Review Article

Innate Immune Regulation of the Allergic March: Using House Dust to Validate the Hygiene Hypothesis

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Abstract

Over the last several decades, it has become increasingly clear that innate immune responses to microbes are mediated by several families of pattern recognition receptors (PRRs) constitutively expressed by a wide range of hematopoietic and non-hematopoietic cell types. These receptors respond to molecules and enzymatic byproducts generated by fungi, parasites, bacteria, and viruses that are often referred to as microbe associated molecular patterns (MAMPs). Not unexpectedly, my laboratory has found that PRRs also play a dominant role in innate responses to non-infectious immunostimulatory materials present in house dust extracts (HDEs) and the living environments they represent. However, our investigations challenge the commonly held view that microbial products in ambient air protect against the allergic march by promoting protective Th1 biased adaptive responses to inspired aeroallergens. Instead, all HDEs studied to date have preferentially promoted the development of Th2 biased airway hypersensitivities when used as adjuvants for intranasal (i.n.) vaccination of mice. In contrast, daily low dose i.n. HDE delivery was found to promote the development of aeroallergen tolerance. This article will review these experimental findings as evidence to propose a new paradigm by which airborne MAMPs and other stimulants of innate immunity may influence aeroallergen specific immunity and the genesis of allergic respiratory diseases.

Keywords: Hygiene hypothesis; Toll-like receptor; LPS; House dust extract; Asthma; Allergy; Tolerance

Abbreviations:

MAMP: Microbe Associated Molecular Pattern; TLR: Toll-Like Receptor; HDE: House Dust Extract; LPS: Lipopolysacchride; BMDDC: Bone Marrow Derived Dendritic Cell; OVA: Ovalbumin; i.n.: Intranasal; BLN: Bronchial Lymph Node

Introduction

Asthma and other allergic diseases have become far more common in industrialized countries in recent decades [1-3]. Moreover, while atopy rates remain low in underdeveloped countries, with modernization, increases have been reported within their cities as well [4,5]. Although reasons for these trends remain speculative, the rapidity with which allergic disease prevalence has increased in affected areas strongly suggests environmental factors are responsible. Adaptive responses associated with allergen tolerance and hypersensitivities appear to become imprinted early in life [6,7]. Therefore, as infants/toddlers spend a majority of their time indoors, there is a great deal of interest in determining how home exposures impact on allergic risk.

It is generally accepted that allergen exposure is a prerequisite for sensitization and for some allergens (i.e. cockroach and house dust mite), the risk for developing hypersensitivities increases significantly when levels in the home exceed a quantifiable threshold [8-10]. However, for other allergens (i.e. dogs, cats), increased levels of home exposure appear linked to a decreased risk of sensitization, both to the allergen of interest, and to unrelated allergens [10-12]. In a meta-analysis of 12 relevant studies, we found the odds ratio (OR) for the development of allergic stigmata during childhood was 0.83 (CI 0.73-0.96) in children raised with versus without household pets [13]. These and other findings suggest that aside from allergens themselves, living environments contain additional molecules that influence the immunological balance between allergen tolerance and hypersensitivity.

Endotoxin, a molecule that activates immunocytes via toll like receptor 4 (TLR4), has been reported to be present at higher concentrations in homes with regular animal exposures than in homes with none [14,15]. Moreover, in several published reports, infants raised in high endotoxin homes were found to have a reduced incidence of atopic diseases [14,16]. In our meta-analysis of 16 pertinent studies, the OR for allergen sensitization was 0.85 (CI 0.77-0.93) for infants raised in homes with high versus low ambient endotoxin levels [13]. In consideration of these relatively weak associations, it is important to recognize that endotoxin-rich environments generally contain increased levels of other immunostimulatory microbial products as well [17,18]. Furthermore, several manmade pollutants promote development of allergic hypersensitivities [19]. Although, much has been learned in recent years, the molecular complexity of ambient exposures has hampered efforts to develop a holistic understanding of their impact on atopic risk. Consistent with this theme, in additional meta-analyses, living on a farm (OR 0.74; CI 0.61-0.91; 9 studies), living in close proximity with livestock (OR 0.54; CI 0.36-0.81; 7 studies), and unpasteurized milk consumption during the first years of life (OR 0.67; CI 0.59-0.75; 5 studies) were all found to be far more negatively associated with allergic stigmata during childhood, than were home endotoxin levels [13]. These meta-analyses and other epidemiological evidence strongly suggest that endotoxin levels are only one of a number of molecular variables influencing the allergenic potential of homes.

