



House dust mite exposure attenuates influenza A infection in a mouse model of pulmonary allergic inflammation

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ABSTRACT

Environmental allergens elicit complex immune responses in the lungs that can promote the development of asthma or exacerbate preexisting asthma in susceptible individuals. House dust mites are one of the most common indoor allergens and are a significant driver of allergic disease. Respiratory infections are known factors in acute exacerbations of asthma but the impact of allergen on the pathogen is not well understood. We investigated the pathogenesis of influenza A infection following exposure to house dust mites. Mice exposed to house dust mites lose less weight following infection and had more transcription of interferon-lambda than controls. These data correlated with less transcription of the influenza polymerase acidic gene suggesting diminished viral replication in house dust mite exposed mice. Altogether, these data suggest that exposure to environmental allergens can influence the pathogenesis of influenza infection.

1. Introduction

Asthma and allergic rhinitis are common allergic diseases impacting millions of people worldwide [1–3]. Atopic individuals are sensitized to environmental allergens, proteins in the environment that can elicit an immune reaction upon subsequent exposure [4]. The nature of the pulmonary immune response to environmental substances is dictated by the composition, physical properties of the material, and host-intrinsic properties. In the case of atopic individuals, re-exposure to environmental allergens can lead to an acute exacerbation of disease. Exacerbation of pulmonary allergic inflammation is characterized by physiologic changes including airway hyper responsiveness, goblet cell hyperplasia and mucus hyper secretion, airway edema, and exaggerated allergic inflammation [4].

The allergic immune response is typically dominated by type-2 cytokines such as interleukins (IL) –4, 5, 13, and 33. Additionally, allergen specific IgE and type-2 cellular responses involving innate lymphocytes, eosinophils, mast cells, and T-cells are hallmarks of pulmonary allergic inflammation [5]. However, it is not uncommon to have a mixed inflammatory response following allergen exposure that includes IL-17 and a mixed eosinophilic-neutrophilic response [6]. Both host-intrinsic and allergen-specific factors likely influence the nature of the immune response to environmental allergens.

House dust mites (HDM) are the most common indoor

environmental allergens [7–9]. A number of allergenic proteins have been identified in dust mites and common house dust contains these HDM proteins among other molecules known to activate innate immune responses [10–12]. In mouse models of allergic inflammation, HDM are known to induce robust type-2 inflammatory responses [13]. Interestingly, HDM are also known to induce a pattern of gene expression that has the features of an interferon-induced antiviral response in rodents and humans [14–18]. Type I interferon (IFN- α/β) and type III (IFN- λ) are the major innate antiviral cytokines responsible for inducing a program of gene expression that provides initial protection from viral infection.

Viral and bacterial microbes are known to have a significant interplay with the lung influencing the development of asthma or acute asthma exacerbations [19]. For example, severe early childhood respiratory syncytial virus-induced bronchiolitis is strongly linked with subsequent asthma development [20]. Moreover, rhinovirus infection is the most frequent cause of acute exacerbations of asthma and asthmatics are at higher risk of serious complications from infection with seasonal influenza [19]. Infection with *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*, are common atypical bacterial infections known to exacerbate asthma [21]. Microbial products can also significantly impact the development or worsening of allergic inflammation. We previously reported that the Community Acquired Respiratory Distress Syndrome toxin produced by *M. pneumoniae* induced allergic-

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type inflammation in naïve rodents and primates [22–24]. Many environmental allergens, including HDM, are complex mixtures that include both the allergenic proteins and other molecules such as lipopolysaccharide capable of stimulating immune responses [11,12]. The innate immune stimulus provided by the non-allergen components of an allergen mixture potentiate allergic immune responses and likely have yet to be defined influences on the pathogenesis of asthma.

Although it is clear that infection can exacerbate the clinical manifestations of allergic disease, the influence of allergen exposure on the pathogenesis of subsequent pulmonary infections are poorly understood. In particular, the influences on pathogen burden and overall disease are not well understood. To further investigate the impact of HDM exposure on viral pathogenesis, we evaluated H1N1 influenza A infection after a single or multiple exposures to HDM in a mouse model of asthma-associated seasonal influenza infection.

2. Materials and methods

2.1. Mouse strains and procedures

Male and Female 5 week-old BALB/cJ, C57BL/6 J, C3H/HeJ, or C3H/HeOuJ mice were purchased from Jackson Laboratory (Bar Harbor ME). Mice were acclimatized to the vivarium for a minimum of 10 days after arrival and prior to experimental use. All animals were housed in an ALAAC-approved facility under specific pathogen-free conditions in isolator cages with soft bedding, a 12-hr light/dark schedule, and free access to food and water. All procedures were approved by the Institutional Animal Care at the University of Texas Health Sciences Center San Antonio. During experimental procedures, animals were monitored by laboratory staff daily and general health was evaluated by monitoring body weight, coat appearance, and mobility. Mice were considered moribund if they lost 30% of their body weight for two consecutive days. Mice maintaining a 30% body weight loss were subsequently euthanized by carbon dioxide exposure. Animal suffering was minimized by providing free access to food and water, animals had soft bedding for the duration of the experiments and all procedures were done under inhaled general anesthesia, 3% isoflurane and oxygen.

2.2. Allergen exposure and infection with influenza

The *D. pteronyssinus* house dust mite extract (HDM) was obtained from Greer Laboratories (Lenoir, NC) and was resuspended in sterile PBS at a concentration of 1 mg (protein)/ml. Groups of male or female 6–7 week old mice were anesthetized with 3% isoflurane in oxygen (1 L/min) and randomly assigned to be treated intratracheally with HDM (100 µg per dose in 100 µl) or PBS (100 µl per dose) (Fig. 1). Influenza A (PR8/H1N1) challenges were performed intratracheally with 400 plaque forming units (PFU) in 100 µl PBS. Mice were challenged either 18 h following HDM exposure for single exposures or 48 h after the last HDM exposure for the multiple exposure studies. Depending on the experiment, body weight was monitored for 10 or 14 days following infection.

