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# An Improved Model of House Dust Mite Allergic Sensitization

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*An Improved Model of House Dust Mite Allergic Sensitization*

**Abstract**

The existing mouse models for allergic asthma are incapable of reproducing all of the immunogenic and physiological features of allergic asthma as seen in humans. Additionally, these models induce some changes inconsistent with human disease. Reasons for these inconsistencies could be the amount of allergen to which the mice are exposed, the way they are exposed, and inadvertent stress brought about through animal handling. To address these issues, we attempted to create a novel mouse model of allergic asthma induction in which mice were persistently (for 6 weeks) exposed to zero, low (1 $\mu$ g/g), medium (10 $\mu$ g/g), and high (100 $\mu$ g/g) doses of house dust mite extract based on the Derp1 content/g inoculated into their bedding nestlets. For this model, we based the amount of allergen inoculated into the bedding on epidemiological studies, which indicate that 5.5% of children living in homes containing a concentration of 10 $\mu$ g Derp1/g of carpet dust are allergically sensitized to house dust mite [1]. Therefore the doses of HDM delivered to the mice were more physiologically relevant and similar to what humans are exposed to. The mice were not handled except for routine veterinary care and they were exposed to HDM through normal breathing instead of intranasal instillation. At the end of the exposures, airway hyperresponsiveness was assessed and immunophenotyping was performed. We found no significant differences in the methacholine hyperresponsiveness, the production of IgG1, IgE, and IgG2a, or the cytokine production between the treatment groups. Since our model did not elicit methacholine hyperresponsiveness or HDM-specific immune responses, future studies will determine whether allergen sensitization elicited through a single intranasal

instillation of 1  $\mu\text{g}$  HDM extract, followed by the 6-week persistent exposure to varying concentrations of HDM in the manner described above, elicits changes consistent with those found in human allergic asthmatics.

## **Introduction**

Asthma is a common chronic disorder of the airways that involves a complex interaction of airway reactivity and inflammation [2]. About 300 million people suffer from asthma worldwide [3], and the US asthma prevalence is approximately 1 in 12 adults and 1 in 11 children [4]. The development of allergic asthma is linked to allergen sensitization, which is the act or process of inducing an acquired allergy.

Asthma has been recognized as a set of symptoms related to difficulty in breathing since ancient times in China, Mesopotamia, and Greece [5]. Over the course of centuries the complexity of the disease has become more appreciated. In an attempt to explain the mechanism behind asthma, the Th2-inflammation paradigm was described and explored by investigators in order to gain a better understanding of allergic asthma. In this process, exposure to an allergen in combination with genetic susceptibility leads to a heightened Th2-immune response that upregulates eosinophilic inflammation and tissue damage, causes airway hyperresponsiveness, and eventually leads to symptoms. However, this hypothesis is too simplistic and can't account for variations in the clinical presentation of asthma or responses to treatment [6].

Today there is an understanding that asthma is a very heterogenic disorder with affected individuals displaying a wide variety of clinical symptoms, pathologies, and responses to treatments [7]. The development and manifestation of asthma is the result of

complex interactions between genetic features that make individuals more susceptible to atopy and airway hyperresponsiveness, the environment, and the natural course of the disease. The clinical presentation of asthma can be separated out into different phenotypes and then further into endotypes, or subgroups, based on the pathophysiologic features, including clinical characteristics, biomarkers, lung physiology, genetics, histopathology, epidemiology, and response to treatment [7]. It is important to consider different endotypes of asthma because they can provide insight into the mechanisms behind different physiologies that cause the symptoms characteristic of asthma. It is also important to create mouse models to align with the different endotypes seen in humans to gain a better understanding of the specific pathologies so that effective treatments can be designed.

Using mouse models has been an important strategy for learning about the mechanisms and pathology associated with allergic asthma [7]. Traditional animal models utilized Alum/OVA with a standard time course for sensitization and challenge. In an attempt to better model human asthma, the traditional models have been supplemented with many physiologically relevant sensitizing agents, allergens and triggers; chronic time courses that better represent the natural history of asthma; and measures of lung remodeling that represent the diverse range of clinically observed indications of asthma [7]. The Th2 Hypothesis was one of the first targets of asthma research that utilized technology for genetically modified mice and molecular profiling methods. The result is that the mechanisms of Th2 driven asthma are now understood down to the molecular level [8]. Understanding all of the potential mechanisms of

asthma in this level of detail is the goal of current research and this is why the creation of animal models that represent the different endotypes of asthma is vitally important.

While progress has been made to better align animal models with human endotypes of asthma, existing mouse models are still insufficient. The flaws of these models come from the allergic sensitization of the mice depending on irrelevant means, routes, doses, and duration of antigen exposure; the imposition of stress on the mice due to excessive handling; and prohibitive costs due to the amount of time laboratory personnel must spend exposing the mice to antigens. Additionally, the existing models lack the chronic inflammatory and epithelial changes of asthma seen in humans. They can also result in pulmonary parenchymal inflammation, alveolitis, and pneumonitis [9] along with vascular remodeling and fibrotic changes that are inconsistent with human asthma [10]. Another problem is that most models of acute allergen exposure result in airway pathologies that peak 24-72 hours after the allergen challenge and resolve in 1-2 weeks, making it difficult to study the effects of drugs designed to control the chronic pathologic processes of asthma [11]. As a result of these imperfect models, it has been very difficult to develop therapeutic treatments for humans because treatments that appear to work in mouse models more often than not fail in human trials due to the differences in physiology [12].

House dust mite (HDM) is a ubiquitous perennial allergen source and a major cause of allergic asthma worldwide. It is estimated that as much as 1-2% of the general population is sensitized to HDM and as many as 50% of asthmatic patients are reactive to HDM [13]. The major antigens found in the two most prevalent species of HDM are Derp1 and Derf1 (from *Dermatophagoides pteronyssinus* and *D. farinae*).