Rationale for the Study of House Dust Extract Bioactivities

While consensus opinion supports the view that living environments have a major educational influence on developing immune systems, understanding of how ambient exposures modify allergic risk remains highly speculative. By necessity, a majority of laboratory investigations aimed at characterizing how living environments affect host immunity have made *a priori* assumptions about which molecules are important. As an alternative, we reasoned that the immunological "ether" associated with homes might be better understood by characterizing the immunostimulatory properties of clinically relevant but unpurified environmental samples. Logic suggests that gravity concentrates immunostimulatory particulates into settled dust. Moreover, house dust allergen and endotoxin concentrations have previously been found to be predictive surrogate markers of allergic risk. Therefore, for the last decade, my laboratory has characterized the immunostimulatory properties of sterile house dust extracts (HDEs). Studies conducted to date have yielded provocative and reproducible results, which will be the focus of this paper [20-26].

HDEs Activate Bone Marrow Derived Dendritic Cells (BMDDC)

For initial studies, dust samples were collected from the bedrooms of 15 suburban homes in San Diego, California and were processed by suspension in PBS, physical agitation, and sterile filtration [21,23]. HDEs prepared in this manner were found to elicit BMDDC cytokine production in a concentration dependant manner. Cytokines readily detected by ELISA included IL-6 and IL-12p40 [21]. However, HDE induced BMDDC secretion of bioactive IL-12p70 was weak compared to responses induced by ligands for TLR4, TLR7, and TLR9 [23]. In more recent unpublished experiments we observed that HDEs also induce IL-1β and IL-23 synthesis by BMDDCs. Furthermore, HDEs were found to induce increased surface expression of MHC Class II and other co-stimulatory molecules by BMDDCs [21].

Consistent with other studies, mean endotoxin levels of dust samples obtained from homes with pets were more than twice that of samples obtained from homes without pets [21]. Moreover, a significant correlation was found between HDE endotoxin levels and their BMDDC cytokine inducing activity. However, the correlation coefficient for this association was less than 0.6, suggesting that additional molecules contribute to the bioactivities of HDEs. Moreover, when analyses were replicated with 200 HDEs derived from the bedrooms of children living in Cincinnati Ohio, little correlation was found between HDE endotoxin levels and their BMDDC cytokine inducing activity (correlation coefficients between 0.085 and 0.112) [26]. These observations suggest that endotoxin may provide a greater proportion of the net immunostimulatory activity of house dust in some regions of America than in others.

To more specifically assess the contribution of TLRs to HDE responsiveness, HDE induced cytokine production by BMDDCs prepared from wild type (WT) and TLR and MyD88 knock out (ko) mice was compared [21]. In experiments with dust samples derived from multiple homes (n=10), TLR4 ko BMDDCs consistently demonstrated reductions in HDE induced cytokine production and co-stimulatory molecule expression but residual responsiveness remained. TLR2 and TLR9 ko BMDDCs also demonstrated reductions in HDE responsiveness, supporting the view that TLR4, TLR2 and TLR9 independently contribute to HDE mediated BMDDC responses. Finally, while MyD88 ko BMDDCs were far more compromised in their ability to respond to HDEs than BMDDCs from mice with individual TLR deficiencies, residual cytokine production and co-stimulatory molecule up-regulation remained [21]. These results establish that MyD88 dependent signaling pathways play a central but not an exclusive role in mediating BMDDC responsiveness to HDEs.

