Characterization of Innate Immune Responses to House Dust Mite Allergens: Pitfalls and Limitations

Alain Jacquet*

Center of Excellence in Vaccine Research and Development, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Whereas house dust mite (HDM) allergy results from a dysregulated Th2-biased adaptive immune response, activation of innate immune signaling pathways is a critical prerequisite for the initiation of HDM sensitizations. Such innate sensing is mainly controlled by the airway epithelium and the skin. The resulting release of epithelial-derived proinflammatory cytokines and innate alarmins such as GM-CSF, IL-25, IL-33 and TSLP mediates the activation of ILC2 cells and cDCs to promote Th2-biased inflammation. Significant progress in the elucidation of HDM innate immune activation has been made in the past decade and highlighted key roles of the LPS/TLR4 axis, chitin-dependent pathways together with HDM protease allergens. However, the precise mechanisms by which HDM allergens are sensed by the innate immune system remain largely unknown. Such investigations are made difficult for several reasons. Among these are (1) the natural association of HDM allergens with immunostimulators from the mite exoskeleton as well as from environmental microorganisms/pollutants or endosymbiotic bacteria; (2) the purification of individual HDM allergens from extracts in sufficient amounts and devoid of any microbial and protein impurities; (3) the production of correctly folded recombinant HDM allergens which could display the same biological activity than their natural counterparts; (4) the accessibility to human epithelial samples with cellular heterogeneities and inter-donor variations; (5) the translation of experimental data from mouse models to humans is almost missing. The goal of the present mini-review is to emphasize some important limitations and pitfalls in the elucidation of innate immunostimulatory properties of HDM allergens.

Keywords: house dust mite, allergen, innate immunity, epithelium, protease

INTRODUCTION

The initiation of the HDM allergic response is dependent on skin/mucosal innate immune receptor engagement(s) to install a Pro-Th2 environment mediated by epithelial-derived proinflammatory cytokines and innate alarmins such as IL-25, IL-1b, IL-33, GM-CSF, TSLP (1). These mediators orchestrate critical cross-talks between epithelium, innate lymphoid type 2 (ILC2) and conventional dendritic (cDCs) cells to promote HDM-induced skin/airway inflammation (2).

The HDM allergome comprises at least 35 IgE-inducing allergen groups and consolidated allergenicity studies evidenced the serodominance of mite allergen groups 1, 2, and 23 (3, 4). In contrast, the hierarchy of HDM allergens capable to activate
innate immune signaling pathways remains to be fully elucidated. In this context, we need to hypothesize that any HDM allergen including the minor ones could participate to these events. Whereas, key roles of LPS/TLR4 axis and PAR-2/PAR-4 signaling by HDM protease allergens have been evidenced, the complete elucidation of the innate immune mechanisms triggered by HDM allergens is a goal difficult to achieve (5). One of the main reasons is that HDM allergens are naturally associated with multiple innate immune activators as chitin from the mite exoskeleton, endosymbiotic bacteria together with microbial/environmental (bacteria, fungi, pollutants, viruses, microparticles) compounds present in the house dust.

The goal of the present narrative mini-review is to highlight the pitfalls and the limitations associated with studies evaluating innate immunomodulation by HDM allergens.

**HDM ALLERGENIC MATERIALS**

**House Dust**

HDM sensitizations are mediated by aerosolized mite feces or fragmented bodies present into the household dust. Mite fecal pellets, with a 20–30 mm average diameter (6), can reach the small airways following fragmentation as these airways are only accessible to small particles in the size range 2–6 μm (7). Analysis of the particle-size distribution of airborne Der p 2 confirmed substantial allergen association with small particles (<4.7 μm) (8). Household dust typically contains a large spectrum of environmental factors/pollutants, such as bacteria, fungal spores, particulate matter (as PM 2.5) (9, 10). All these components in association with HDM allergens, elicit innate immune activation and thus represent adjuvants to promote sensitization to HDM.