2.3. Influenza A virus

The H1N1 influenza A virus (A/PR/8/34) was purchased from the American Type Culture Collection (Manassas, VA). Mice were infected intratracheally under sedation with 400 or 4000 PFU of virus, 400 PFU is at the LD₅₀ for this PR8 isolate in BALB/cJ mice.

2.4. Poly I:C

Polyinosinic:polycytidylic acid (poly (I:C)) was purchased from Millipore (Darmstadt, Germany; 528906) and resuspended in sterile PBS at a concentration of 1 mg/ml.

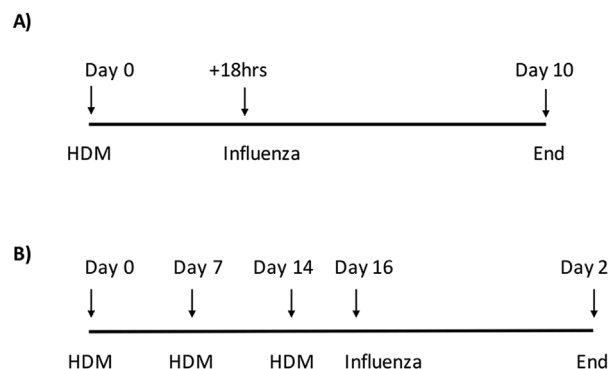


Fig. 1. House dust mite exposure scheme **A)** short term exposure model, mice are exposed once to 100 µg of HDM IT and are infected with 400 PFU of influenza 18 h later. Control mice are treated with 100 µl saline IT and are then infected with influenza. **B)** long term exposure model, mice are exposed IT to 100 µg of HDM once a week for three weeks and are infected with 400 PFU of influenza virus 48 h after the last HDM treatment. Control mice are exposed three times IT with 100 µl of saline and infected with 400 PFU 48 h after the last exposure to saline.

2.5. Histopathological analysis

BALB/cJ (3 mice/treatment/time point) mice were treated once or 3 × with HDM or saline. Mice treated once with HDM were infected with 400 PFU influenza A 18 h after HDM exposure and mice treated 3 × were infected with 400 PFU of influenza 48 h after the last HDM exposure. On days 2, 5, 8, and 10 after infection, lungs were harvested and fixed 2–3 days in 10% formalin, and embedded in paraffin. Lungs were cut (4 µm thin sections) and stained with hematoxylin and eosin by Histology & Immunohistochemistry Laboratory of the University of Texas Health Sciences Center San Antonio. Histopathology was evaluated by two individuals blinded to the treatments. Images of the representative conducting airways were taken on a Zeiss microscope using an Axiocam HR color digital camera and the Axiovision suite of software V.4.5. Pulmonary pathology was quantitatively evaluated using the panel of standards method as we have previously described [23–25].

2.6. qRT-PCR

Female 6–7 week-old BALB/cJ mice were randomly assigned into groups, they were anesthetized with isoflurane and challenged with HDM or PBS once or three times with the dose described above. Eighteen hours following HDM exposure, lungs were harvested and preserved in Invitrogen RNeasy Lysis Solution as per manufacturer's instructions and stored in liquid nitrogen prior to RNA extraction. Total RNA from each lung was extracted by TRI reagent (Sigma-Aldrich, MO) and chloroform (Sigma-Aldrich, MO) phase separation method and further purified with RNeasy Mini Kit (Qiagen, MA). Nanodrop ND-1000 (NanoDrop Technologies, USA) was used for RNA quality and concentration determinations. The cDNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, NL) and PCT-200 Peltier Thermal Cycler. Real-time qRT-PCR was performed in StepOnePlus Real-Time Pcr System (Applied Biosystems, NL) with TaqMan gene expression assays (Applied Biosystems, NL) for the following genes: GAPDH, IFN-λ and IFN-β. Data was analyzed by using the comparative ΔCt method and control condition were used for comparison.

2.6.1. Determination of viral burden

To determine viral burden, three mice from each group were euthanized four days after infection and total RNA was prepared from their lungs as described above. The RNA was used for determining viral

load with Real-Time PCR as described [26,27]. The H1N1 influenza PA gene on plasmid pHW:193 was used to generate standard curves for calculating the copies of the polymerase (PA) genes per 1 mg lung cDNA. The pHW:193 plasmid was a kind gift from Dr. Richard Webby at St. Jude's Children's Hospital. The following primers were used: forward primer, 5'-CGGTCCAAATTCCTGCTGA-3'; reverse primer, 5'-CATTGGG TTCCTCCATCCA-3'; probe, 5'-6-FAM-CCAAGTCATGAAGGAGAGGGA ATACCGCT-3'.

2.7. Statistics

Results are expressed as the mean $\pm$ S.D. Statistical differences were determined using a two-way ANOVA and post test analysis by using GraphPad Prism version 5.04 (GraphPad Software, San Diego CA).

3. Results

3.1. Exposure to HDM induced expression of interferon β and λ

Others have reported that airway cells exposed to HDM respond with a pattern of gene expression consistent with an antiviral response [17]. To test expression of antiviral genes after lung exposure to HDM, C57BL/6 J mice were exposed either once or three times intratracheally to 100 μ g of HDM extract as outlined in Fig. 1. Lungs from singly exposed mice were collected at either 8 or 18 h following HDM exposure. Lungs from mice exposed three times to HDM were collected 48 h following final HDM exposure. At 8, 18 or 48 h after the last exposure to HDM, lungs were harvested and RNA prepared for qRT-PCR. The

expression of the major antiviral interferons (IFN), β and λ were determined using Taqman qRT-PCR. As shown in Fig. 2, exposure to HDM results in significant changes in IFN expression. IFN- β expression is not significantly different than controls at 8 and 48 h after exposure to HDM (Fig. 2A and C) and is significantly down regulated in the lungs of mice 18 h after one exposure to HDM extract (Fig. 2B). In contrast, IFN- λ is significantly up-regulated at all the time points tested. These data suggest that mice exposed to HDM up-regulate the expression of IFN- λ in vivo while not changing expression or modestly down-regulating IFN- β expression at the time points tested. Importantly, to confirm our ability to detect IFN expression in the lungs of mice at early time points, mice were treated IT with 50 μ g the TLR3 agonist polyI:C (Fig. 2A). PolyI:C is a potent stimulator of IFN and antiviral gene expression. The polyI:C treated mice expressed readily detectable IFNs β and λ suggesting the lack of IFN- β expression was not due to an inability to detect expression. As shown in Fig. A1, BALB/cJ showed similar patterns of interferon expression following HDM exposure suggesting this pattern of gene expression occurs in several species of mice.