Epidemiological studies in humans have linked the prevalence of these two proteins in household dust to the likelihood of developing allergic sensitization. When homes contained a concentration of 10 $\mu$ g Derp1 and Derf1/g of carpet dust, a peak level 5.5% of children from 0-3 years old were found to be allergically sensitized to house dust mite. Concentrations below 0.1g Derp1 and Derf1/g of carpet dust were associated with a low risk of allergic sensitization [1]. Other studies have predicted the greatest risk of HDM sensitization to occur when people are exposed to 3.5-23.4 $\mu$ g Derp1/g of bed or airborne dust, with levels lower than 2 $\mu$ g/g considered safe [13, 14].

We attempted to create an improved model for allergically sensitizing mice to house dust mite that promotes an asthmatic physiologic and immunologic phenotype and also better reflects the natural route, dosing, and duration of allergen exposure experienced by humans. Based on the epidemiological studies, we inoculated low, optimal, and high doses of house dust mite, based on Derp1 content, into the nestlets that mice shred to prepare their bedding nests so that the allergen exposure would be persistent and through a route of exposure similar to that of humans. The mice were handled only during cage changes and for routine veterinary care. Consequently, the personnel time required for the work was substantially reduced compared to other models. The success of this model was evaluated by measuring the major parameters associated with allergic asthma, including methacholine responsiveness, airway inflammation, lung histology, and the adaptive immune response, as well as measures of systemic distress. Results from this model were compared to the Poynter laboratory's existing data from a frequently used model of intranasal house dust mite extract exposure.

## Materials and Methods

### *Mice*

3 week old female BALB/cJ and C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and acclimated for one week prior to initiating allergen exposure. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care (AALAC)-approved facility, maintained on a 12h light/dark cycle, and provided food and water ad libitum. The University of Vermont Institutional Animal Care and Use Committee approved these studies.

BALB/cJ mice were used for these experiments because they are particularly sensitive to developing airway hyperresponsiveness in response to methacholine [15]. C57BL/6J mice were used for these experiments because they are the preferred strain for making genetic models.

### *Develop an optimized model of house dust mite exposure*

Freeze-dried and pulverized extracts of whole *D. pteronyssinus* (Greer, Lenoir, NC which provides lot-specific information on total protein, endotoxin, and Derp1 content) were resuspended in sterile water at a concentration of 1mg Derp1/1mL of H<sub>2</sub>O. The resuspended HDM extract was combined with sterile water to create low (1µg/g), optimal (10µg/g), and high (100µg/g) doses based on the Derp1 content/g of nestlet. The varying doses of HDM were then inoculated onto the nestlets that mice shred to prepare their bedding nests, which were subsequently air-dried. Nestlets were also inoculated with only water to serve as a control and then air-dried. Nestlets were added to the cages of 4-week-old mice (female, C57BL/6J and BALB/cJ) for 6 weeks and replaced twice each week throughout the exposure period. Half way through the exposure, used nestlet

material was collected, weighed, and frozen until subsequent aqueous extraction and quantitation of Derp1.

*Aqueous extraction of HDM from bedding and Derp1 ELISA*

Used and unused bedding was soaked in 10mL of sterile water per gram of bedding for 30 minutes as previously described [16]. Subsequently, the water was pushed out of the bedding using a syringe. The Derp1 content of the bedding was assessed by ELISA (Indoor Biotechnologies, Charlottesville, VA). The wells of a high-binding 96-well microtiter plate (Corning, Kennebunk, ME) were coated with mAb at 10 $\mu$ L/10mL in 50mM carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plate was washed three times with PBS-0.05% Tween 20 (PBS-T) and then blocked for 30 minutes with 1% BSA, PBS-T. After this, the plate was washed three times and the standards and samples were added for 1 hour. The plate was then washed three times with PBS-T and the detection antibody was added to the plate for 1 hour. The plate was again washed three times with PBS-T and streptavidin-HRP was added for 30 minutes. The plate was then washed 4 times and chromogenic substrates A and B (R&D Systems, Minneapolis, MN) were added in a 1:1 ratio. Once the plate was developed, the reaction was stopped with 2N H<sub>2</sub>SO<sub>4</sub>. The plate was read on a BioTek PowerwaveX (Winooski, VT) instrument at 450nm with a  $\lambda$  correction of 570nm using the program Gen5 1.1. The absorbance of the samples were compared to the absorbance of the standards of known concentrations to calculate the concentrations of Derp1 present in the bedding.

*Perform in vivo assessment of methacholine responsiveness*



Mice were anesthetized by intra-peritoneal injection of sodium pentobarbital (90 mg/kg), and the trachea was dissected free of surrounding tissue and cannulated with a blunted 18-gauge needle. The mice were then connected to a SciReq *flexiVent* small animal ventilator for analysis, as has been previously described [17]. Airway resistance ( $R_N$ ), tissue damping (G), and tissue resistance (H) were calculated at baseline and after challenge with three incremental doses of aerosolized methacholine (12.5, 25, and 50 mg/ml) in saline. The peak average was calculated by taking the average of maximum values from each animal at each dose with the values directly preceding and following.

#### *Assess lung inflammation*

Following methacholine responsiveness measurements, mice were removed from the ventilator, lungs were lavaged with 1 mL of DPBS (Sigma-Aldrich, St. Louis, MO), the BAL fluid was centrifuged, and all cells in the pellet were resuspended in saline and enumerated by counting with an Advia 120 Hematology System. Differential analysis was performed by cytopspin and H&E stain from approximately 200 cells per slide.

#### *Serum Immunoglobulin ELISAs to measure house dust mite-specific immune responses*

Blood was collected by cardiac puncture, serum was prepared, and it was stored for subsequent analysis of House Dust Mite-specific IgE, IgG1, and IgG2a (BALB/c) or IgG2c (C57BL/6) by ELISA. These ELISA assays were performed using 1:4 serial dilutions over the range of 1:10-1:163,840 for the positive control samples and over the range of 1:640 for the serum samples. The serum samples from the IgG1 plate were transferred to the coated and blocked IgE plates after they had been on the IgG1 plates for 2 hours. The experimental serum samples were compared to a positive control sample of mice that had been sensitized and challenged intranasally with HDM.