Experimental approaches based on chronic exposures of epithelial cells/animals to house dust samples are technically feasible. However, the low allergen concentration together with the presence of contaminating immunostimulators in house dust make them virtually impossible to elucidate the innate sensing of specific HDM allergens. Large differences in allergen and microbial/chemical component composition and concentration are commonly observed in house dust samples as well (9, 10). Moreover, seasonal, environmental as well as geographical variations in house dust composition negatively affect the data interpretation.

**HDM Allergen Extracts**

The use of commercial or in-house HDM allergen extracts provided key insights into fundamental mechanisms of HDM allergic response (1, 5). Whole mite, mite bodies or feces extracts are prepared from in-vitro HDM exhausted cultures. Large variabilities in allergen composition, contaminating microbial and environmental components as well as mite microbiome are observed between extracts, introducing reproducibility and data interpretation issues (11, 12) (Figure 1). A recent study evaluating several commercial HDM allergen extracts highlighted the importance to provide the lot characteristics, the concentration of major HDM allergens and LPS as well as the methods used for dosing normalization in order to compare results across different publications (13).

Proteomic analysis identified large differences between the allergen content of mite fecal pellets and mite bodies. Serodominant allergens Der p 1, Der p 2 and Der p 23 together with Der p 3, Der f 6 like allergen, Der p 9, Der p 15 and Der p 28 accumulated in mite feces mainly (3). As fecal particles penetrate more efficiently the lower lung tissues than fragmented mite bodies, mite fecal allergens must represent the first ones sensed by innate immune receptors in the lungs. Interestingly, Der p 1, 2, 5, 14 and Der f 6 like allergen were identified as the most abundant allergens in house dust, confirming that HDM allergens restricted to mite bodies as Der p 5 and Der p 14 are released from mite bodies degradation (3).

The complexity of the HDM allergen extract composition makes challenging investigations on the role to a given allergen in the activation of innate immune signals. To our knowledge, only the key roles of Der p 1, Der p 10 and Der p 13 were directly evidenced in experiments using HDM extracts treated with Der p 1-specific cysteine protease inhibitor, depleted in Der p 10 or Der p 13 or incubated with Der p 10/Der p 13-specific neutralizing antibodies, respectively (14–16).
a reducing agent (as L-cysteine) prior to in-vivo administration or in-vitro cell activation. This reduction is optimal as well for the interactions with specific Der p 1 protease inhibitors (E-64 notably) (14). This procedure was not systematically adopted in studies involving HDM extracts (5). It is hypothesized that the airway redox environment, through high glutathione levels and the role of human glutathione-S-transferase pi, maximizes the Der p 1 cysteine-protease activity (20, 21).

Although natural Der p 2 could be isolated from mite allergen extracts (22), most of the Der p 2 characterization was performed with different recombinant forms of this allergen. Der p 2 displays structural homologies with myeloid differentiation factor-2 (MD-2), the LPS binding co-receptor of TLR4 (23). Recombinant Der p 2 (rDer p 2) produced in insect cells and in the presence of trace amount of LPS was capable to reconstitute TLR4 signaling in the absence of MD-2, demonstrating that Der p 2, by its capacity to interact with LPS, displays auto-adjuvant properties (24). However, a more recent study showed that rDer p 2 alone produced in yeast can cause a strong Th2-biased response in a TLR4-independent manner (25). Moreover, lipid binding assays evidenced that rDer p 2, contrary to rDer f 2, bound weakly to LPS (26, 27). In this study, rDer p 2 was produced as inclusion bodies in E. coli, suggesting that incomplete refolding process affects the conformation of the Der p 2 hydrophobic pocket and impairs interactions with LPS. More recent findings suggested that cholesterol, originated from dietary source of mite culture and from flakes of human skin in house dust, could represent the natural ligand of Der p 2 (28). Again, MS analysis of lipids extracted from rDer p 2 produced in E. coli did not evidence the presence of LPS. Taken together, the LPS binding capacity of
natural Der p 2 remains to be more deeply investigated together with the identification of the endogenous Der p 2 lipid ligand(s). We can hypothesize that the large Der p 2 hydrophobic pocket can accommodate lipid cargos with multiple structures. Their nature will be dependent on the expression system used for the Der p 2 production or the culture conditions for allergen extract preparation (Figure 1). We can speculate that innate activations triggered by Der p 2 are not limited to the unique Der p 2-LPS/TLR4 axis and particularly as lipid ligands can activate other PRRs as heterodimeric TLR1-TLR2 or TLR2-TLR6 (29).