3.2. Mice treated with HDM are partially protected from H1N1 influenza A infection

IFN expression following HDM exposure suggests that these animals are more resistant to respiratory viral infections. To test this hypothesis, C57BL/6 J mice were exposed to HDM and then 18 h later they received a sub-lethal dose of H1N1 influenza A. Mice were then observed for changes in body weight as a surrogate measure of general health. As shown in Figs. 3A and A2, male and female mice treated with saline and then infected with influenza lost significantly more weight than mice

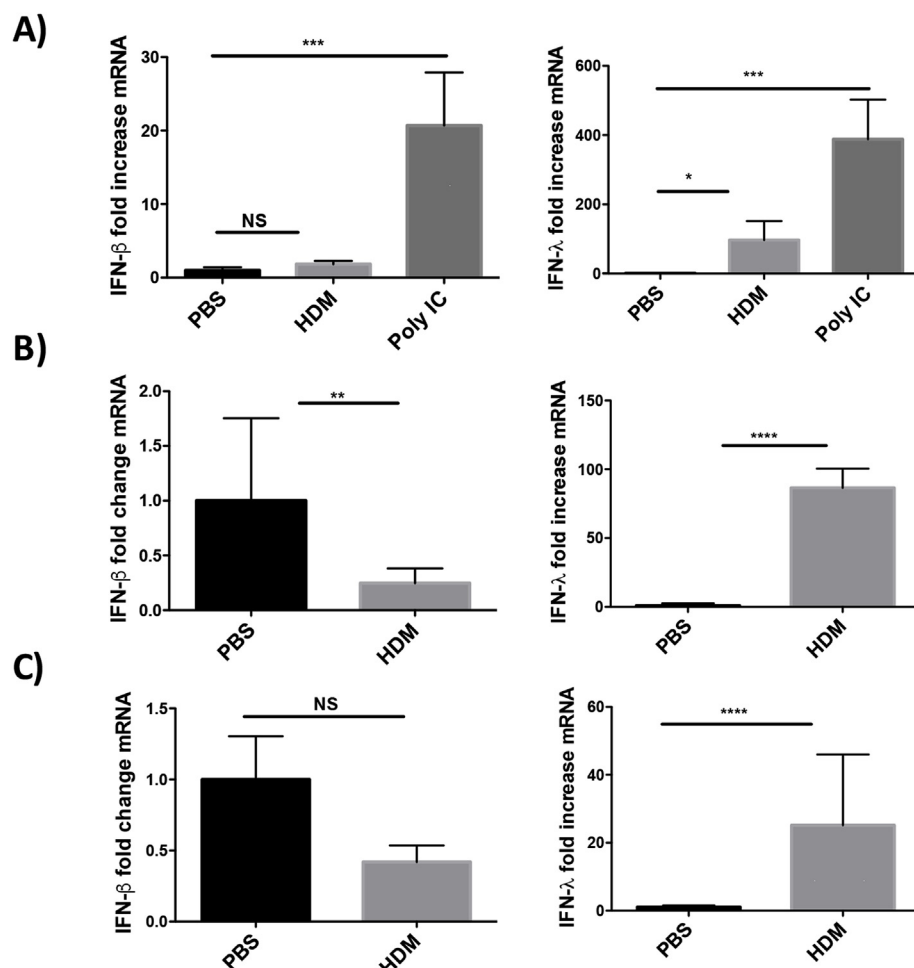


Fig. 2. House dust mite exposure induces interferon expression. C57BL/6 J mice were treated once with 100 μ g of HDM or 100 μ l of saline and lungs were harvested at 8 h (A), 18 h (B), or three times weekly (C) and lungs were harvested 48 h after the last HDM treatment. Lungs were prepared for qRT-PCR and the expression of IFN- β and IFN- λ was determined using Taqman qRT-PCR. For the 8hr time point a positive control was included and mice were treated IT with 50 μ g of Poly I:C. Data is representative of two independent experiments with 3 mice per group. Data was analyzed by ANOVA with Dunnett's multiple comparisons test **p = 0.005, ***p = 0.0005, NS not significant.

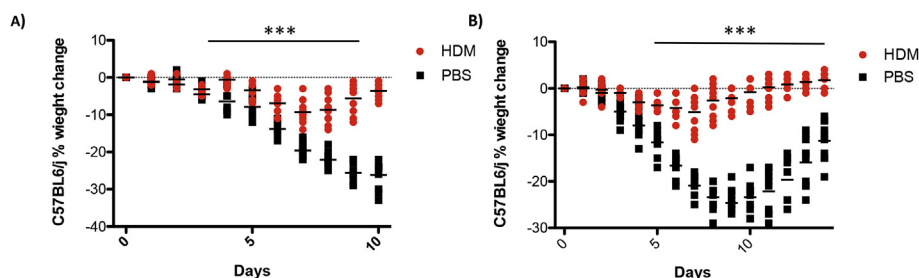


Fig. 3. House dust mite exposure protects against influenza A infection. C57BL/6 J mice were treated once for 18 h (A) or three times (B) with 100 μ g of HDM or 100 μ l of saline. Mice were challenged IT with 400 PFU of influenza and body weight was determined daily. Data represents 10 mice and was analyzed by ANOVA *** p = 0.005, **** p = 0.0005.

exposed to HDM. Similar results were observed when mice were exposed to HDM three times prior to influenza exposure (Fig. 3B). Mice treated three times with HDM extract lost substantially less weight than mice treated once. Altogether these data suggest that mice exposed to HDM are more resistant to influenza infection than control animals.