The wells of high-binding 96-well microtiter plates were coated with 1  $\mu\text{g/ml}$  HDM extract (Greer, Lenoir, NC) in PBS overnight. The next day, the plates were blocked with 1% BSA for 1 hour, washed three times with PBS-T, and loaded with serum diluted 1:10 for 2 hours. After this, the plates were washed 3 times and 2  $\mu\text{g/ml}$  of biotinylated anti-mouse isotype-specific antibodies (BD Biosciences, San Jose, CA) were added for 1 hour. The plates were again washed three times and streptavidin-HRP was added for 30 minutes. The plates were then washed 4 times and chromogenic substrates A and B (R&D Systems, Minneapolis, MN) were added in a 1:1 ratio. Once the plate was developed, the reaction was stopped with 2N  $\text{H}_2\text{SO}_4$ . The plates were read on a BioTek PowerwaveX (Winooski, VT) instrument at 450nm with a correction of 570nm using the program Gen5 1.1.

*In vitro antigen restimulation and cytokine quantitation*

Following mechanical disruption of the spleen through a 70  $\mu\text{m}$  mesh filter, splenocytes were isolated using Lymphocyte Separation Media (MP Biomedicals, Solon, OH), as previously described [18]. Cells were counted with an Advia 120 Hematology System and  $4 \times 10^6$  cells/ml were cultured in RPMI-1640 supplemented with 5% FBS (Cell Generation, Fort Collins, CO), 1 mM sodium pyruvate, 2500  $\mu\text{g/ml}$  glucose, 100U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, 2 mM L-glutamine, 10  $\mu\text{g/ml}$  folic acid, and 50  $\mu\text{M}$  2-mercaptoethanol and treated with 15  $\mu\text{g/ml}$  HDM extract (Greer). Supernatants were collected after 96 hours of incubation at 37°C in 5%  $\text{CO}_2$ . Analysis of cytokine content from cell supernatants was performed using a custom Magnetic Luminex Screen Assay kit for IL-5, IL-13, IL-4, IL-10, IL-17A, and  $\text{IFN}\gamma$  (R&D Systems, Minneapolis, MN).

*Magnetic Luminex Screening Assay*

All reagents, standards, and samples were prepared according to the manufacturer's instructions. The resuspended, diluted microparticle cocktail was added to the 96 well microplate. The standards and sample were then added to the plate and incubated at room temperature for 2 hours. The plate was then washed three times and diluted biotin antibody cocktail was added for 1 hour. Following this, the plate was washed three times, Streptavidin-PE was added for 30 minutes, and then washed again three times. Finally, the microparticles were resuspended in wash buffer and the plate was read using a Bio-Plex Multiplex System (Bio-Rad, Hercules, CA).

*Quantitate stress and distress elicited from allergen exposure*

The stress hormone cortisol was measured using competitive ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Serum was diluted 1:400. Calibrator diluent, standards, diluted serum samples, cortisol conjugate, and primary antibody were added to the plate and incubated for 2 hours at room temperature. The plate was then washed three times, substrate solution was added for 30 minutes, and then the reaction was stopped. The plates were read on a BioTek PowerwaveX (Winooski, VT) instrument at 450nm with a correction of 570nm using the program Gen5 1.1. The absorbance of the samples were compared to the absorbance of the standards of known concentrations to calculate the concentrations of cortisol.

*Statistical Analysis*

Results were analyzed by one-way ANOVA with Tukey's post hoc test and by two-tailed unpaired parametric t test with Welch's correction using GraphPad Prism 6 for

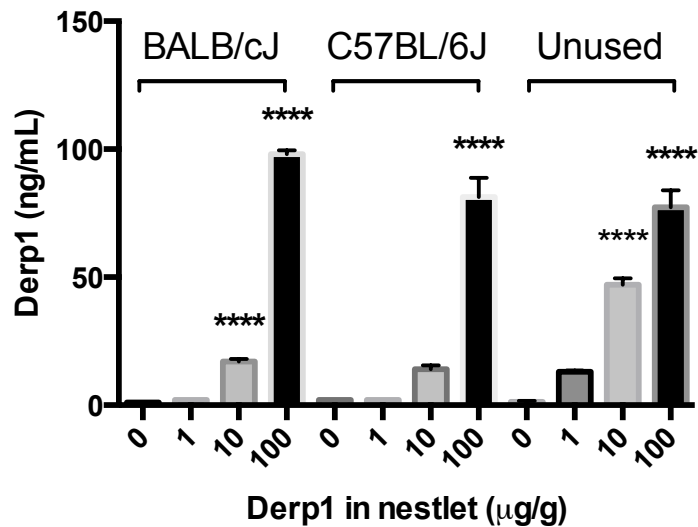
Macintosh (GraphPad, La Jolla, CA). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ,

\*\*\*\* $p < 0.0001$  compared to the 0 HDM dose unless otherwise noted by bracket.

## Results

*Derp1 was inoculated into nestlets in the desired physiologically relevant doses and did not degrade significantly with use.*

To ensure that the house dust mite extract was successfully inoculated into the nestlets in the desired physiological relevant doses and that it did not degrade significantly over time with use and freezing, an ELISA assay was performed to measure the Derp1 content. Nestlets used by BALB/cJ mice and C57BL/6J



