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## Proteases in Helminth- and Allergen-Induced Inflammatory Responses

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### Abstract

Proteolytic activity is a central biochemical property that endows molecules with intrinsic allergenicity. Thus, the cysteine protease of dust mite, Der p1, the aspartic protease of cockroach, Bla g 2, the serine protease of *Aspergillus fumigatus* and the bacterial subtilisins are all major allergenic molecules responsible for the increase in asthma and atopic conditions worldwide. These proteases induce Th2-driven inflammatory responses in the airways by disrupting the epithelial cell junctions so that these, and other molecules, gain access to, and alter the function of, underlying cells of the innate immune system (dendritic cells, mast cells, basophils and macrophages) and B and T cells. Helminth parasites secrete proteases to gain entry into their hosts, and to feed on and migrate through tissues. Their action leads to tissue damage and the activation of inflammatory responses dominated by elevated IgE, eosinophilia and Th2 cells, much like allergic responses. In certain situations, such as in acute infections (especially with zoonotic helminths), proteases secreted by helminths may sensitise individuals to allergens. However, the anti-inflammatory responses observed in chronic helminthiasis, involving IL-10 and TGF $\beta$ , that are primarily responsible for controlling immune-mediated damage to the host that is initiated by secreted proteases, coincidentally protects against similar inflammatory damage by allergens.

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What makes an antigen an allergen and how do these induce allergic reactions? The answers to these questions are critical to the understanding of the nature of molecules that initiate and sustain allergic inflammation and to the design of strategies to prevent this condition. Our current assessment of the potential for a protein to be allergenic focuses on three main aspects: (1) inherent allergenicity, which refers to the likelihood of an immunoglobulin E (IgE)

antibody or T-cell response developing to a particular molecule; (2) cross-reactivity, which refers to the ability of an IgE clonotype or a T cell clone previously induced by one allergen to react with another molecule, and (3) clinical symptoms, i.e. the manifestation of the allergic response, such as eczema, rhinitis, conjunctivitis, asthma and anaphylaxis [1].

Clinical and functional data have been extensively reported for common allergens for many years. More recently, in an attempt to identify the structural signatures of these allergens, a number of databases have been generated that contain molecular information, including primary sequence, secondary and tertiary structure and T cell/IgE binding epitopes, for known allergens (table 1). Researchers can use basic bioinformatic tools to interrogate these databases for common 'allergen' motifs and to predict the potential allergenicity of unknown or query molecules, much like the standard 'blasting' of general protein databases. Indeed, the present recommendation for the prediction of allergenicity and allergic cross reactivity sanctioned by the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) is a scheme based on an assessment of sequence similarity between the query protein and known allergens. Thus, the query protein is considered potentially allergenic if it has (a) >35% sequence similarity over an 80 amino acid segment, or (b) an identity of six or more contiguous amino acids when compared with established allergens [1].

While sequence comparisons may reveal similarities in contiguous residues (linear epitopes) they clearly fail to disclose structural characteristics of proteins that may be essential in the induction of IgE or in the determination of IgE cross-reactivity. Since surface exposure is important for certain epitopes of allergens to induce IgE, structural analysis tools are also required to identify secondary or tertiary structures that may impact on allergenicity. However, extensive analyses of allergenic proteins for which the protein folds are known, or can be predicted on the basis of homology, indicate that allergens have few, if any, structural features in common [1]. Nevertheless, most allergens are grouped into a small number of rather general structural classes: (1) antiparallel  $\beta$ -strands; (2) antiparallel  $\beta$ -sheets associated with one or more  $\alpha$ -helices; (3)  $\alpha + \beta$  structures in which the  $\alpha$ - and  $\beta$ -structural elements are not intimately associated; (4)  $\alpha$ -helical, and (5) parallel  $\beta$ -strands in combination with an  $\alpha$ -helix linking the two strands (allergens with these structural arrangements seem to be underrepresented) [1].

The lack of a characteristic structural feature between allergens indicates that factors other than sequence similarity and 3-dimensional conformation are a prerequisite for a protein to exhibit allergenicity. The accumulation in the databases of allergens that possess proteolytic activity may be leading to the conclusion that this is one central biochemical property that confers a protein