3.3. Mice treated with HDM have lower viral burdens than controls

Mice treated with HDM and subsequently infected with influenza lose less body weight than control animals. These observations suggest that HDM-induced antiviral gene expression is impacting viral burdens in the lung. To evaluate viral burdens in the lungs of mice infected with influenza virus, we determined the levels of polymerase acidic (PA) gene mRNA in infected lung tissues by Taqman qRT-PCR. Mice were treated once or three times with HDM or saline as a control, and subsequently infected with 400 PFU of influenza A virus. Four days after infection, lungs were harvested for qRT-PCR analysis of PA mRNA. As shown in Fig. 4, animals treated with saline had significantly more PA mRNA present in lung tissues than animals treated with HDM suggesting that HDM treatment reduces viral burden.

3.4. HDM-mediated partial protection from viral infection is dose and timing dependent

Although HDM exposure partially protects mice from influenza after one or three exposures, it was unclear if animals exposed to high-dose influenza, with chronic HDM exposure, or re-exposed to allergen would maintain protection. To test protection from high-dose influenza infection, mice were exposed to HDM or saline three times and then challenged with a lethal dose of influenza (4000 PFU). As shown in Fig. 5, when mice are challenged with a lethal dose of influenza virus, pre-exposure to HDM does not protect against disease and animals lose weight with kinetics similar to the saline-treated control mice. To evaluate HDM-mediated protection from influenza virus in the context of chronic allergic inflammation, mice were treated once a week for eight weeks with HDM or saline and then challenged with 400 or 4000 PFU of influenza. As shown in Fig. 6A, mice with chronic allergic inflammation are significantly protected from infection with 400 PFU of influenza. When challenged with 4000 PFU mice that had been exposed

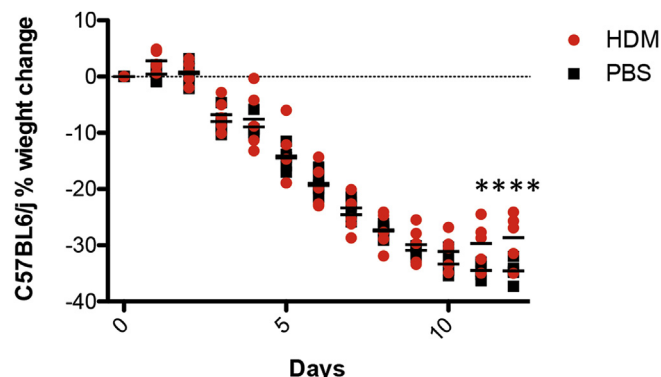


Fig. 5. House dust mite exposure does not protect against a lethal influenza challenge. C57BL/6 J mice were exposed three times to 100 μ g of house dust mites or saline as a control. Forty-eight hours after the last exposure to HDM or saline mice were infected with 4000 PFU of influenza A. Body weight was monitored daily. N = 5 mice/group, ANOVA determined statistical significance **** p = 0.0005.

to HDM eight times initially lost weight with the same kinetics as the saline controls but began to recover faster (Fig. 6B). These data did not reach statistical significance but animals exposed to HDM demonstrated a rate of recovery in animals treated 8X with HDM that was faster than animals treated 3X (Figs. 3 and 5). These data suggest that additional exposures to HDM provide limited benefit to mice challenged with high-dose influenza compared to the significant protection provided against sub-lethal infection.

It is clear that viral infections are a critical driver of acute exacerbations of human asthma [19]. To evaluate the effect of HDM exposure after virus infection, mice were sensitized to HDM through two exposures to HDM. Mice were subsequently infected with 400 PFU of influenza virus and challenged with HDM five days later (Fig. 7A). Most of the mice in both the HDM and control groups had to be euthanized between days 6 and 7 due to excessive body weight loss. One animal in each group survived past 7 days with the PBS treated animal ultimately recovering while the HDM treated animal required euthanasia (Fig. 7B and C). Altogether, these data suggest that HDM exposure does not

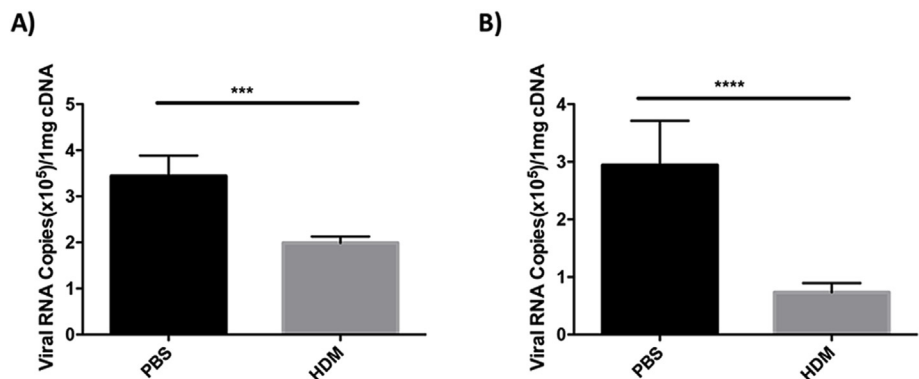


Fig. 4. Exposure to house dust mite reduces viral load. C57BL/6 J mice were exposed to HDM or saline IT once (A) or three times (B). Mice were infected IT with 400 PFU of influenza A and lungs were harvested 4 days after infection and prepared for Taqman qRT-PCR. As a marker for viral replication we determined the levels of polymerase acidic (PA) gene mRNA in the lungs of infected mice. Data is representative of two experiments N = 3/group. Statistical significance was determined by ANOVA *** p = 0.005, **** p = 0.0005.

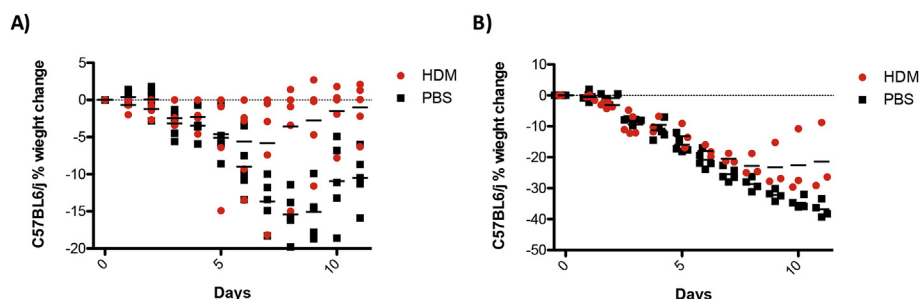


Fig. 6. Chronic exposure to house dust mite protects against influenza infection. Mice were exposed to HDM or saline weekly for eight weeks prior to infection with 400 or 4000 PFU of influenza A. Body weights were monitored daily. $N = 5$ mice/group ANOVA determined statistical significance **** $p = 0.0005$. Note that statistical significance with a multiple measures ANOVA could not be determined in (B) due to unequal group sizes after day 5.

protect against high-titer viral challenge and that challenge with allergen shortly after viral infection exacerbates disease.