Single allergen-based experimental conditions can overestimate the specific contribution of individual HDM allergens in the activation of innate sensors. Indeed, HDM allergic patients are never exposed to only a single allergen. Moreover, HDM allergen exposures could simultaneously activate identical innate immune signaling pathways, considering the functional redundancy of some HDM allergen groups (protease or lipid-binding proteins at least). Remarkably, the Prothrombinase activity of Der p 1 was shown to generate subsequently TLR4 ligands in airway epithelium resulting in TLR4-dependent ROS production whereas native Der p 2 failed to trigger ROS production (30). Consequently, Der p 1 could be top-ranked in the hierarchical classification of HDM allergens eliciting innate immune activation, raising uncertainties on the key contribution of Der p 2 in the TLR4 stimulation.

The present human protein substrates targeted by HDM protease allergens include soluble (as prothrombin, antitrypsin, elafin, SP-A, IL-33) or receptor/membrane associated molecules (occludin, ZO-1, PAR-2, CD23, CD25, CD40) (30, 31). The challenging elucidation of the complete substrate degradation of HDM proteases was surprisingly not explored up to now.

The glycan structures of HDM allergens could affect their innate immune recognition through interactions with C-type lectin Receptors (CLR) (32). Der p 1 and Der p 2 are naturally glycosylated and interact with mannose receptor and DC-SIGN, respectively (33–36). Recombinant Der p 7 produced in P. pastoris could bind to DC-SIGN as well but the glycosylation and the binding to CLR were not evidenced for native Der p 7 (37).

The only HDM allergen showing binding activity to epithelial Dectin-1 was the structural tropomyosin Der p 10 allergen, however devoid of glycosylation (16).

Chitin, present in the mite exoskeleton as well as in the peritrophic membrane of fecal pellets displays size-dependent immunomodulatory properties. Large chitin structures (>40 μm) activated typical Th2 responses through notably the induction of epithelial-derived cytokines IL-25, TSLP and IL-33 (38). HDM allergens from group 12 (Blo t 12) and group 23 (Der p 23) display sequence homologies with chitin-binding peritrophins whereas those from groups 15 (Der p 15) and 18 (Der p 18) have some similarities with glycosyl hydrolase family 18 chitinases. Consequently, these allergens could mediate innate immune activation through chitin transport or chitin processing (degradation and release of chitin from the peritrophic membrane of inhaled fecal pellets). However, chitin binding assays with commercial chitin beads or shellfish chitin evidenced interactions with Blo t 12 only (39). The chitin binding capacity of Blo t 12 needs not only confirmation but the immunostimulatory properties of this allergen-chitin complex remain unraveled yet. It would be interesting to further investigate chitinase or chitin binding activities of these allergens using HDM-derived chitin (40).

HDM-INDUCED ASTHMA MOUSE MODELS OR in-vitro CELL ACTIVATION ASSAYS

In-vivo Studies
Most of the in-vivo studies used conventional or conditional knock-out (KO) mice to investigate the role of a specific innate molecule in a defined HDM model of airway inflammation. A key report from Hammad’s team evidenced the crucial role of TLR4 signaling in lung structural cells to initiate the HDM allergic response (41). To our knowledge, the rare in-vivo studies deciphering the role of unique HDM allergens were focused on protease allergens. The proteolytic activity of Der p 1 was essential for the initiation of HDM sensitization together with PAR-2 activation by serine protease allergens (15, 42–44). Although mice models are convenient, it is important to recognize their limitations to recapitulate human innate immunity (Figure 1). Notably, classical monocytes, crucial cells for innate immune responses in humans, do not respond to TLR7/8 (R848) and TLR4 (LPS) ligand in mice (45). Sequence variations in TLR4 and MD-2 between human and mice can help to explain the molecular basis of the species differences in TLR4:MD-2 response to lipid A stimulation (46).