**Table 1.** Allergenic proteases from various sources

| Allergen        | Species                               | Biochemical nature | Accession No. |
|-----------------|---------------------------------------|--------------------|---------------|
| <i>Mites</i>    |                                       |                    |               |
| Der p 1         | <i>Dermatophagoides pteronyssinus</i> | cysteine protease  | U11695        |
| Der p 9         |                                       | serine protease    | AF409110      |
| Der f 1         | <i>Dermatophagoides farinae</i>       | cysteine protease  | X65196        |
| Blo t 1         | <i>Blomia tropicalis</i>              | cysteine protease  | AF277840      |
| Eur m 1         | <i>Euroglyphus maynei</i>             | cysteine protease  | X60073        |
| <i>Animals</i>  |                                       |                    |               |
| Fel d 1         | <i>Felis domesticus</i>               | protease           | X62477        |
| <i>Fungi</i>    |                                       |                    |               |
| Asp f 5         | <i>Aspergillus fumigatus</i>          | metalloprotease    | Z30424        |
| Asp f 10        |                                       | aspartic protease  | X85092        |
| Asp f 13        |                                       | serine protease    | M99420        |
| Asp f 18        |                                       | serine protease    | P87184        |
| Asp fl 1        | <i>Aspergillus flavus</i>             | serine protease    | AF137272      |
| Asp fl 13       |                                       | serine protease    |               |
| Asp n 18        | <i>Aspergillus niger</i>              | serine protease    | AAA32702      |
| Asp o 13        | <i>Aspergillus oryzae</i>             | serine protease    | CAA35594      |
| Cur l 1         | <i>Curvularia lunata</i>              | serine protease    |               |
| Epi p 1         | <i>Epicoccum purpurascens</i>         | serine protease    | P83340        |
| Pen ch 13       | <i>Penicillium chrysogenum</i>        | serine protease    | AAF23726      |
| Pen ch 18       |                                       | serine protease    | AAF71379      |
| Pen c 1         | <i>Penicillium citrinum</i>           | serine protease    |               |
| Pen c 2         |                                       | serine protease    | AF098517      |
| Pen c 13        |                                       | serine protease    |               |
| Pen o 18        | <i>Penicillium oxalicum</i>           | serine protease    | AAG44478      |
| Tri r 4         | <i>Trichophyton rubrum</i>            | serine protease    | AF282514      |
| <i>Bacteria</i> |                                       |                    |               |
| Alcalase        | <i>Bacillus subtilis</i>              | serine protease    |               |
| Esperase        | <i>Bacillus licheniformis</i>         | serine protease    |               |
| Savinase        |                                       | serine protease    |               |
| <i>Insects</i>  |                                       |                    |               |
| Api m 7         | <i>Apis mellifera</i>                 | serine protease    | AY127579      |
| Bom p 4         | <i>Bombus pennsylvanicus</i>          | protease           | A56338        |
| Bla g 2         | <i>Blattella germanica</i>            | aspartic protease  | U28863        |
| Pol d 4         | <i>Polistes dominulus</i>             | serine protease    | P81656        |
| <i>Foods</i>    |                                       |                    |               |
| Act c 1         | <i>Actinidia chinensis</i>            | cysteine protease  | P16785        |
| Ana c 1         | <i>Ananas comosus</i>                 | cysteine protease  | AAK54835      |
| Cuc m 1         | <i>Cucumis melo</i>                   | serine protease    | D32206        |
| Car p 1         | <i>Carica papaya</i>                  | cysteine protease  | AAB02650      |
| Gly m 1         | <i>Glycine max</i>                    | thiol protease     | P24337        |

Compiled from allergen-specific data sources: [www.allergen.org](http://www.allergen.org), [www.allergenonline.com](http://www.allergenonline.com), <http://fermi.utmb.edu/SDAP/index.html> and [www.allergome.org](http://www.allergome.org).

with the ability to generate IgE and Th2-type cytokine responses. Also emerging in the literature is the idea that proteases, both endogenous and exogenous/environmental, are critical in the regulation of the immunological environment at epithelial surfaces, and in the pathophysiology of allergic respiratory disease [2]. Proteases are major components amongst the profile of antigens secreted by helminth parasites during infection and perform a number of essential functions, including modulation of the host immune response [3]. Could these enzymes be key to the relationship between allergy, atopy and parasitic infections?

### **Proteases Are Major Allergens Derived from Various Organisms**

Of the diverse biochemical agents associated with classified allergens, proteolytic enzymes appear to be particularly potent in allergy-susceptible individuals. These allergens are generally endoproteases, i.e. they cleave peptide bonds within the protein structure, and most belong to the cysteine, aspartic or serine class of proteases (table 1). Endoproteases are ubiquitous in nature and hence it is not surprising that proteolytic allergens are found in many organisms, both plant and animal.

The significance of protease activity in allergy was first highlighted more than a decade ago when it was discovered that the major allergen from the house dust mite (HDM), *Dermatophagoides pteronyssinus* (Der p 1), was a cysteine protease. In mites, proteases such as Der p 1 are involved in food digestion and found in faecal pellets from where they become aerosolized and inhaled. A dose-response relationship can be demonstrated between the levels of Der p 1 inhaled, the prevalence of sensitization and the risk of asthma [4]. Der p 1 is the most immunodominant dust mite allergen involved in the induction of IgE-mediated hypersensitivity and is now considered the most common cause of allergen-linked asthma worldwide [4]. However, because of the increasing attention given to allergies in recent times, other allergens, such as the aspartic protease Bla g 2 of the cockroach, are growing in clinical relevance [5]. As much as 25–37% of asthmatic children in the United States are allergic to Bla g 2 [5].

Bacterial-derived proteases used in the manufacture of detergent products were also among the first identified allergens and are associated with occupational outbreaks of rhinitis and asthma. IgE-mediated respiratory allergies correlate with exposure to bacterial enzymes, such as the serine protease subtilisins (alcalase, savinase and esperase) (table 1). Surveillance of enzyme-specific IgE

antibodies before the onset of allergic symptoms has minimized the risk of occupational allergy in the detergent industry [6].