3.5. HDM-mediated protection from influenza virus infection is independent of TLR4 signaling and occurs in multiple strains of mice

House dust and HDM extracts are complex mixtures with numerous molecules capable of stimulating innate immune responses. One of the most potent innate immune-stimulating components of HDM extracts is lipopolysaccharide (LPS). To test if LPS-stimulated responses were responsible for HDM-mediated protection from influenza infection, we evaluated responses in C3H/HeJ mice. C3H/HeJ mice have a spontaneous mutation in TLR4 (*Tlr4^{Lps-d}*) that inactivates the signaling functions of TLR4 [28]. C3H/HeJ mice were sensitized three times to HDM or saline and were then challenged with 400 PFU of influenza virus. As shown in Fig. 8A, C3H/HeJ mice respond similarly to the TLR4 sufficient C3H/HeOuJ mice (Fig. 8B). C3H/HeOuJ are genetically very similar C3H/HeJ mice but they are capable of signaling through TLR4. These data suggest that TLR4 signaling does not significantly contribute to HDM-mediated resistance to influenza infection. As shown in Fig. 3, C57BL/6J mice display HDM-mediated resistance to influenza infection. However, BALB/cJ mice are more susceptible to developing allergic inflammation relative to the C57BL/6J mouse [13]. To test the ability of HDM to protect BALB/cJ mice, BALB/cJ mice were treated once or three times with HDM or saline and were subsequently challenged with 400 PFU of influenza virus. As shown in Fig. 9, exposure to HDM provided significant protection from influenza infection in BALB/cJ mice. Similar to what was observed with C57BL/6J mice, BALB/cJ mice exposed to HDM express significantly more IFN- λ than controls while the levels of IFN- β are not substantially different at the time points tested (Supplemental Fig. 1). Altogether, these data suggest that TLR4 is not required for HDM-mediated protection from influenza virus

infection and that HDM can induce protection in multiple strains of mice (BALB/cJ, C57BL/6J, C3H/HeJ, and C3H/HeOuJ).

3.6. Mice exposed to HDM and influenza A virus have histological evidence of mixed inflammatory responses

As described above, mice exposed to HDM extracts have better outcomes compared to animals exposed to saline. To further test the pulmonary response to influenza A infection following HDM exposure, BALB/cJ mice were treated once or three times with HDM or saline and then challenged with 400 PFU influenza A virus. Mice were randomized and cohorts of HDM treated or controls were euthanized on days 2, 5, 8, and 10. Lungs were removed and prepared for histology. Thin sections (4 μ m) were stained with hematoxylin and eosin and inflammatory pathology was evaluated microscopically (Figs. 10 and A3). Animals treated once with HDM and then infected with influenza virus had no evidence of allergic inflammation. These animals developed evidence of viral pneumonia including inflammatory infiltrates, denuded epithelium, areas of consolidation, and exudate in the conducting airways by day 5 post-viral infection. Animals treated with saline had more severe disease that encompassed greater areas of lung tissue however, these data did not reach statistical significance (Fig. A3A). Animals treated with HDM three times, as expected, had histology consistent with mild allergic inflammation including, peribronchiolar and perivascular lymphocytic and leukocytic cellular inflammation, eosinophilia, and epithelial hyperplasia. Following infection with influenza virus, both the HDM-treated and control mice had evidence of viral pneumonia. However, the control animals had more severe pneumonia based on the extent of tissue involvement and damage that was statistically significant on day 5 (Fig. A3B). These findings are consistent with the differences in viral burdens and body weight maintenance.

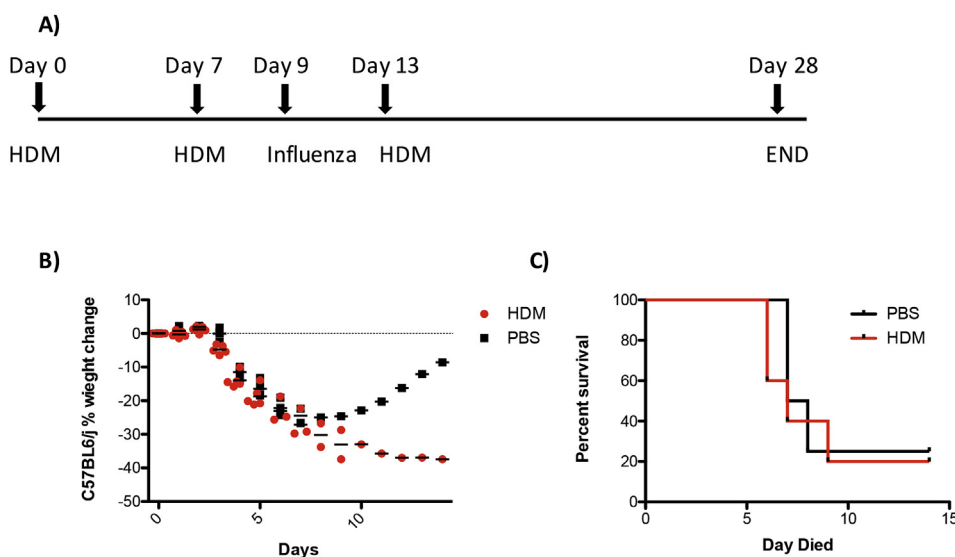


Fig. 7. House dust mite exposure of sensitized animals after influenza infection worsens outcomes. Mice were exposed to 100 μ g of HDM or 100 μ l of saline twice a week apart. Mice were then infected IT with 400 PFU of influenza virus 48 h after the second exposure to HDM or saline. Four days after influenza virus infection mice were challenged with 100 μ g of HDM or 100 μ l of saline. Body weight was monitored daily. Mice were euthanized if body weight loss $\geq 30\%$ of the initial body weight. (A) schematic of experiment (B) Body weight loss (C) data from (B) plotted as a survival graph. $N = 5$ mice for the HDM group and $N = 4$ for the saline group.

