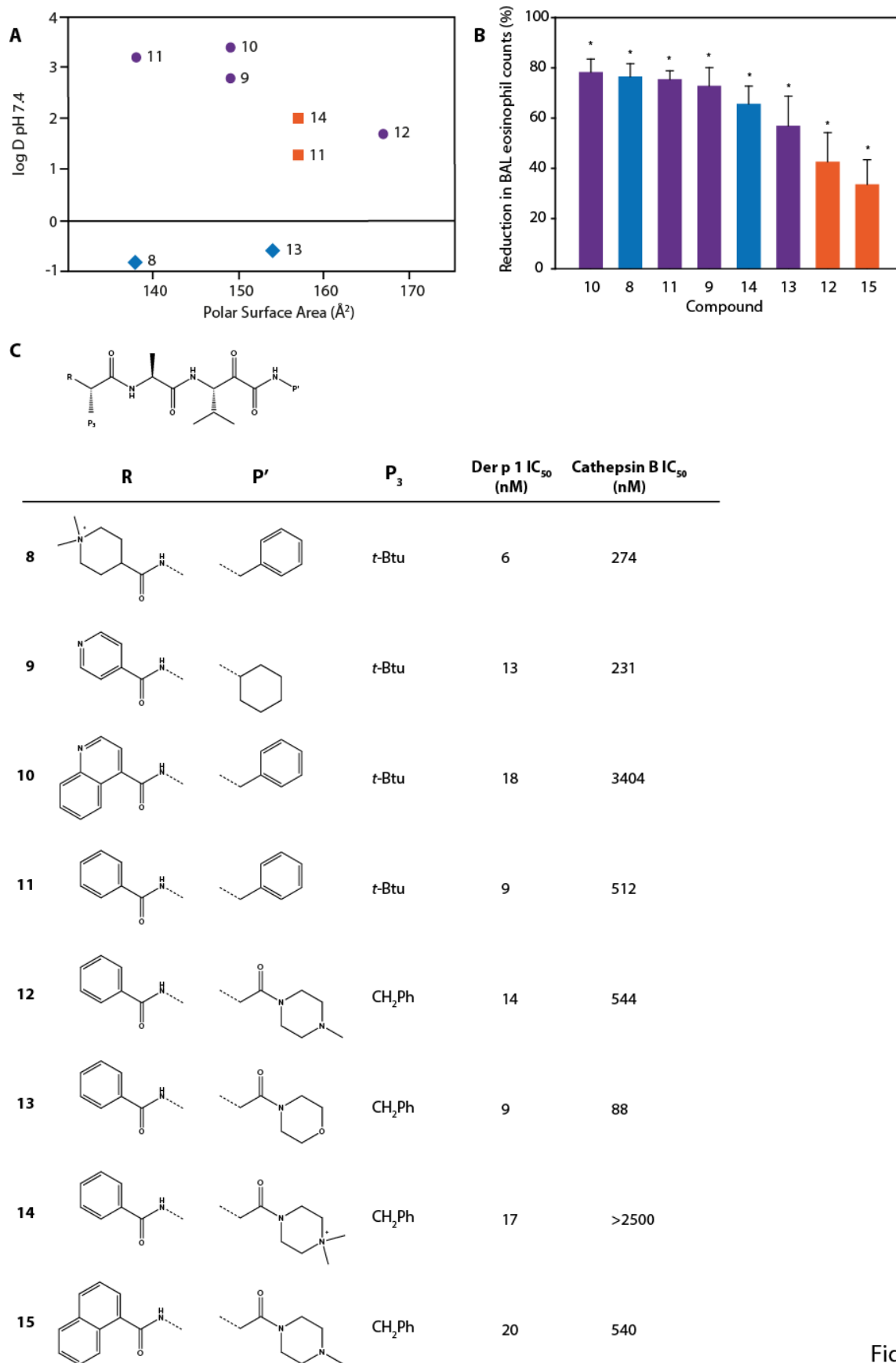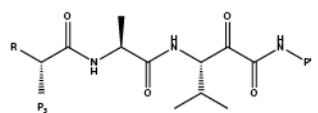
Figure 7

Figure 8
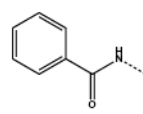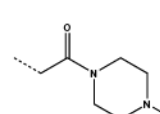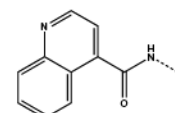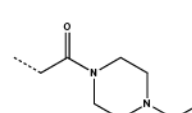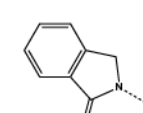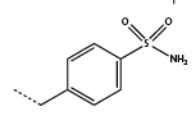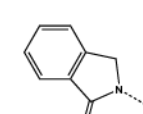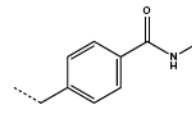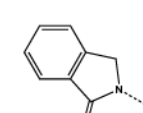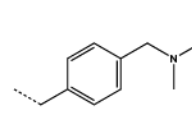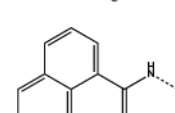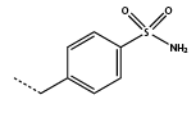
	R	P′	P₃	Der p 1 IC$_{50}$ (nM)	Cathepsin B IC$_{50}$ (nM)
16			Adamantan-1-yl	5	>2500
17			t-Btu	3	>2500
18			t-Btu	3	3049
19			t-Btu	4	3571
20			t-Btu	4	2847
21			t-Btu	4	532

Figure 9

A

Inhaled allergens

- ● Der p I
- ● Bystander allergen

Tight junctions opened by Der p1

Chemokines e.g. CCL-2 CCL-20

Allergen delivery

Inflammatory cell recruitment

Dendritic cell recruitment

Dendritic cell recruitment

CD252 induction

Blood vessel

● ST2 receptor
● OX4OL/CD252

GMCSF IL-25 TSLP IL-33

Basophil

Eosinophil

T cell

Cell migration and activation with allergy bias

Mast cell

IL-5 IL-6 IL-13

iH2 nuocytes

B

Inhaled allergens

- ● Der p 1
- ● Bystander allergen
- ○ ADI

Tight junctions

Chemokines e.g. CCL-2 CCL-20

Allergen delivery

Inflammatory cell recruitment

Dendritic cell recruitment

Dendritic cell recruitment

CD252 induction

Blood vessel

● ST2 receptor
● OX4OL/CD252

GMCSF IL-25 TSLP IL-33

Eosinophil

Basophil

T cell

Cell migration and activation with allergy bias

Mast cell

IL-5 IL-6 IL-13

iH2 nuocytes