Airborne spores of *Aspergillus* and *Penicillium* fungi are commonly implicated in allergic disease and are considered important causative agents of extrinsic bronchial asthma [7]. Extracts from the dermatophyte fungus, *Trichophyton rubrum*, also induce both immediate and delayed-type hypersensitivity reactions. The causative agents are now believed to be active proteases, predominantly of the serine class but also aspartic and metallo-proteases that are liberated by these fungi [7].

Food allergic reactions are causing increasing concern as approximately 5% of children worldwide under the age of 4 years exhibit IgE-mediated food allergic reactions. Moreover, food hypersensitivity has been related to moderate or severe atopic dermatitis in 35% of children with these skin symptoms. Some of the components of food that cause sensitization are serine and cysteine proteases, for example, those present in melon, kiwi and papaya fruit [8].

### **How Do Proteases Act as Allergens?**

Considerable advances have been made in elucidating the mechanisms by which proteolytic enzymes may function as allergens (the majority of investigations have used Der p 1 as the model allergen). A review of the main literature reveals that allergens may intervene at various steps (or even several) in the allergic inflammatory cascade including: (a) the degradation of epithelial barrier cells to permit passage of the allergen throughout tissues; (b) cleavage of receptors on the surface of innate immune cells eg. dendritic cells; (c) cleavage of receptors on B and T cells; (d) activation of mast cells via protease-activated receptors (PARs), and (e) alteration of the protease/anti-protease balance of mucosal epithelia.

#### *Disruption of the Epithelial Barrier*

The primary risk factor for the development of allergic sensitization is the delivery of allergen across the mucosal epithelium. Paracellular channels of the epithelial layer are normally sealed by tight junctions, macromolecular assemblies of proteins that form contiguous rings at the apices of epithelial cells. Der p 1 has been shown to degrade the transmembrane proteins occludin and claudin involved in tight junction adhesion and sealing resulting in increased permeability of the bronchial epithelium [9]. This digestive action may facilitate the passage of allergens across the mucosa and enhance the accessibility to antigen presenting cells residing beneath the epithelial barrier.

### *Modulation of Dendritic Cell (DC) Function*

Passage across the mucosal surface would bring the allergen in direct contact with dendritic cells (DC), key players in the development of allergic inflammation. A pivotal role in the activation and function of DC is played by the CD40 surface ligand. When DC are exposed to Th1 inducing antigens, such as LPS, CD40 expression becomes elevated and production of IL-12 increases in response to CD40 engagement [10]. However, DC matured in the presence of proteolytically active Der p 1 produce significantly less IL-12 in response to LPS stimulation and the balance of IFN $\gamma$  and IL-4 production by T cells is modulated such that a Th2 response is promoted [10]. This alteration in the T cell cytokine balance results from proteolytic cleavage of CD40 by Der p 1, as inactive Der p 1 has no effect on DC [10].

### *Modulation of B and T Cell Function*

Der p 1 can influence peripheral blood T cells to promote a pro-allergic Th2 type response by cleaving CD25, the 55-kDa  $\alpha$ -subunit of the IL-2 receptor [11]. Since this receptor is pivotal for the propagation of Th1 cells, removal of CD25 by Der p 1 leads to diminished proliferation and reduced secretion of IFN $\gamma$  in response to mitogenic stimulation. There is a consequential polarisation of T cells towards a Th2 phenotype, with increased secretion of IL-4 and IL-13 (cytokines responsible for inducing IgE synthesis). In addition, Der p 1 cleaves CD23, which is involved in the negative feedback regulatory cycle of IgE synthesis, from the surface of cultured human B cells and thus potentially disrupts this mechanism to cause excessive IgE synthesis and subsequent development of the allergic phenotype [12].

### *Activation of Mast Cells Via Protease Activated Receptors*

The production of mediators of allergic inflammation from mast cells follows the antigen-mediated aggregation of IgE that is bound to high affinity Fc epsilon receptor I (Fc $\epsilon$ RI) on the cell surface. However, the activation of mast cells by allergens can also occur *via* a non-IgE-mediated pathway, and proteases from mites, fungi and insects are among those molecules that can generate allergic inflammation in this way [2]. For example, when the serine protease (Der f) from the HDM *Dermatophagoides farinae* is administered into the airways of mice it induces the degranulation of mast cells and up-regulation and secretion of a number of pro-allergic cytokines, including IL-4, IL-6, IL-9 and IL-13, without IgE aggregation [13]. It has been suggested that this mast cell activation is initiated by cleavage of cell surface protease activated receptors (PARs) [2].