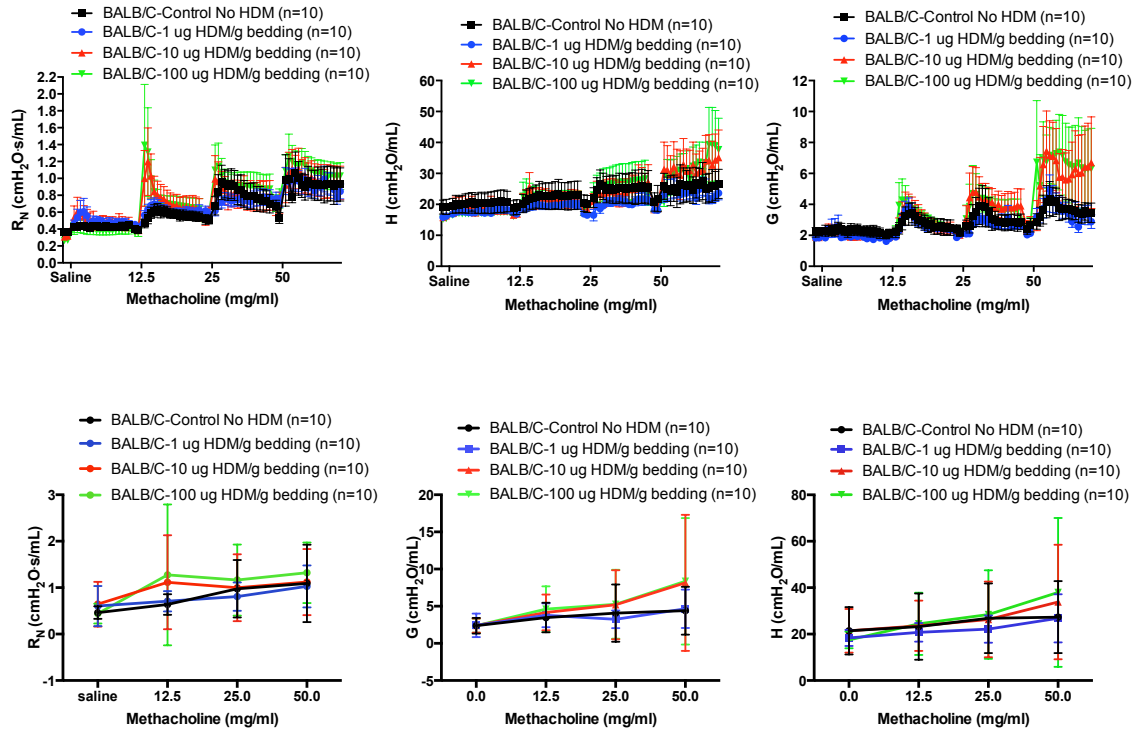
**Figure 1: Derp1 ELISA to assess HDM content in nestlets.** HDM was extracted from frozen unused nestlets and samples of nestlets used by BALB/cJ and C57BL/6J mice by resuspending in 10mL of sterile water per 1g of bedding material. Triplicate samples of bedding were used.

mice along with unused nestlets of each HDM dose were soaked in sterile dH<sub>2</sub>O for 30 minutes to resuspend the HDM extract. The liquid was then squeezed from the bedding material and used for analysis. There was a significant and dose-dependent difference in the Derp1 content present in the unused nestlets and the nestlets used by the BALB/cJ and C57BL/6J mice based on the dose of HDM with which the nestlets had been inoculated (**Figure 1**). The measured content of Derp1 also matched the physiologically relevant doses that we were attempting to inoculate into the nestlets. Therefore, it was

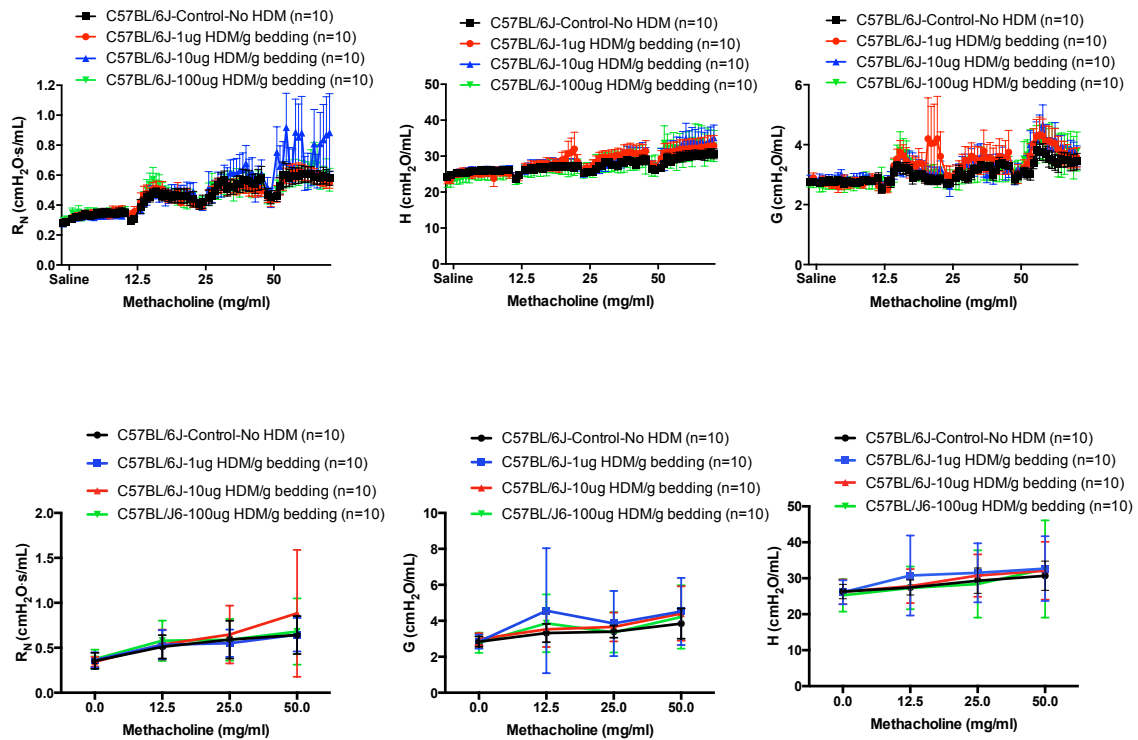
determined that the method for inoculating nestlets with varying doses of HDM was successful.