HDEs Contain Invariant Natural Killer T (*iNKT*) cell Antigens

Recognizing that HDEs contain MAMPs capable of activating BMDDCs through several TLRs we have considered the possibility that they might also contain MAMPs capable of activating host immunity through additional PRR families. *iNKT* cells express a highly restricted T cell receptor (TCR) and only respond to glycolipid antigens presented on the non-classical MHC class 1 like molecule, CD1d [27,28]. Unlike classical T cells, *iNKT* cells respond rapidly to glycolipid antigens without the requirement of clonal expansion and differentiation [29]. Therefore, *iNKT* cells are generally considered to contribute to the innate, rather than the adaptive immune response [30,31]. Select bacteria are known to produce glycosphingolipid antigens capable of activating iNKT cells through their TCR [31-33]. Recently we found that *iNKT* cell antigens could be detected in HDEs with a simple CD1d coated plate assay using a panel of 3 *iNKT* cell hybridomas with unique TCRs [25]. Responses could not be duplicated with purified TLR ligands and were CD1d dependent, establishing that many HDEs contain antigens specific for *iNKT* cells. However, these *iNKT* cell hybridomas, which have subtle differences in glycolipid specificity, due to their unique Vβ segments, did not show the same patterns of reactivity, suggesting that more than one *iNKT* cell antigen may be common in living environments. Two human *iNKT* cell lines expanded from PBMCs were also found to recognize antigens within HDEs, providing additional evidence that this observation is relevant to human immunity [25].

Other Pathways by Which HDEs May Activate the Innate Immune System

Recent experimental observations suggest that in addition to MAMP exposures, as traditionally considered, the airways are also exposed to microbial products with enzymatic activities that have the potential to activate the innate immune system indirectly. The best studied are proteinases of fungal and house dust mite origin [34-36]. In fact the capacity of fungi to induce Th2 biased airway hypersensitivity responses in mice was found to be highly dependent on this proteinase activity. A recent paper by Millien and colleagues further demonstrated that airway exposure to a proteinase derived from *Aspergillus* species induced Th2 biased airway inflammation that was TLR4 dependent [35]. Moreover, these proteinases were found to convert fibrinogen into split products that acted as endogenous TLR4 ligands. Given that house dust contains fungi and house dust mite allergens [37], it stands to reason that proteinase activities associated with these aeroallergens are also likely to contribute to the net immunostimulatory potential of HDEs.

Type 2 innate lymphoid cells have recently been identified as playing an important role in mediating allergic responses in the airways [38,39]. However, these cells may not respond directly to allergens or microbes, as they lack antigen recognition receptors and have not been found to respond to TLR ligands [40]. Nonetheless, type

2 innate lymphoid cells are known to produce cytokines at high levels in response to cytokines produced by activated airway epithelial cells, including IL-25, IL-33, and TSLP [38,41-43]. Therefore, it is likely that these cells contribute to the immunostimulatory potential of inspired air but further study will be required to fully elucidate their role in immune homeostasis within the lungs.

HDE Adjuvant Activities

To assess the adjuvant activities of HDEs, mice received intranasal (i.n.) immunizations with ovalbumin (OVA) alone or with HDEs from multiple homes, three times, at weekly intervals [20]. The HDE dose selected for these experiments was found to provide optimal adjuvant activity and experiments were replicated with HDEs derived from 10 different homes. Additional control groups were i.n. immunized with OVA and Pam-3-Cys (TLR2), LPS (TLR4), or CpG DNA (TLR9), according to the same vaccination schedule. Mice i.n. immunized with OVA and HDE had far stronger adaptive responses, than mice i.n. immunized with OVA alone, establishing that HDEs have mucosal adjuvant activities. In addition, HDEs used in these studies were consistently found to act as Th2 biasing adjuvants, as they induced strong allergen specific IgE and Th2 polarized cytokine responses but weak IgG2a and IFNγ responses. Furthermore, mice immunized with OVA and HDEs consistently developed an eosinophil rich airway inflammatory response and increased bronchial responsiveness to methacholine after i.n. OVA challenge [20]. Finally, HDEs were found to be more effective at inducing OVA specific, Th2 biased, airway hypersensitivities than Pam-3-Cys and low dose LPS, both of which have previously been described as Th2 adjuvants [20,44].

The adjuvant activities of HDEs were initially found to be dependent on signaling through MyD8820, additional evidence of their relative dependence on signaling through TLRs. However, in more recent studies, iNKT cells were also found to contribute to the adjuvant potential of HDEs [25]. These results establish that multiple PRRs contribute to the adjuvant activities of HDEs and the living environments they represent. Moreover, they challenge the commonly held belief that microbial products protect against the allergic march by inherently favoring the development of Th1 biased responses towards inspired aeroallergens. Considered together, these observations beg the question, why are mice and humans not universally allergic to inspired aeroallergens?