PARs are G protein-coupled transmembrane receptors that mediate cellular responses and are activated by cleavage of an N-terminal domain [2, 14]. Four

PARs have been identified; PAR-1 is expressed on neutrophils, platelets, endothelial cells and fibroblasts, while PAR-2 is found on T cells, neutrophils, epithelial cells, endothelial cells, smooth muscle cells and neurons. PAR-1, -3 and -4 are activated by thrombin, while PAR-2 is activated by trypsin [2]. Many allergenic proteases have similar specificities to the mammalian serine proteases thrombin and trypsin, and therefore allergenic proteases may function by activating PARs. This activation leads to G-protein signalling cascades generating transcriptional responses through ERK (extracellular signal related kinases), mitogen-activated kinases and NF $\kappa$ B and ultimately to the production of chemokines and cytokines that enhance IgE production and airway hyperresponsiveness [2, 14].

Der p 1 can induce IL-6 and IL-8 cytokine release from bronchial epithelial cells via the activation of PAR-2 receptor [15] and the serine proteases of HDM, Der p 3 and Der p 9, cleave PAR-2 on lung epithelial cells causing the activation of phospholipase C which is the initial step in the transcription of cytokine genes [16]. Similarly, the cockroach protease allergen, Bla g 2, activates PAR-2 on bronchial epithelial cells resulting in the activation of ERK and the subsequent induction of IL-8 transcription [17]. Whether these allergens also modulate PAR-3 or PAR-4 remains unknown, although given the involvement of PAR-4 in the induction of IL-6 and IL-8 secretion this has been suggested [2, 17].

#### *Alteration of the Protease/Anti-Protease Balance*

Mucosal epithelia are protected from damage by endogenous proteolytic enzymes, like elastase, by the presence of anti-proteases such as  $\alpha_1$ -antitrypsin, elafin and secretory leukocyte proteinase inhibitor (SLPI). Maintaining the balance of proteases and anti-proteases at the epithelial matrices is critical to protecting lung tissues and an upset in this balance results in conditions such as emphysema and asthma [18]. Clinical studies indicate that subjects with a deficiency in  $\alpha_1$ -antitrypsin are more prone to developing asthma. Furthermore, the administration of exogenous proteases such as papain, proteinase 3, human cathepsin B and human cathepsin L into mouse lungs results in the development of many features of pulmonary disease that is attributed to the inactivation of anti-proteases [18]. Recently, Der p 1 was shown to cleave and inactivate several anti-proteases including  $\alpha_1$ -antitrypsin, elafin and SLPI [18]. Inactivation of these in vivo would modulate the elastase/anti-elastase balance in favour of elastase and result in the upregulation of IL-8 from lung epithelial cells and a shift in the immune environment to one that facilitates the development of a pro-inflammatory allergic response [19].

Squamous cell carcinoma antigen (SCCA) proteins are cross-class serine and cysteine protease inhibitors found in the peripheral blood of bronchial

asthma patients and are induced by IL-4 and IL-13 [20]. SCCAs inhibit Der p 1 protease activity and are produced in the lung in response to the immunomodulatory effects of this allergen. These inhibitors have been suggested to specifically target exogenous cysteine proteases (and possibly other proteolytic allergens) to protect the lungs against allergic reactions [20]. On the other hand, it is noteworthy that some protease inhibitors, such as the cystatins from cat skin and potato and an aspartic protease inhibitor from potato [www.fermi.utmb.edu/cgi.bin/SDAP], have been identified as allergens and, therefore, altering the protease/anti-protease balance in either direction may leave the lung-epithelium open to events that lead to allergy.

### **Proteases Provoke Allergic Responses to Non-Peptidolytic Molecules**

All of the processes outlined above whereby proteolytic allergens may induce allergic inflammation depend on the enzyme being functionally active. The requirement for protease activity in the induction of allergic inflammation has been demonstrated extensively through the use of protease specific inhibitors such as E-64, phosphoramidon and antipain. For example, complete inactivation of protease activity in *Aspergillus oryzae* serine protease allergen prevented both airway inflammation and Th2 activation in an experimental murine model of asthma [14]. Moreover, selective inhibition of Der p 1 protease activity by the synthetic peptide based inhibitor, PTL11028, resulted in the suppression of both airway hyper-responsiveness and inflammation and correlated with reduced levels of allergen-specific IgE in the blood of rats [21].

It is well known that many atopic patients develop an allergic reaction to antigens that lack protease activity and hence this property would not seem to be an exclusive requisite for allergenicity. On the other hand, this does not rule out the possibility that these individuals were concomitantly or previously exposed to a proteolytic allergen. Of importance in this regard is that serine protease allergens derived from *Aspergillus* sp. are capable of conferring allergenic potential, as judged by the recruitment of IL-4 producing cells and induction of antigen specific IgE, to otherwise innocuous antigens, such as ovalbumin, applied to the respiratory tract [14]. Furthermore, co-injection of Der p 1 enhanced the IgE antibody response to chicken egg ovalbumin [22] and promoted the Th2 response to *Schistosoma mansoni* Sm28-GST by reducing IFN $\gamma$  responses and antigen specific IgG2a [23]. Therefore, proteases may destabilise or alter the microenvironment within target tissues making them more susceptible to pro-allergic inflammation and IgE responses which are then driven by non-peptidolytic molecules.