*Mice sensitized with varying doses of HDM inoculated into their bedding did not display airway hyperresponsiveness.*

Measuring the response to inhaled methacholine is a well-characterized method for assessing airway hyperresponsiveness (AHR), one of the characteristic symptoms of allergic asthma [19]. To assess this important variable, mice sensitized with the varying doses of HDM based on Derp1 content underwent methacholine challenge and were measured using forced oscillations. There were no differences in the levels of Newtonian airway resistance ( $R_N$ ), tissue damping (G), or tissue stiffness (H) between any of the groups in either BALB/cJ or C57BL/6J mice. Additionally, the slope of the methacholine dose dependent curves did not increase dramatically (**Figures 2 & 3**). This indicates that none of the doses of HDM inoculated into the bedding nestlets for sensitization promoted the development of airway hyperresponsiveness.



**Figure 2: Assessment of airway hyperresponsiveness to inhaled methacholine in BALB/cJ mice.** The mice were exposed to 0, 1, 10, or 100 µg/g doses of HDM based on the Derp1 content. Forced oscillations by the flexiVent were used to assess measures of  $R_N$ , G, and H as well as the responsiveness to inhaled methacholine. n = 10 mice per/group.



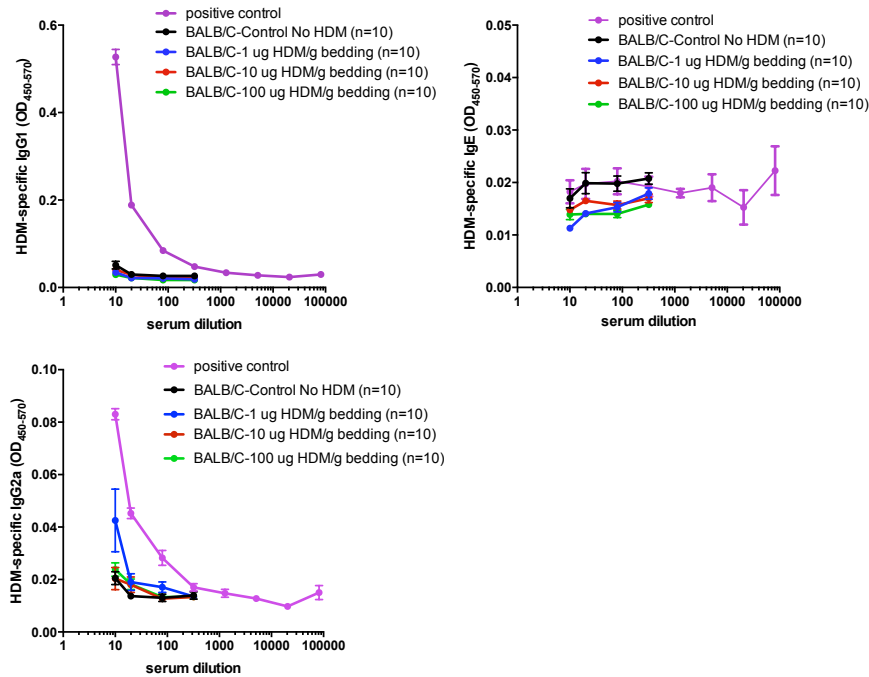
**Figure 3: Assessment of airway hyperresponsiveness to inhaled methacholine in C57BL/6 mice.**

The mice were exposed to 0, 1, 10, or 100  $\mu\text{g/g}$  doses of HDM based on the Derp1 content. Forced oscillations by the flexiVent were used to assess measures of  $R_N$ , G, and H as well as the responsiveness to inhaled methacholine.  $n = 10$  mice per/group.

*There were no differences in the house dust mite specific immune responses of the mice based on what dose of HDM they were sensitized with.*

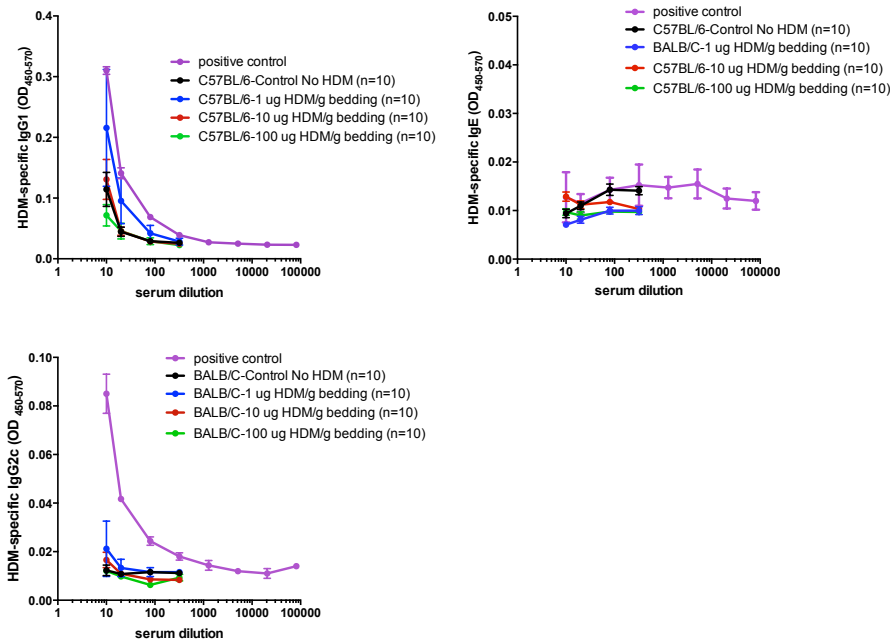
Blood from BALB/cJ and C57BL/6J mice exposed to the different doses of HDM was collected upon death and serum was prepared. Serum immunoglobulin ELISAs were performed to measure the concentration of IgG1, IgG2a/IgG2c, and IgE. These immunoglobulins were also measured in serum samples from a mouse that was sensitized to HDM intranasally. There were no significant differences in the concentrations of the different immunoglobulins in either strain of mice based on the dose of HDM that they were exposed to. There was no statistically significant difference

in the concentrations of each type of immunoglobulin between the positive control and the serum samples (**Figures 4 & 5**).