HDE Tolerogenic Activities

Experiments just discussed might be construed to suggest that many, if not all, living environments intrinsically promote the development of allergic asthma. However, in these studies, mice were airway exposed to the immunostimulatory contents of HDEs at weekly intervals and at levels likely to be in great excess of daily physiological exposures. In contrast, individuals are thought to inhale air laced with far lower concentrations of immunostimulatory molecules, on a semi-continuous basis [45]. Therefore, additional experiments were designed to better model real world exposures. First a HDE standard (HDEst) was prepared from individual HDEs with high levels of adjuvant activity. Mice received 3 weekly i.n. OVA immunizations, as in previously described experiments, while low dose HDEst (1/7th weekly dose) was i.n. delivered daily, beginning 1 week before the first and ending with the last dose of OVA, weekly with OVA (as in experiments discussed in the previous section), or both [20].

Daily i.n. HDEst delivery had little adjuvant effect on OVA specific responses. More importantly, this delivery schedule prevented mice concurrently receiving weekly i.n. OVA and HDEst (adjuvant dose) from developing both Th2 biased adaptive responses and experimental asthma [20]. Additional studies, further established that i.n. OVA vaccinations delivered in conjunction with daily i.n. HDEst induced allergen specific tolerance that lasted more than two and a half months [24]. Analogous results were obtained when LPS was used in place of HDEst for these experiments. Likewise, Peters et al. found that daily airway exposures to barn dust induced allergen tolerance in another murine model of asthma [46]. These observations provide direct evidence that stimulants of innate immunity, which are traditionally considered to be adjuvants, when used under distinct experimental conditions, also serve as potent inducers of allergen tolerance.

Along with allergen naive infants developing primary immunity (hypersensitivity versus tolerance), airway exposures to stimulants of innate immunity are likely to impact greatly on the clinical status of patients with pre-existing allergic respiratory diseases. Therefore, we have also determined how i.n. HDEst delivery modifies the aeroallergen challenge responses of previously Th2 sensitized mice [22]. One group of OVA sensitized mice received a high dose HDEst bolus concurrently with each airway OVA challenge, while another group received low dose i.n. HDEst (1/7th bolus dose) on a daily basis beginning seven days before the first and ending with the final OVA challenge. Both daily and bolus i.n. HDEst delivery induced neutrophilic airway inflammation, and both delivery schedules attenuated features of the allergen induced Th2 biased airway hypersensitivity response. However, daily delivery was significantly more effective. Moreover, daily but not bolus HDEst delivery attenuated Th2 cytokine production by harvested bronchial lymph node (BLN) cells stimulated with OVA in vitro and desensitized mice to additional airway challenges a month later with allergen alone. Given that daily airway exposures to stimulants of innate immunity contained in HDEs attenuated both effector and memory responses to aeroallergens in previously Th2 sensitized mice, its stands to reason that immunostimulants in inspired air could have an analogous effect on children with allergic respiratory diseases.

Mechanisms by Which Daily Airway HDE Exposures Might Induce Immunological Tolerance

To better assess CD4 cell outcomes in previously described mouse models of Th2 biased airway hypersensitivity and tolerance, CFSE labeled, naïve, OVA transgenic TCR, CD4 cells (DO11.10 TG X RAG-1 KO CD4 cells) were adoptively transferred into mice receiving i.n. OVA vaccinations [24]. Select mice also received daily low dose HDEst beginning 1 week before and ending with OVA delivery or as a single adjuvant dose of HDEst delivered with OVA. Ten days later, mice were i.n. challenged with OVA alone and 24 hours after this, BLNs were harvested. While BLN cells from mice co-immunized with high dose of HDEst displayed marked increases in the proliferation and expansion of adoptively transferred OVA TCR transgenic CD4 cells, BLNs from mice receiving i.n. OVA alone or OVA plus daily low dose HDEst did not. Interestingly, FOXP3 expression by transferred OVA TCR transgenic CD4 cells did not increase in any of the experimental groups. These findings demonstrate that the tolerogenic influence of daily i.n. HDE delivery on allergen specific immunity, is explained, at least in part by the failed clonal expansion of naïve allergen specific CD4 cells but not by their preferential differentiation into allergen specific adaptive Tregs.