Another key parameter affecting HDM-induced innate immune response is the constant interactions between microbiota and lung/gut mucosa (47). As microbiota composition and functions are dependent on environmental and dietary factors, co-housing, dietary control and breeding of independent mouse lineages (KO, wild-type) in the same facility would minimize microbiota differences. These conditions optimize the reproducibility of the results and allow correct interpretations on the impact of innate marker deletion in such KO animal models. Another method to normalize microbiota between mouse groups is to design littermate-controlled experimental setup (48).

In-vitro Cell Activation Assays
Several cellular sources of airway and skin epithelium were commonly used in in-vitro cell assays: primary nasal, bronchial cells or skin keratinocytes (commercial sources or isolates), epithelial cell lines or reporter cells expressing a specific Pathogen Recognition Receptor (PRR). All these cell systems offer advantages but have limitations as well (Figure 1). The airway epithelium is not made by a single type of cells. In the contrary, the core of the epithelial structure is pseudostratified and consists of basal, club, ciliated, and goblet cells. Each of these cells play unique roles in defense of the host (49). Moreover, rare cell types are also present and include among others pulmonary neuroendocrine cells and tuft cells. The barrier function of the epithelium is dependent on the formation of intercellular junctions (tight junctions [TJs], adherens junctions [AJs]) for the control the epithelial sheet integrity (50). The complexity of the airway epithelial structure clearly indicates that epithelial cell
lines represent simplified biological systems for studies on the airway innate immune activation by HDM allergens, even if they offer controllable, versatile and reproducible setups. Moreover, the expression profile of PRRs and TJ proteins can greatly differ between different airway epithelial cells and do not correspond to their natural in-vivo expression (51). Whereas, the cell surface localization of TLR4 is important to sense LPS, this receptor can be localized intracellularly or on the basolateral side of the cells under homeostatic conditions (52). MD-2 expression is often low explaining partly the in-vitro hyporesponsiveness of primary epithelial cells to LPS (53). To our knowledge, whereas HDM-derived b-glucans/LPS induced cell surface expression of TLR2 and TLR4 in nasal and bronchial epithelia, respectively (54), effects of individual HDM allergens on the expression, cell-surface transfer and localization of TLR2 and TLR4 remains to be explored. The accessibility of TLR2/TLR4 (particularly on the basolateral side of epithelia) could be dependent as well on the degradation of TJ proteins by HDM protease allergens, suggesting that innate immune activation induced by HDM lipid binding proteins could be largely dependent on the initial intervention of protease allergens. The contribution of CD14, LBP, and CD36, important accessory molecules for TLR2 and TLR4 activation, in the initiation of the HDM-induced epithelial activation remains uninvestigated to this day (55).

Primary nasal or bronchial cell cultures from HDM allergic patients developing allergic rhinitis or allergic asthma is the optimal cell system to represent the complex architecture of the airway epithelium in the context of HDM allergy (56, 57). Such cell isolates can display a range of abnormalities at the level of epithelial integrity and innate immunity, commonly observed in HDM allergic patients (50). Moreover, it allows comparative studies on HDM component-resolved innate stimulation in relation to the donor atopic status. Unfortunately, important limitations make it difficult the use of primary epithelial cells: complex acquisition of cell samples, limited number of cells and passage, patient availability issues, cost, challenging genetic modification as well as in-vitro differentiation in air–liquid interface (ALI) conditions. All these issues together with the inherent biological variability between human samples affect the reproducibility of the results.