**Figure 4: Immunoglobulin ELISAs on serum from BALB/cJ mice.** The mice were exposed to varying concentrations of HDM inoculated into their bedding and compared to a positive control serum sample from mice sensitized to HDM intranasally to study the immune response to HDM. n = 10 mice per/group.



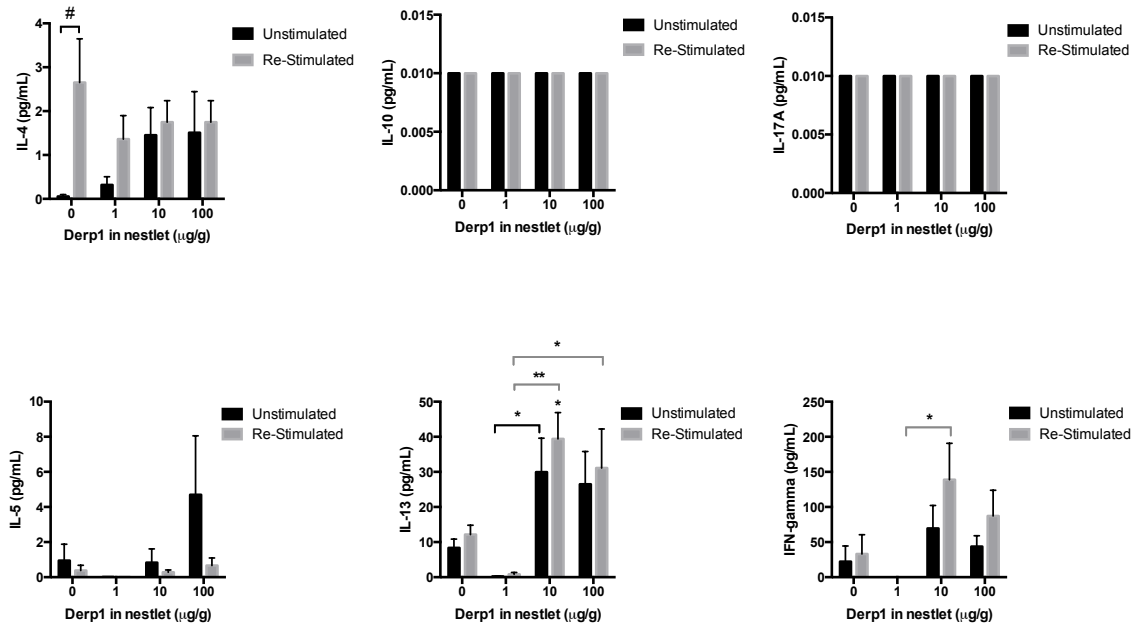


**Figure 5: Immunoglobulin ELISAs on serum from C57BL/6J mice.** The mice were exposed to varying concentrations of HDM inoculated into their bedding and compared to a positive control serum sample from mice sensitized to HDM intranasally to study the immune response to HDM. n = 10 mice per/group.

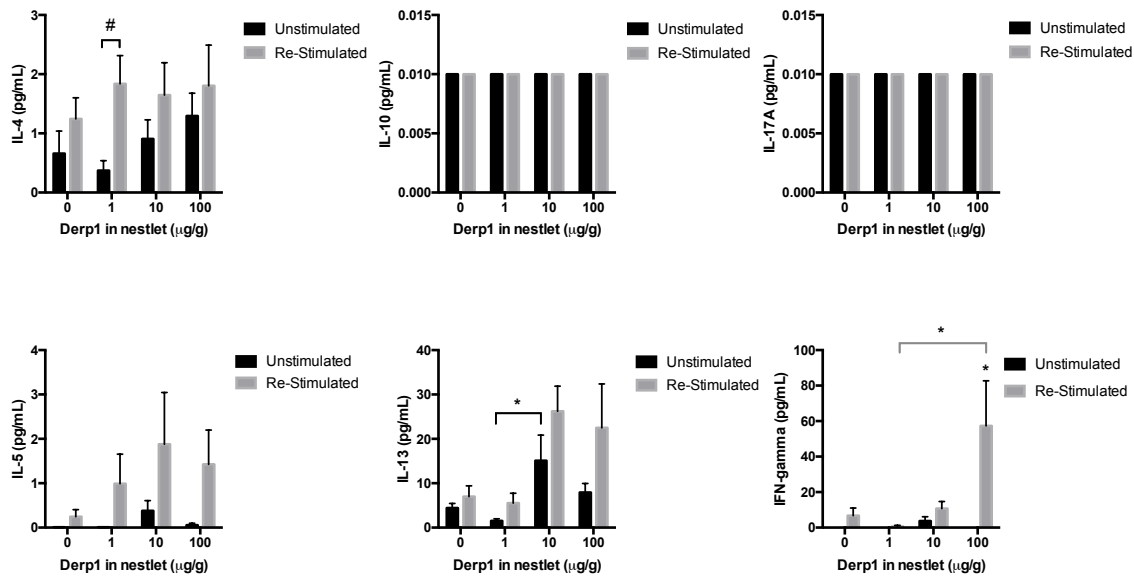
*T cell stimulation was not influenced by the dose of Derp1 and antigen stimulation does not promote cytokine production.*

Splenocytes were isolated from BALB/cJ and C57BL/6J mice exposed to varying amounts of HDM in their bedding. The splenocytes were cultured in the absence or presence 15  $\mu$ g/ml HDM extract for 96 hours. The concentrations of the cytokines IL-10 and IL-17A were undetectable in both strains of mice. While there were some dose dependent differences in the production of IL-13 and IFN $\gamma$  in both strains there was no consistent trend indicating that the varying doses of HDM promoted T cell stimulation. The only observed difference in cytokine production between the stimulated and unstimulated T cells was in the production of IL-4 for one HDM dose in each strain

(Figures 6 & 7). Since the difference between stimulated and unstimulated cells was not more wide spread, this indicates that antigen stimulation does not promote cytokine production in this model.