While OVA HDE vaccination did not induce the differentiation of naive allergen specific CD4 cells into adaptive Tregs , we did find that i.n. HDEst delivery increased the frequency of natural Tregs recruited to the lungs and BLNs of recipient mice [24]. Moreover, these increases were about twice as large in mice receiving daily i.n. HDEst for a week compared to mice receiving a single bolus dose of HDE and they persisted for a week or longer. A final series of experiments further established that Treg depletion attenuated the tolerogenic influence of daily i.n. HDE exposure in our murine asthma model [24]. Taken together, these observations strongly suggest that daily i.n. HDE exposures induce allergen tolerance by mechanisms that involve the local recruitment of natural Tregs to the airways.

Epidemiological Evidence that HDE Immunostimulatory Activities Predict Allergic Risk

Building on our previous work we recently determined whether parameters of HDE bioactivity were predictive of allergen skin prick test (SPT) reactivity for high allergic risk infants participating in the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) [26]. A nested case-control study was conducted with 99 children that were SPT reactive to at least one aeroallergen at age three and 101 SPT negative subjects. HDEs were prepared from dust samples collected from their homes at age one. Murine splenocytes and BMDDCs were incubated with HDEs and supernatant cytokine concentrations determined by ELISA. HDE endotoxin levels were determined by standard methods. HDEs derived from the homes of SPT positive (cases) and negative (controls) children were found to have similar bioactivities. However, when cases were considered in isolation, HDE bioactivity levels correlated directly with the number of allergens to which subjects reacted. Analogous statistical analyses failed to identify any association between HDE endotoxin levels and the aeroallergen sensitization profiles of children included in the study. Taken together these findings suggest that HDE immunostimulatory activities predict the aeroallergen sensitization status better than HDE endotoxin levels and that the net immunostimulatory activity of homes has the potential to attenuates the allergic march in a dose dependent manner.

Conclusions

Investigations reviewed in this paper demonstrate that pulmonary exposures to allergen non-specific microbial products ubiquitous in living environments have the potential to impact significantly on allergic risk. Nonetheless, understanding of the molecular variables and immunological mechanisms responsible is far from complete. Correlations between pet, farm, animal, unpasteurized milk, and endotoxin exposures during childhood and a reduced incidence of allergic manifestations have been found in many studies but these trends have been inconsistently reported, and in select studies, associations were relatively weak, non-existent, or reversed [13]. Moreover, results from the many epidemiological studies conducted to date provide little insight as to the cellular and molecular mechanisms by which living environments influence allergic risk.

Although ubiquitous, the concentrations of TLR ligands, *iNKT* cell antigens, and other MAMPs in the ambient air of most micro-environments, are likely to be very low. For example, one study of pediatric exposure calculated that the median amount of endotoxin inhaled by children each day is from 46-220 picograms [45]. However, endotoxin can be readily detected in essentially all air samples tested and concentrations can vary by as much as 5 logs [45,47]. Given this lack of homogeneity, estimates of daily exposure based on air sampling, are unlikely to account for episodic brief periods of exposure to air containing extremely high concentrations of endotoxin and/or other immunostimulants of microbial origin. Moreover, despite evidence that living environments contain a wide range of molecules that induce innate immune activation, the only assay commercially available for their detection at a molecular level is the limulus lysate assay for endotoxin. This deficiency in assays for the detection of molecules with immunostimulatory potential is a major impediment for efforts aimed at characterizing how living environments affect allergic risk.

As an alternative to measuring concentrations of specific immunostimulatory molecules in HDEs our research group has used well-defined bioassays and animal models of allergic disease to better characterize the immunostimulatory content of HDEs and the living environments they represent. HDEs have been found to contain biologically significant levels of various TLR ligands, as well as *iNKT* cell antigens [21,23,25]. Intranasal vaccination experiments have further revealed that weekly airway exposures to adjuvant doses of HDEs induce Th2 biased airway hypersensitivities to a co-administered allergen, while daily HDE exposures promote the development of long lived allergen tolerance [20,22,24,25].