## Do Parasites Exacerbate or Protect against Allergy?

Immune responses to helminth parasites are similar to those observed in allergy in that they are typified by a polarised Th2 immune response, involving high levels of IL-4, IL-5 and IL-13 accompanied by mastocytosis, eosinophilia and potent IgE production [24]. This would suggest that a helminth infection predisposes an individual to allergic reactions; indeed, in a number of infections including anisakiasis [25], enteric infection with the dog hookworm [26] and echinococcosis [27] increased allergic reactivity is a symptom of infection. On the other hand, field studies suggest that helminth infections may, in fact, protect people from allergies. For example, children in the Gabon who are infected with *Schistosoma haematobium* exhibit a 63% lower risk of developing reactivity to HDM: removal of the parasites by drug treatment results in increased atopic responses [24, 28]. In South America, the presence of intestinal worms such as hookworm, *Ascaris* and *Trichuris*, all of which induce high IgE levels, also correlate with lesser skin reactions to HDM [24, 28]. However, a study in Venezuela noted that individuals with light helminth infections exhibit raised allergic reactions which were relieved following drug treatment, whereas those with high infection were protected from atopy which became exacerbated on drug treatment [24, 28].

Helminth parasites have complicated life cycles and go through various developmental stages in their host before establishing chronic infections that can last a long time (many years even). At the same time as skewing the immune system toward a Th2-driven response, they have a general suppressive effect on the host immune system that prevents their elimination and reduces immune-mediated tissue damage. It has been suggested that cytokines of the anti-inflammatory network, particularly IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ), that are produced in response to continual stimulation of the immune system by parasite antigens, are pivotal to regulating the damage they cause and that, coincidentally, these have a bystander protective affect against allergic reactions [24]. In support of this idea, individuals that are prone to allergies tend to have lower IL-10 levels while people with *S. haematobium* and other helminths have raised IL-10 levels which decrease with drug therapy and, in turn, results in increased atopy [28].

In contrast to anthrophilic infections of humans, zoonotic helminth infections, such as with the fish parasite *Anisakis simplex* and the dog hookworm *Ancylostoma caninum*, are often of short duration. A relative absence of suppressor cytokines are observed to these zoonoses, probably because humans are non-permissive and unadapted hosts, and consequently are more likely to evoke allergic responses [25, 26]. Therefore, whether a parasitic infection predisposes or protects an individual against allergies appears to be influenced by

parasite species, whether infection is a zoonosis or not, parasite load, stage of infection, length of infection and host genetic factors (including ability to produce IL-10).

### **Helminth Parasites Secrete Proteases**

Helminth parasites synthesise proteases of all four major mechanistic classes, (cysteine-, serine-, aspartic and metallo-proteases), although the relative importance of each class varies between parasites [3]. Proteases often represent a major component of the parasite total protein production; for example, 4–5% of ESTs characterised for *S. mansoni* encode proteases (cysteine and aspartic), whereas almost 10% of the ESTs described for the related flukes *Clonorchis sinensis* and *Fasciola hepatica* encode cysteine proteases [29]. Proteases are also abundant in parasitic nematode transcriptomes; for example, 17% of *Haemonchus contortus* gut-derived ESTs encode cathepsin B-like enzymes, large gene families encoding cysteine proteases are found, and filariae and both cysteine and aspartic proteases are highly expressed in hookworms. The functions of these proteases are varied, but include essential roles in parasitism such as facilitating entry and penetration of host tissues, digestion of host tissue for nutrients, tegumental (trematodes) and cuticular (nematodes) turnover and immunoregulation. To perform these functions proteases are actively secreted or excreted by the parasite, usually from their guts or surface, and often in copious amounts [3].

Proteases are essential for parasites that move or migrate through tissues and, therefore, it is easy to understand how these would be delivered directly into host tissues (notably mucosal barriers) during the acute stages of infection. After reaching maturity, many helminths reside in spaces outside the circulation or main visceral tissues, for example in the intestines, lungs or bile ducts. However, these parasites generally feed on the walls of these tissues or blood drawn through them and, therefore, proteases still gain access to the host circulation. In short, helminth-infected individuals are continuously exposed to parasite-secreted proteases during acute and chronic infection.

### **Can Parasite Proteases Sensitise Individuals to Environmental Proteases Via Cross-Reactive IgE?**

A number of reports have shown that excretory/secretory (ES) products of helminths collected from in vitro cultures can induce Th2 responses, involving enhanced levels of IgE, when injected into mice [24, 28]. Proteases are associated

with these ES products and there is evidence that these can directly induce IgE responses. For example, using affinity-purified IgE antibodies from patients with acute tropical pulmonary eosinophilia, transpeptidases from *Brugia malayi* and *Wuchereria bancrofti* were identified as major filarial allergens [30]. Similar methods were used to isolate two allergens secreted by *Paragonimus ohirai*, which were subsequently found to be cysteine proteases [31]. Furthermore, cysteine proteases secreted by *Nippostrongylus brasiliensis* [32], *Onchocerca volvulus* [33], *Ancylostoma caninum* [34] and *S. mansoni* [35] have also been shown to be among the target antigens to which IgE antibodies associated with Th2 immune responses are reactive. Therefore, parasite protease-specific IgE is induced by helminth infections, however, it is not known whether this antibody is capable of sensitising individuals to protease allergens in the environment.