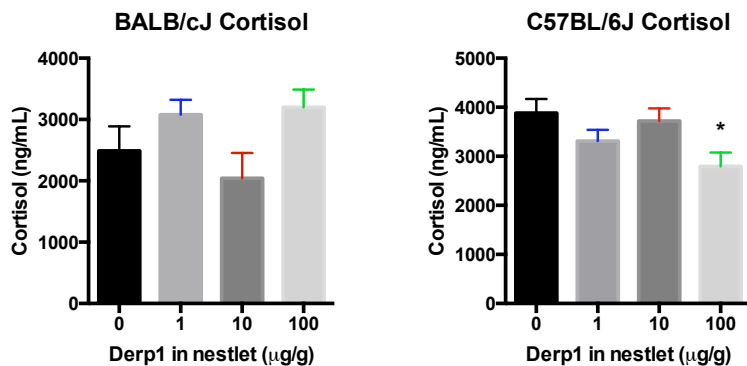
**Figure 6: Cytokine panel of re-stimulated and unstimulated splenocytes from BALB/cJ mice to assess inflammation.** The mice were exposed to 0, 1, 10, or 100 µg/g doses of HDM based on the Derp1 content. Positively selected splenic CD4<sup>+</sup> T cells from BALB/cJ mice were cultured in the presence of antigen-presenting cells isolated from naive BALB/cJ mice and 1 µg of *HDM* extract for 96 hours. Cytokine concentrations were measured by Luminex. n= 10 mice/group.



**Figure 7: Cytokine panel of re-stimulated and unstimulated splenocytes from C57BL/6J mice to assess inflammation.** The mice were exposed to 0, 1, 10, or 100 µg/g doses of HDM based on the Derp1 content. Positively selected splenic CD4<sup>+</sup> T cells from C57BL/6J mice were cultured in the presence of antigen-presenting cells isolated from naïve C57BL/6J mice and 1 µg of *HDM* extract for 96 hours. Cytokine concentrations were measured by Luminex. n= 10 mice/group.

*Cortisol production was not influenced by the dose of Derp1 that mice are exposed to.*

Cortisol is a hormone that is associated with the natural stress response [20]. Serum from BALB/cJ and C57BL/6J mice that were exposed to different doses of HDM was

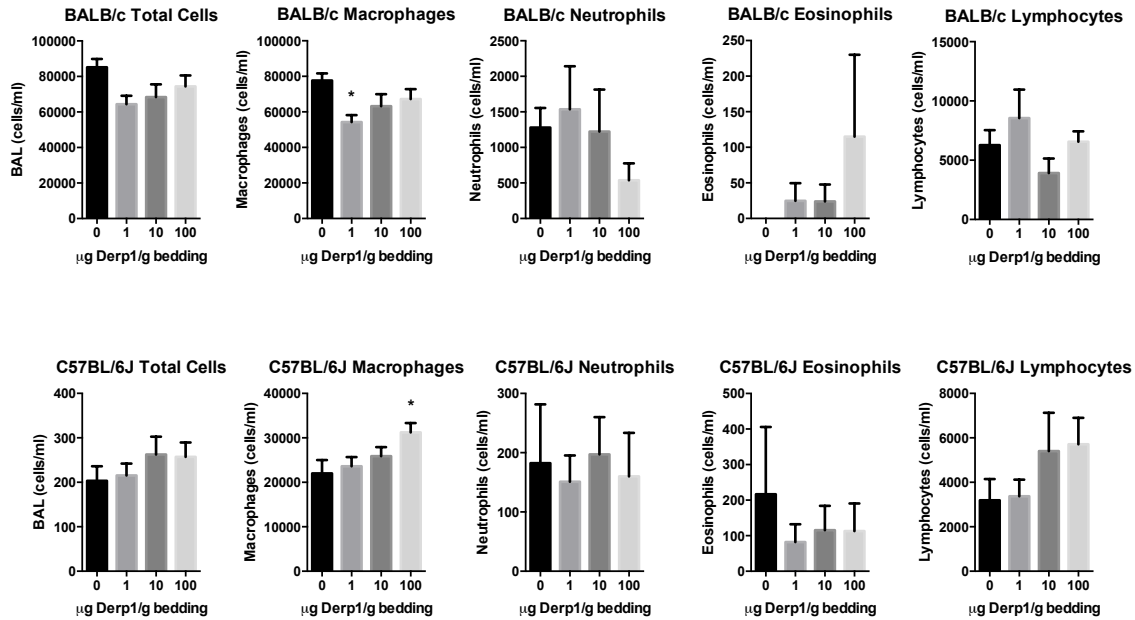


**Figure 8: Cortisol ELISAs to assess stress.** Cortisol ELISAs were performed on the serum of BALB/cJ and C57BL/6J mice treated with 0, 1, 10, or 100 µg/g doses of HDM based on the Derp1 content. n = 10 mice per/group.

analyzed by cortisol ELISA. While there was one statistically relevant difference in the cortisol levels between the different doses of HDM, the overall values in both strains were very similar (**Figure 8**). Therefore, mice exposed to varying doses of HDM do not have different stress levels.

*The concentrations of leukocytes in the lavageable airspaces were not dependent on the dose of Derp1 to which mice were exposed.*

BAL cells were isolated from the BAL fluid collected during the harvest of BALB/cJ and C57BL/6J mice that were exposed to different concentrations of HDM based on Derp1 content. While there was one difference in the concentration of macrophages in the BAL between doses in both strains, the differences were between two different doses (**Figure 9**). Therefore, there is no indication that the concentration of house dust mite based on Derp1 content inoculated into bedding nestlets causes differential production of immune cells.