If reflective of real world exposures, our HDE studies strongly suggest that ambient immunostimulatory molecules within inhaled air do not inherently drive the development of "protective" Th1 biased responses to aeroallergens, as commonly suggested in the literature. Instead, our experimental findings lead us to speculate that these airway exposures have far greater potential to promote tolerogenic and/or Th2 biased immune responses, depending on additional variables [20,22,24,25]. These variables are likely to include the relative and absolute concentration of each individual MAMP contained in inspired air during the course of the day, and in particular, the frequency and duration of exposures to air laced with high concentrations of these immunostimulants. The observation that molecules that activate innate immunity and act as mucosal adjuvants (i.e. HDEs and LPS) also function as tolerogenic agents is novel and likely to be of great clinical significance. However, while mechanistic studies are ongoing, current understanding of this phenomenon is limited and based principally on indirect evidence.

Along with attenuating the adjuvant activities of HDEs, we found that the innate airway inflammatory response that develops 24 hours after i.n. bolus HDE challenge (neutrophilic inflammation and cytokine release) can be inhibited by pre-treating mice with daily i.n. low dose HDE [20]. This phenomena of reduced responsiveness with repetitive exposure has previously been described with LPS and other TLR ligands, and is commonly referred to as LPS tolerance [48-50]. In unpublished studies we further observed that daily i.n. HDE delivery led to significant increases in local mRNA expression for molecules that mediate LPS tolerance (IL-10, STAT3, IRAKM, SHIP) [48,50-52] when compared to bolus i.n. HDE delivery. Finally, daily airway HDE delivery was found to selectively recruit natural Tregs to the lungs and draining BLNs of mice [24]. These observations may explain why human lungs remain un-inflamed despite the continuous inhalation of air laced with stimulants of innate immunity [53]. Moreover, they provide a potential mechanistic explanation for why daily airway immunostimulant exposures promote the development of allergen specific tolerance.

In field studies we further determined if parameters of HDE bioactivity were predictive of allergen SPT reactivity for high allergic

risk infants participating in the CCAAPS [26]. While the magnitude of HDE responses did predict the number of allergens to which atopic children reacted, no association was found between the HDE endotoxin levels and the aeroallergen sensitization profiles of children included in this study. These findings suggest that along with endotoxin, homes contain a variety of other immunostimulatory molecules that also influence the outcome of the allergic march.

While much remains to be learned of the molecular and immunostimulatory content of homes, experimental results discussed herein lead us to propose a new paradigm by which ambient exposures might modulate innate immune homeostasis in the airways, promote the development of aeroallergen tolerance or hypersensitivity, and modify the respiratory status of previously aeroallergen sensitized patients. The tenets of this model are as follows: 1) Basal levels of exposure to MAMPs present in ambient air are generally not sufficient to provoke an inflammatory reaction in the airways or to provide adjuvant activity for co-inspired aeroallergens, 2) Physiological exposures to ambient air laced with low but adequate concentrations of these immunostimulatory molecules leads to a state of relative airway hypo-responsiveness to these molecules, 3) Airways receiving inadequate basal innate immune stimulation remain far more responsive to airborne MAMPs than airways that are regularly exposed to these molecules, 4) If dampening of immune responsiveness is inadequate, episodic exposures to ambient air laced with super-physiologic concentrations of these immunostimulatory molecules have the potential to provide Th2 adjuvant activity for the development of hypersensitivities to co-inspired aeroallergens.

Although far from proven, the model is testable and consistent with current understanding of the regulation of innate and adaptive immunity in the airways. In addition, this paradigm provides a rational mechanistic framework for understanding why semi-continuous airway exposures to aeroallergens and molecules that activate innate immunity only prime a subset of individuals to develop Th2 biased allergic respiratory diseases, while a majority become aeroallergen tolerant and their airways remain un-inflamed. In the years to come, the continued study of the interface between host immunity and the environment will give rise to a far more comprehensive understanding of the genesis of allergic respiratory diseases and other diseases of immune dysregulation and ultimately, may lead to the development of better therapeutic strategies for their prevention and treatment.

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