Many parasite cysteine proteases and common allergenic proteases are members of the same clan – clan CA of the C1 family of cysteine proteases – and are structurally related [3]. Using the current recommendation for the prediction of allergenicity by WHO/FAO, we compared the primary sequences of cathepsin L and cathepsin B cysteine proteases from a number of helminths, including *S. mansoni*, *Fasciola hepatica* and hookworms, to allergens within the SDAP allergen database (<http://Fermi.utmb.edu/SDAP>). In summary, our analyses revealed that the overall similarity in amino acid sequence between the parasite and common allergenic cysteine proteases is relatively low (data not shown); the highest match found was between the parasitic cathepsin Ls from *F. hepatica* and *S. mansoni* and other cysteine protease allergens in foods such as kiwi fruit (Act c 1, 38%), papaya (Car p papain, 36%) and soybean (Gly m 1, 35%), and the dustmite allergen, Der p 1 (30%). Moreover, this similarity is dispersed throughout the primary sequences except for one region of eight identical amino acids, from residues 22–29 in mature Der p 1 (fig. 1). While this match fulfilled the criteria of allergenicity as defined by the WHO/FAO, the regions of high identity/similarity fall within the active site groove of the proteases and would not be accessible to binding free or mast cell-bound IgE.

Analysis of tertiary structure has been used to determine whether allergens have common or conserved features that are recognised by the immune system with particular interest in mapping linear IgE epitopes. While there does not appear to be a consensus allergen structure [1], comparative molecular modelling of a number of clan CA cysteine proteases indicated that they display a putative IgE binding epitope, previously identified on Der p 1, although this varied in shape and degree of accessibility depending on the protease. The core of the epitope consists of a centrally located conserved tyrosine (residue 154 in Der p 1) flanked on both sides by accessible amino acids [36]. While the corresponding regions in *S. mansoni* and *F. hepatica* cathepsin Ls (fig. 1) and



## Parasite Peptidolytic Activity May Sensitise Individuals to Allergens

As discussed above, functional activity is necessary for the induction of allergic inflammation by fungal, mite and insect peptidolytic allergens. Given that parasite and allergenic proteases exhibit conservation in the active sites, which would endow them with similar/overlapping substrate specificities, the potential of parasite proteases to induce sensitivity to environmental allergens may be related to their action on certain cell types or immune responses that are also targeted by allergens. This mode of sensitisation would not depend on the activation of cross-reactive IgE. Parasite proteases can modify host immune responses in various ways – as described below.

### *Degranulation of Mast Cells and Basophils*

Mast cells and basophils both possess high-affinity receptors for IgE (FcεRI) which when cross-linked by bivalent or multivalent antigens are activated to release various mediators, including IL-4 and IL-13, that influence IgE production and the pathophysiology of allergies [2]. Basophils are also elevated in the blood of people infected with hookworm (*Necator americanus*) and filariae (*B. malayi*), and are considered a major source of IL-4 that serves to amplify Th2 responses [37, 38]. Recently, basophils that accumulated in tissues of mice, mainly lung and liver, during an infection with *N. brasiliensis*, were shown to be a principle source of IL-4. However, stimulation of these basophils was independent of FcR cross-linking [39, 40]. Both the secreted cysteine proteases of *N. americanus* and the cysteine protease allergen Der p 1 have been shown to induce basophils to degranulate and secrete IL-4 and IL-13 even in the absence of IgE. Importantly, the action of these proteases on basophils was dependant on their proteolytic activity [41].

### *Modulation of T Cell Responses*

The secretion of proteases by helminth parasites into the circulation ensures that their effects are systemic and it is likely that they contribute to the potent induction of Th2 responses that are observed in these infections. Our studies with *F. hepatica* cathepsin L cysteine proteases have shown that an intravenous injection of active proteases can suppress Th1 responses to a respiratory infection of *Bordetella pertussis* and delay clearance of the bacterial pathogen [42]. These proteases also suppressed the development of Th1 responses to a *B. pertussis* vaccine or keyhole limpet haemocyanin (KLH) given intra-peritoneally [43]. The capacity of cathepsin L to downregulate Th1 responses differs to that of Der p 1, since cathepsin L is unable to cleave the T cell receptor CD25 (but like Der p 1, it does not cleave various other T cell receptors including CD3, CD4, CD7, CD8, CD18, CD28, CD47 and CD69).

Nevertheless, a systemic suppression of Th1 responses may predispose individuals to Th2 responses when exposed to allergens.

Antigens delivered remote from the lungs can influence the reactivity of these tissues to allergens, an example being the experimental induction of allergy to chicken egg ovalbumin. When administered intra-pulmonarily, the antigen induces a tolerogenic response in the lungs, but when administered following priming of the immune response with intra-peritoneal injections of the antigen in alum it induces all the hallmarks of allergy [2, 14]. By analogy, the secretion of parasite proteases may prime the lungs to respond to other proteases, and to non-protease molecules, that would not otherwise induce allergy.