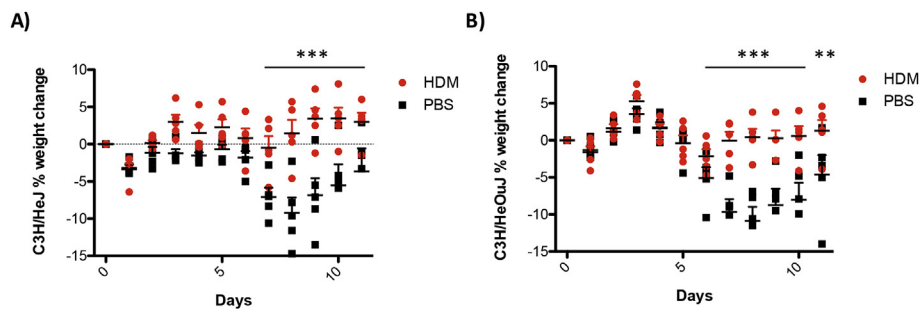


Fig. 8. House dust mite-mediated protection from influenza infection is independent of TLR-4. (A) TLR-4 deficient C3H/HeJ mice were exposed IT to 100 µg of HDM or 100 µl of saline weekly for three weeks. Forty-eight hours after the last exposure, mice were challenged with 400 PFU of influenza A. (B) TLR-4 sufficient C3H/HeOuJ were treated similarly. Body weight was monitored daily. N = 5 mice/group ANOVA determined statistical significance. **p = 0.05, ****p = 0.0005.

4. Discussion

The lung is an organ that is in constant contact with the outside environment. This exposes lung tissues to a variety of insults including potentially allergenic materials, chemical pollutants, and pathogenic organisms amongst others. How the respiratory system responds to these encounters depends on the nature of the material, genetics, immune status, and comorbid conditions. Allergic disease affects a significant proportion of the population with an estimated 40–50% of children worldwide being sensitized to at least 1 common allergen [1–3,29]. Although these rates encompass all manifestations of allergic disease, allergic diseases impacting the respiratory tract, allergic rhinitis and asthma, account for a large fraction of allergic disease in the US. There are an estimated 26 million Americans with asthma [1]. Many of these individuals will have an acute exacerbation of their disease associated with a microbial infection, most often a rhinovirus infection.

Although it is clear that infection can exacerbate features of allergic disease [19,22–25], the consequences for the pathogenesis of the infectious agent are less understood. Others have suggested that exposure to HDM inhibits antiviral responses but this was not tested with an actual viral infection in vivo [30]. To investigate responses to a natural environmental allergen, we utilized a mouse model of HDM mucosal sensitization. This was coupled with sub-lethal influenza A infection to model viral infection secondary to allergen exposure. Although the most common virus linked to asthma exacerbation is rhinovirus [31,32], the mouse poorly models human rhinovirus infection. The mouse model of influenza virus infection recapitulates many aspects of human seasonal influenza but also has some limitations in terms of human disease pathogenesis [33]. The goal of this study was to determine the impact of allergen exposure on viral infection. We did not investigate physiological aspects of asthma exacerbation such as hypoxia, changes in pulmonary function, and changes in airway remodeling.

Surprisingly, we observed that a single exposure to HDM significantly reduced the weight loss associated with influenza infection. This correlated with decreased viral mRNA expression and the increased expression of antiviral interferon λ in the lungs of animals exposed to HDM with no change or diminished expression of interferon β at the time points tested. These data suggest that dust mite induced

antiviral responses are mediated by IFN- λ yet this needs to be formally tested. The ability of HDM to induce both IFN- λ and antiviral gene expression has been reported by others and suggests innate immune sensors recognize materials in HDM [14,16]. IFN- λ is predominantly expressed by the epithelium in a TLR3-dependent manner [34]. Although IFN- λ is an antiviral cytokine, it is also known to have anti-inflammatory properties [14–16,18,35–37] and it is currently unknown if the improved outcomes observed with HDM-exposed mice infected with influenza virus is due to the antiviral effects of interferon or reduced inflammation. The pathogen associated molecular pattern molecule (PAMP) present in HDM that induces IFN is currently not known. However, both HDM extracts and house dust contain LPS which is known to induce type I and II interferons and antiviral gene expression from monocyte derived cells.

To test the impact of TLR4 signaling on HDM-mediated resistance to influenza infection, we treated TLR4 deficient C3H/HeJ mice with HDM or saline and then infected the mice with influenza. Similar to the very closely related TLR4 sufficient C3H/HeOuJ mice, as well as BALB/cJ, C57BL/6J, C3H/HeJ mice showed significantly less body weight loss when treated with HDM and infected with influenza relative to controls. These data suggest LPS is not a significant factor in HDM-mediated protection from influenza infection. These data are consistent with the limited expression of IFN- β following HDM exposure since the TLR4/TRIF pathways predominantly mediate LPS-dependent type-I IFN responses [38].