**DISCUSSION**

The direct innate immune activation by HDM allergens results from a myriad of interactions with epithelial innate sensors (Table 1). Research deciphering the intrinsic role of HDM allergens in the innate immune receptor engagement is challenging as these proteins are naturally associated with lipidergic and polysaccharide components. Despite multiple pitfalls and limitations described in this mini-review, consolidated data highlighted the key roles of HDM protease allergens, LPS and chitin to shape innate responses leading to the HDM allergic inflammation (5). In contrast, the innate sensing of lip binding proteins and peritrophin/chitin binding protein/chitinase needs to be extensively investigated. This would allow to draw conclusion on their contributions in the initiation of the HDM allergic response. The HDM allergens from groups 2, 5, 7, 13, 14, 21, 22, 31, 35, exhibiting similarities with lipid binding protein and/or showing capacity to interact with lipid cargos, are all susceptible to promote pleiotropic effects on the innate immune system through interactions with TLR2, TLR4 or other receptors as TLR1, TLR6, RAGE, CD14, CD36 (24, 28, 58–60). Whether these proteins play redundant or unique role(s) in the innate immune activation remains to be investigated.

Airway epithelium is continuously in interactions with the lung microbiome and alteration of lung microbiota is commonly observed in allergic asthmatics, leading to inappropriate inflammatory response (47). Moreover, lung epithelial cells and alveolar macrophages (AMs) communicate continually with each other to maintain lung homeostasis (61). Consequently, future studies should test individual HDM allergens in double or triple epithelial coculture model systems (epithelial cells and bacteria in the presence or absence of AMs) to mimic more closely the allergic nasal/lung environment (62). Nevertheless, these complex coculture models will inevitably generate challenging issues in experimental design and analysis.

Described as the first cell layers in contact with HDM allergens, airway epithelium and skin keratinocytes were considered as the primary cellular sensors of HDM allergens. However, recent findings shed a new light on the initiation of HDM allergic response. Transient receptor potential vanilloid 1 (TRPV1) expressing sensory neurons are activated by cysteine protease activity of HDM extracts and this early event is critical for the allergic skin inflammation (63). Intradermal administration of papain in mice triggered the release of substance P by TRPV1+ neurons which subsequently induces CD301b+ DC migration through Mas-related G-protein coupled receptor member A1 (MRGPR A1) (64). Nevertheless, substance P alone was unable to induce a Th2 cell response indicating that additional signal(s) mediated by alarmins/cytokines are required. Nevertheless, these results suggest that these nociceptors participate to the Der p 1-induced initiation of HDM allergic response. Additional works are required to support this hypothesis, notably through ablation or silencing of airway nociceptors in mouse model of Der p 1 sensitization (65).

With few exceptions, the direct translation of murine experimental data on innate immune activation by HDM allergens to human pathological events is missing. Notably, the association between TLR2, TLR4, PAR-2 and atopic diseases remains controversial or unknown (66). Clinical studies based on topical skin or intranasal administration of single purified allergen are not realistic as they would need, for each tested allergen, GMP-grade batch of highly purified protein. On the other hand, ex-vivo studies based on single-cell transcriptomics of human lung/skin tissue samples treated by HDM allergens would render feasible the spatiotemporal mapping of innate activation in airway/skin epithelial cell landscape (67).