#### *Recruitment and Activation of Alternatively Activated Macrophages*

The alternative activation of macrophages is important in the development of allergic inflammation, particularly asthma [44]. Fizz1, a recognised marker for alternatively activated (AA) macrophages, was first identified in secretions from inflamed alveolar epithelium and was implicated in stimulating fibrosis during pulmonary inflammation [45]. The expression of another marker, Ym1, is up-regulated in allergy and is a known chemotactic agent for eosinophils [46]. Interestingly, studies in both humans and mice have demonstrated the recruitment of AA macrophages during a variety of helminth infections, including *B. malayi* [47], *N. brasiliensis* and *Litomosoides sigmodontis* [48], with similar expression profiles (i.e. up-regulated Fizz1 and Ym1) to those observed in allergy. In both disease states AA macrophages have been implicated in the development of the Th2 immune environment, IgE elevation and eosinophilia.

Recently, we demonstrated that infection of mice with larvae of *F. hepatica*, or repeated injection of worm ES products, induced the recruitment and alternative activation of macrophages in the peritoneal cavity and the development of potent Th2 immune responses [49]. As shown in figure 2, the induction of the AA macrophage markers Ym1, Fizz1 and Arginase1, and of the Th2 cytokine IL-4, can be observed in bronchial lavage cells isolated from BALB/c mice that were given a single intra-pulmonary dose of purified *F. hepatica* cathepsin L1. Since inactive mutant cathepsin L1 had no effect on the lungs these studies show that exogenous proteases can set up a pro-inflammatory environment involving AA macrophages in this tissue.

### **Are Helminth Proteases Allergens?**

Using the criteria of the WHO/FAO many helminth proteases could be listed as allergens since they exhibit significant identity with known allergens.

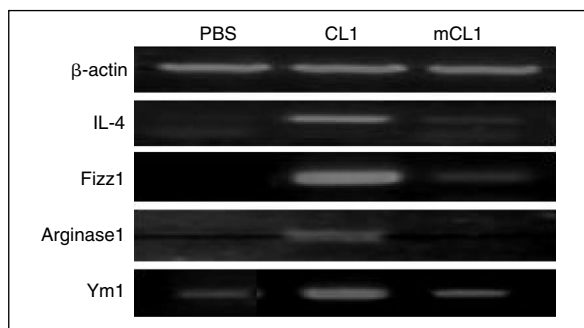


**Fig. 2.** Alternative activation of macrophage in lungs following administration of cathepsin L protease. BALB/c mice were given 50 µg of recombinant *F. hepatica* cathepsin L1 (CL1), inactive cathepsin L1 (mCL1) or PBS intra-tracheally. Twenty-four hours later bronchoalveolar lavage fluids were obtained by injection and aspiration of 0.5-ml volumes (total 4–5 ml) of warm RPMI 1640 medium via cannulation of the trachea. Cells were recovered by centrifugation and resuspended in Tri-reagent for extraction of total RNA. Markers of alternatively activated macrophages (Ym1, Fizz1, Arginase1) and for β-actin and IL-4 were analysed by RT-PCR. The data shown is from a single mouse, and is representative of findings from 4 mice (the expression of each gene was similar in each mouse).

While in a number of helminth infections anti-protease IgE has been detected in patient serum this may simply be part of the overall Th2-driven immune response to these parasites and, as pointed out here, would not be expected to cross react with environmental allergens. Furthermore, there is no clinical evidence indicating that helminth protease induce IgE-mediated allergic-like responses (e.g. skin reactions involving mast cell degranulation, asthma) during infection. Hence, the WHO/FAO criteria for identifying allergens may not be suitably applied to parasite proteases.

The existence of a diverse range of protease allergens of various mechanistic classes (cysteine-, serine-, aspartic- and metallo-proteases) points to the idea that the critical biochemical property for intrinsic allergenicity is ‘proteolytic activity’ (in its broadest sense rather than in a peptide-specific sense). Therefore, both the HDM cysteine protease, Der p 1, and the cockroach aspartic protease, Bla g 2, proteases with distinct peptide bond preferences, can induce allergic responses in the lung that lead to an asthmatic condition. The common event that would initiate the allergic response would simply need to be the degradation of the protein molecules that bind the lung epithelial cells together. Disruption of the lung epithelium would allow these proteases access to the underlying cells of the innate immune system, e.g. dendritic cells, mast cells, basophils, macrophages, and to B and T cells. As discussed in this