Multiple strains of mice (BALB/cJ, C3H/HeJ, C3H/HeOuJ, and C57BL/6J) are protected from influenza infection following one or three exposures to HDM. We tested whether this protection could be bypassed if animals were exposed to a lethal dose of influenza (4000 PFU). HDM-exposed mice challenged with 4000 PFU of influenza had the same rate of body weight loss as control animals losing 25–30% of their initial body weight by day seven post infection. The HDM-exposed animals did begin to regain weight faster than controls but these data suggest that high-dose exposure to influenza can overcome protection provided by three exposures to HDM. Likewise, to test if chronic allergic inflammation impacted resistance to influenza infection, mice were treated eight times with HDM prior to influenza challenge. Mice treated eight times with HDM did significantly better with a reduced peak weight loss compared to animals treated once or three times when challenged with 400 or 4000 PFU of influenza. These data suggest that

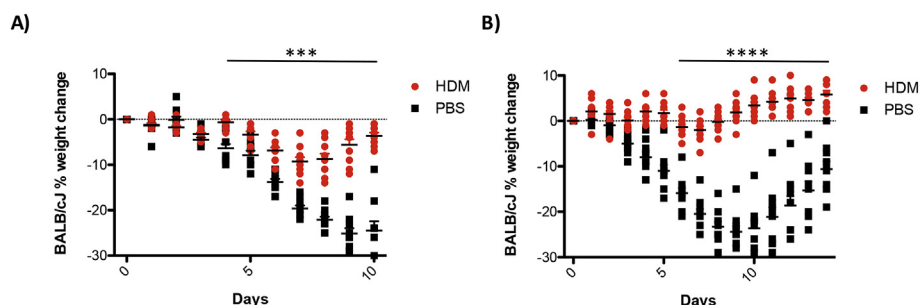


Fig. 9. HDM exposure protects BALB/cJ mice from influenza infection. Mice prone to allergic inflammation, BALB/cJ, were evaluated for protection from influenza infection. Mice were treated as outlined in Fig. 1. Both mice exposed once (A) or three times (B) were protected from influenza A virus infection. N = 10 mice/group statistical significance was determined by ANOVA. ***p = 0.005, ****p = 0.0005.

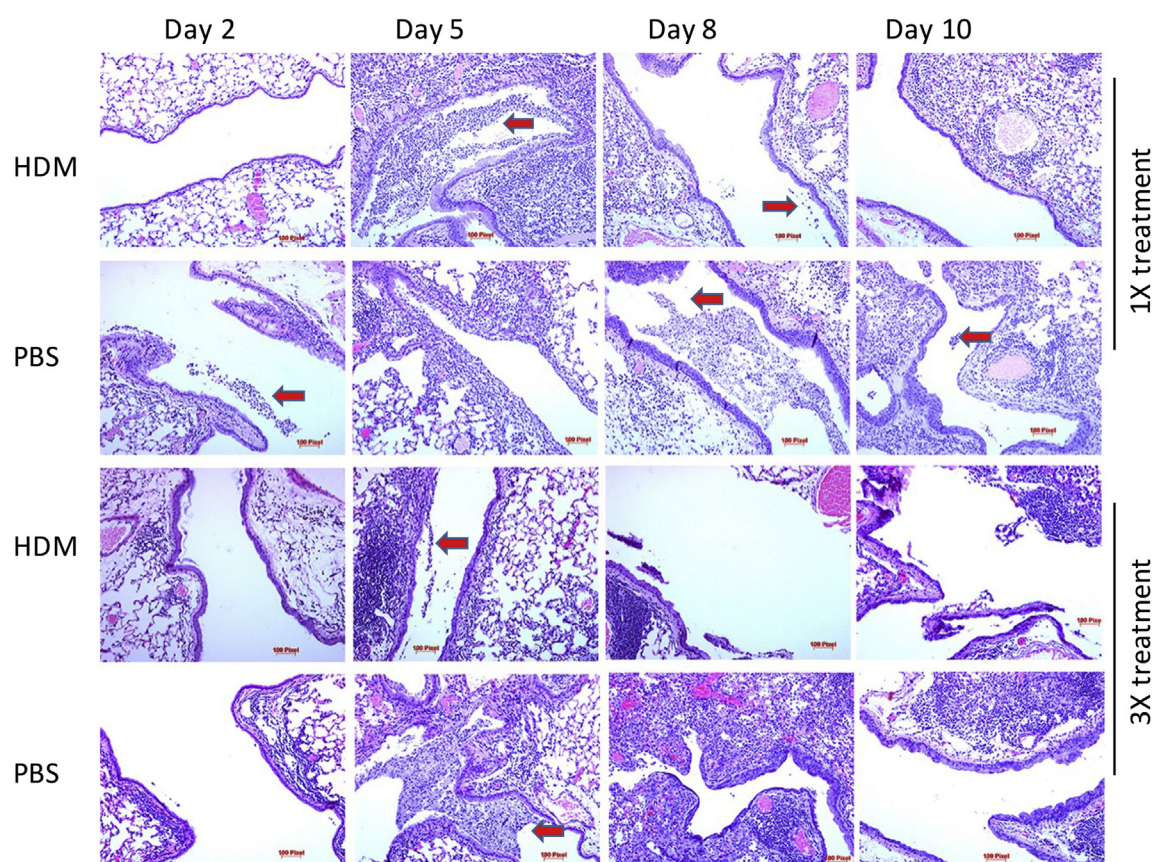


Fig. 10. Inflammatory histopathology of BALB/cJ mice infected with influenza A. BALB/cJ mice were exposed to saline or HDM once or three times prior to being infected IT with 400 PFU of influenza A. Mice were randomized and 3 mice/treatment/time point were euthanized at the indicated time and lungs were removed for histological analysis. Inflammatory pathology was evaluated by investigators blinded to the treatment groups. Images of representative large airways were obtained at 10 $\times$ magnification. Red arrows indicate inflammatory exudate in the conducting airways.

protection from influenza infection is maintained in a chronic model of inflammation. However, when sensitized mice were infected with influenza and then challenged with HDM they developed severe disease and succumbed quickly suggesting that an allergic challenge worsens disease during an active viral infection. It is currently unknown why mice are protected from viral infection if they are exposed to virus after an allergen challenge but succumb quickly if the allergen challenge occurs during active viral infection.

Although using the mouse model of HDM-induced allergic inflammation we observed protection from influenza infection, this mouse model does not reflect human severe refractory asthma [13]. Severe asthmatics are at highest risk of significant complications from respiratory infection [39]. Additionally, these mice were not treated with medications such as inhaled corticosteroids or bronchodilators that are commonly taken daily by individuals with asthma. The severity of disease, medication usage and effectiveness, and other comorbid conditions all impact the ability of individuals with asthma to respond to respiratory infections.