Over the last two decades and despite the experimental issues described in this review, substantial advances have been made in the characterization of innate immune activation by HDM allergens. Future studies are needed not only to disclose all the aspects of these molecular pathways but also to demonstrate their clinical relevance.
| Allergen | Biological activity | PRR activation/Protein target | Effects | Notes |
|---|---|---|---|---|
| Der p 1 | Cysteine protease | PAR-1/4, TLR4 (Pro-thrombinase activity) MRGPRX1 and others MR, DC-SIGN (glycosylation) TJ proteins | Intracellular ROS, Pro-inflammatory cytokine productions IL-33 maturation Unknown Facilitate allergen uptake by DCs and PRR activation by other HDM allergens | Indispensable innate immune activator Complete substrate degradome unelucidated in-vivo CLR activation? Interaction with sensory neurons? |
| Der p 2 | MD-2-like fatty acid binding protein | TLR 4, TLR 2 | Pro-inflammatory cytokine productions | Natural lipid ligand(s) partially identified LPS binding to be confirmed Interaction with other innate receptors/co-receptors? |
| Der p 3 | Trypsin-like serine protease | PAR-2, PAR-4 TJ proteins | Pro-inflammatory cytokine productions, Mast cell activation Facilitate allergen uptake by DCs and PRR activation by other HDM allergens | Complete substrate degradome unelucidated |
| Der p 5 | Lipid/fatty acid binding proteins | TLR2 | Pro-inflammatory cytokine productions | Impossible to isolate enough natural Der p 5 to identify its lipid ligand(s). Mechanism of lipid binding unelucidated: conformational switch or hydrophobic pocket at the Der p 5 dimer interface? |
| Der p 7 | Lipid/fatty acid binding proteins (LBP-like) | TLR2 CLR? (glycosylated natural allergens) | Pro-inflammatory cytokine productions Unknown | Natural Der p 7 accessible but identification of the natural ligand(s) and glycosylations not investigated. |
| Der p 9 | Collagenase | PAR-2 TJ protein degradation | Pro-inflammatory cytokine productions Facilitate allergen uptake by DCs and PRR activation by other HDM allergens | Complete substrate degradome unelucidated |
| Der p 10 | Tropomyosin | Dectin-1 | Regulation of IL-33 release | Mechanism of binding of unglycosylated Der p 10 to Dectin-1 unelucidated |
| Blo t 12 | Chitin binding protein | Unknown | Pro-inflammatory cytokine productions through chitin transport? | Blo t 12 homologs non-identified in Dermatophagoides mite species up to now Chitin binding activity to be confirmed |
| Der p 13 | Cytosolic FABP | TLR2 SAA | Pro-inflammatory cytokine productions Stimulation FPR2-IL-33 axis | Natural ligand unidentified |
| Der p 15 | Chitinase | Unknown | Chitin processing? Release of allergens from fecal pellets? | Chitinase activity to be evaluated Chitin binding activity to be confirmed |
| Der p 18 | Chitinase | Unknown | Chitin processing? Release of allergens from fecal pellets? | Chitinase activity to be evaluated Chitin binding activity to be confirmed |
| Der p 20 | Lipid/fatty acid binding proteins | TLR2 | Pro-inflammatory cytokine productions | Impossible to isolate enough natural Der p 21 to identify its lipid ligand(s). Mechanism of lipid binding unelucidated: conformational switch or hydrophobic pocket at the Der p 21 dimer interface? |
| Der f 22 | Lipid binding protein, Der p 2 homolog | TLR4? TLR2? | Pro-inflammatory cytokine productions? | Similar innate immune activation than Der p 2? |
| Der p 23 | Chitin binding protein | Unknown | Pro-inflammatory cytokine productions through chitin transport? | Chitin binding activity to be confirmed |
| Der f 31 | Cofilin (actin-binding protein) | TLR2 | IL-33, TSLP production, ILC2 activation | Native Der f 31 inaccessible Mode of interaction between actin-binding protein and TLR2 unknown |
| Der f 35 | MD-2-like lipid-binding protein | TLR4? | Pro-inflammatory cytokine productions? | Similar innate immune activation than Der p 2/Der f 22? |

CLR, C-type lectin receptor; DC, dendritic cell; DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; FABP, Fatty acid binding protein; FPR2, N-formyl peptide receptor 2; IL22, Innate lymphoid type 2 cell; LBP, LPS-binding protein; MD-2, Myeloid differentiation factor-2; MR, Mannose receptor; MRGPRX1, Mas-related G-protein coupled receptor member X1; PAR, Protease-activated receptor; PRR, Pathogen recognition receptor; ROS, Reactive oxygen species; SAA, Serum amyloid A; TJ, Tight junction; TLR, Toll-like receptor; TSLP, Thymic stromal lymphopoietin.
AUTHOR CONTRIBUTIONS

AJ designed and wrote the first draft of the manuscript, contributed to the article, and approved the submitted version.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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