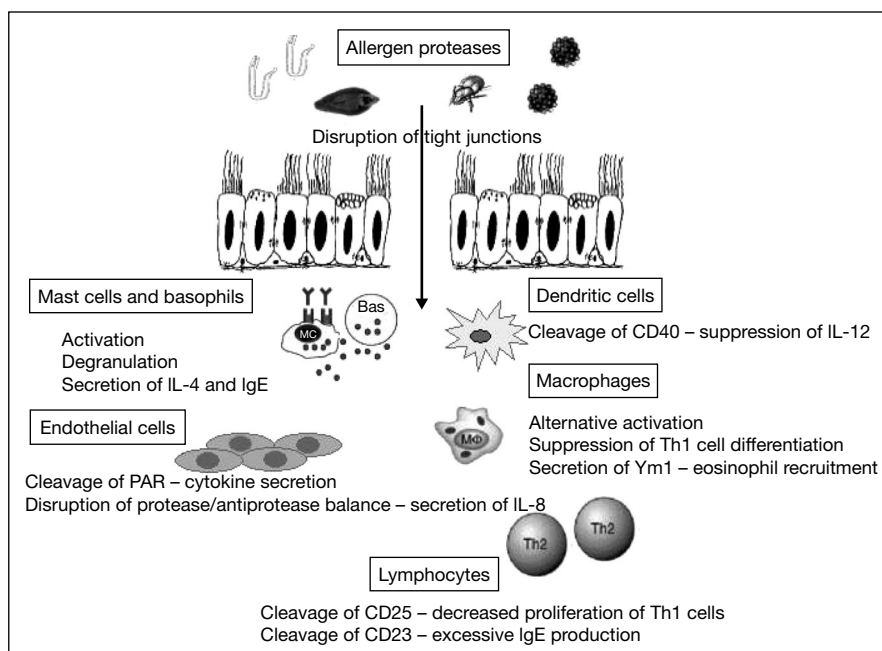


**Fig. 3.** Schematic representation of events that lead to protease-induced inflammatory responses.

review, proteases could modify the activity of these cells by cleaving surface receptors and altering intracellular signalling pathways, and this could be exacerbated by upsetting the protective protease/anti-protease balance at the lung surface (fig. 3). Additionally, proteases would facilitate access for many other molecules in allergenic substances, such as HDM faecal antigens, to host immune cells which may enhance inflammatory reactions in the lungs. While it is well documented that proteases themselves can act as allergens, a recent study showed that adding active proteases (in this case *Aspergillus fumigatus* serine protease) to non-allergenic proteins can precipitate an allergic response [14].

So, could helminth proteases cause allergic reactions? The very fact that these molecules have proteolytic activity would suggest that they can behave in a similar way to allergens. Like allergenic proteases, helminth proteases contribute to development of a Th2-type immune environment by degrading host tissues so that these and other molecules can gain access to the underlying cells of the immune system. Allergic responses induced by helminths are generally associated with acute infections, and are particularly pronounced in zoonotic

infections that induce potent Th2 responses. Take for example, the often life-threatening allergic responses in people that ingest fish contaminated with *A. simplex*. Allergic responses coincide with the degradation of the intestinal wall of the host by larvae, facilitated by proteases released from the parasite [25]. The parasite secretions induce a potent Th2 response with the production of IL-4 and IL-5, elevation of specific and total IgE, the infiltration of eosinophils and hyperplasia of intestinal mastoid tissue. Patients become positive to skin prick tests using *A. simplex* antigen. Thus, the hallmarks of gastro-allergic anisakiasis (GAA) are similar to allergic responses in the lungs.

But could parasite proteases sensitise individuals to protease allergens in the environment? Helminth proteases, if delivered into the lung, would set up a pro-inflammatory environment (as described in this review). Some helminths, for example hookworms, that pass through the lungs during their migration in the mammalian host break through the alveoli and are then coughed up and swallowed into the gut. To get through into the air spaces, they secrete proteases which induces wheezing or productive cough at this stage of infection. However, in most chronic infections, with the exception of helminths that penetrate lung tissues, proteases are not delivered at this site but are usually liberated into tissues such as the liver and intestinal wall or directly into the circulation. Nevertheless, the continual passage of low levels of helminth protease through the lung *via* the circulation may be sufficient to sensitise this tissue making it more susceptible to exogenous protease activity and to other airborne molecules. Proteases could prime the draining lymph nodes to a Th2-driven pro-inflammatory response. This may be mediated by the blocking of Th1 cytokine production, as we have shown that an intravenous injection of cathepsin L cysteine protease suppresses IFN $\gamma$  production in the spleen, hepatic and lung lymph nodes [42, 43].

## Conclusion

The argument concerning the association between helminth infection and the incidence of allergy in the developed and developing worlds is complicated by the fact that many factors, both parasite- and host-derived, influence the clinical manifestations of allergy. However, regulation of the immune responses to chronic helminth infections by anti-inflammatory cytokines, including IL-10 and TGF- $\beta$ , which function to prevent excessive immune-mediated host tissue damage, is suggested to have a bystander dampening effect on allergic responses (these cytokines may block the degranulation of mast cells and possibly basophils, and the activity of T cells) [24, 28]. In situations of acute infections, especially with zoonotic helminth parasites, or where low parasite

burdens exist, allergy and atopy is more pronounced because of the relative absence of anti-inflammatory cytokines. Given that the damage induced by helminth parasites is closely linked with the secretions they produce, a main component of which is proteases, it may not be surprising that mechanisms designed to defend against their action are also effective against immune mediated damage evoked by allergenic proteases.

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