During the 2009 pandemic influenza outbreak it was noted that asthmatics were more likely to be hospitalized but were less likely to have severe outcomes [40–43]. The reasons for these observations are not clear but it has been associated with inhaled corticosteroid use [42] and eosinophilia [44]. We did not evaluate steroids in our model. Eosinophils were shown to be sensitive to influenza infection and to function as antigen presenting cells to promote anti-viral CD8⁺ T-cell responses [44]. We did not specifically test the role of eosinophils in HDM-mediated protection from influenza infection. The protection observed in mice treated three times or eight times with HDM could be consistent with eosinophil-mediated responses as these animals have

moderate to robust allergic inflammation respectively. However, animals treated once with HDM for eighteen hours are protected from influenza infection and there is no histologic evidence of allergic inflammation. Moreover, when we examined expression of cytokines and chemokines critical for recruitment of eosinophils to the lungs, IL-5, eotaxin-1 and 2, and saw no differences in expression levels in mice treated once with saline or HDM for 18 h (not shown). These data suggest that there are multiple factors contributing to the HDM-mediated anti-viral response.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2019.02.019>.

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Appendix

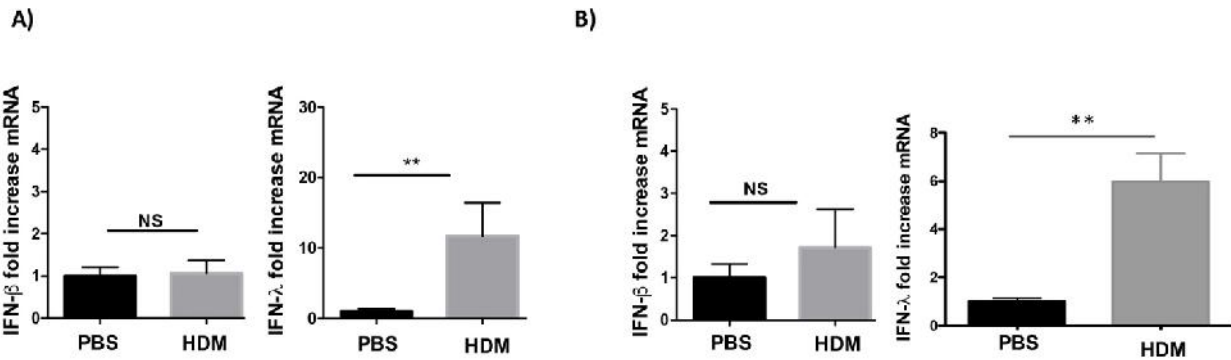


Figure A.1 Antiviral interferon expression in the lungs of BALB/cJ exposed to HDM. Mice were treated once or three times with HDM or PBS as described in Figure 1. Lungs were removed 18hrs after a single exposure (**A**) or 48hrs after three exposures (**B**) and RNA extracted for qRT-PCR. Taqman assays determined relative expression of IFN- β and IFN- λ mRNA.

**p=0.005

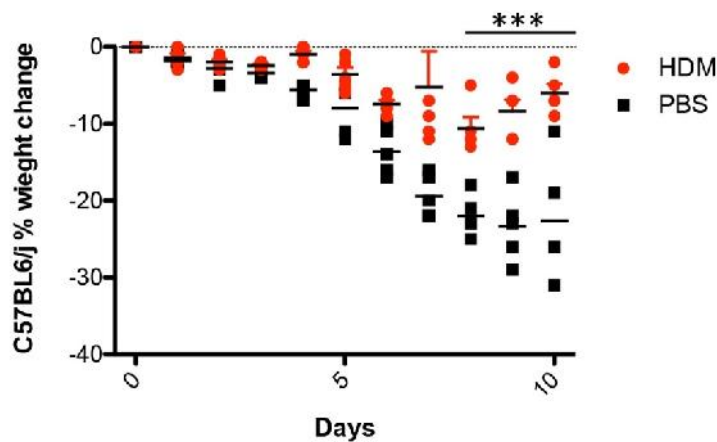


Figure A.2 Male C57BL/6J mice are protected from influenza A infection following exposure to HDM. Male C57BL/6J mice (n=5) were exposed to 100 μ g of HDM or PBS and 18hrs later they were infected with 400PFU of influenza A. Body weights were monitored for 10 days and data are presented as percent body weight loss. ***p=0.0005

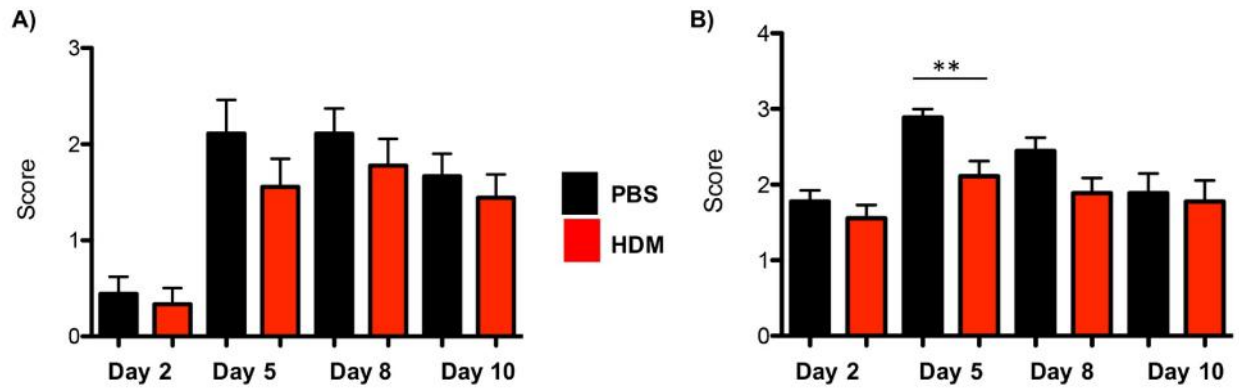


Figure A.3 BALB/cJ mice have less severe inflammatory pathology associated with viral pneumonia following HDM exposure. Inflammatory pathology in Figure 10 was quantitatively evaluated by 3 individuals blinded to treatment groups by the panel of standards method. Scores ranged from 0= least severe pathology to 3= most severe. (A) single exposure to HDM and (B) three exposures to HDM. ** p=0.005