**Figure 9: Differential cell counts of cytopins of BAL cells from BALB/cJ and C57BL/6J mice.**

The mice were exposed to 0, 1, 10, or 100  $\mu\text{g}/\text{g}$  doses of HDM based on the Derp1 content. Upon harvest lungs were lavaged with 1 mL of DPBS, BAL fluid was collected BAL fluid was centrifuged, and all cells in the pellet were resuspended in saline. Differential analysis was performed by cytopsin and H&E stain from approximately 200 cells per slide.  $n = 10$  mice per/group.

## Discussion

There is an existing need for animal models that more accurately display the pathophysiological features of the different endotypes of asthma and chronic nature of the disease [7]. To address the issues of the existing animal models, we attempted to create a model that utilized physiologically relevant doses of allergen, a natural route of exposure, a more relevant duration of allergen exposure, and involved minimal handling of the mice. The doses of HDM inoculated into the bedding nestlets were based on epidemiological studies that indicated that children exposed to Derp1 (a component of HDM) levels of 10 $\mu\text{g}/\text{g}$  of bedding had an increased risk for becoming allergically sensitized to HDM [1].

The physiologically relevant doses of 0, 1, 10, & 100 $\mu$ g Derp1/g of bedding were successfully inoculated into the nestlets (**Figure 1**). Additionally these doses of HDM remained stable over time with use and being stored in the freezer. It would be expected for the samples from the used nestlets to have lower detectable amounts of Derp1 compared to the unused nestlet samples because some HDM would be spread throughout the cage environment. This pattern was seen for the 1 and 10 doses of Derp1, but the Derp1 concentrations in the 100 dose were very similar for both used and the unused samples. It is possible that HDM accumulated in the cage environment in between cage changes as fresh nestlets were added twice weekly and HDM would be spread around the cage upon the shredding of the nestlets. Therefore HDM from previous nestlets could end up in the bedding material.

Measuring the response to inhaled methacholine is a well-characterized method for assessing airway hyperresponsiveness, one of the hallmarks of allergic asthma that is present in most patients [19]. The presence of AHR in humans and animal models has been associated with chronic changes in the structure of airways, including subepithelial thickening, subbasement membrane thickening, smooth muscle hypertrophy, matrix deposition, and altered vascular components [21]. Additionally, the acute effects of inflammation caused by environmental factors can influence AHR and may explain why there is such a variety in the degree of AHR seen in asthmatic patients [21]. In our model, there was no airway hyperresponsiveness in response to inhaled methacholine and no significant differences in airway hyperresponsiveness depending on what dose of Derp1 the mice were exposed to. This indicates that there were no chronic structural airway

changes or environmental factors causing acute inflammation that would have caused AHR.

The serum immunoglobulin isotypes IgG1 and IgG2a/IgG2c are markers of Th1 and Th2 lymphocytes, respectively [22]. The immunoglobulin IgE is the isotype that is primarily associated with allergic (Th2) inflammation [23]. The Th1 response activates macrophages in response to a pathogen, leading to inflammation aimed at eliminating intracellular pathogens. In asthmatics there is an imbalance between the Th1 and Th2 responses, leaning more heavily towards the Th2 response [24]. The high level of activation of the Th2 response causes the increased expression of cytokines that lead to the activation of eosinophils and the subsequent production of the IgE that is responsible for producing the allergy associated inflammation, which under normal situations is adept at eliminating extracellular parasites [25]. Since our model indicated that there were no differences in the production of these immunoglobulins for different doses of Derp1, the dose of HDM inoculated into the bedding nestlets did not heighten either the Th1 or Th2 immune responses (**Figures 4 & 5**).

The Th2 and Th17 immune responses are strongly associated with allergic asthma and a mixed Th2/Th17 response is reminiscent of severe asthma phenotypes [26]. The Th2 response develops in response to environmental allergens. CD4<sup>+</sup> T cells are activated to produce the cytokines IL-4, IL-5, and IL-13, which are responsible for promoting the production of IgE by B cells, eosinophil activation and recruitment, and mucus production [24]. The Th1 response occurs in response to microbial infections and activates macrophages and helps B cells with antibody production (particularly IgG2). The cytokines IFN- $\gamma$  and IL-4 counter-regulate each other [27]. Therefore, the stronger

response should have higher levels of its characteristic cytokines. The effector cytokines of the Th17 response, including IL-17, indirectly promote the recruitment of neutrophils and cause acute inflammation [26]. IL-10 is an anti-inflammatory cytokine that inhibits the Th1 response [28]. Our data showed that there was no differential activation of the Th1, Th2, and Th17 responses based on the dose of Derp1 to which mice were exposed (**Figures 6 & 7**). For IL-10 and IL-17A, all of the values were out of range for detection. Most of the values for all of the other cytokines were beyond the lower level of quantitation and were extrapolated from the standard curve. There was also no widespread difference in cytokine production between the stimulated and unstimulated splenocytes. The purpose of testing this difference was to see whether or not a memory response was elicited. If the mice had been successfully sensitized the administration of HDM *in vitro* should have caused more cytokine production. Taken together with the results of the serum immunoglobulin ELISAs this indicates that a house dust mite specific immune response did not develop in this model.

Corticosterone is the main steroid hormone involved in the stress response in rodents. Both corticosterone and cortisol are present in serum, have similar dynamics under normal non-stressful conditions, and increase during stressful events. Corticosterone is a more adaptation-related biomarker and decreases during chronic stress whereas cortisol responds faster to severe acute stress [20]. A previous study demonstrated that under normal conditions the concentration of cortisol in the serum was under 15ng/mL and peaked around 45ng/mL in response to a chronic stressor [20]. In contrast, the cortisol levels measured in our model were between 2,000 and 4,000 ng/mL. Since these cortisol levels were so high, any observed differences between treatment



groups are irrelevant. This may indicate that our mice were eliciting extremely large stress responses. However, the indicated high levels of stress could instead be unrelated to the stress they experienced as a consequence of their living conditions. Instead, the manner in which the mice were killed, and what they experienced in the flexiVent analysis preceding their euthanasia, could have elevated their cortisol levels. In many studies that examine cortisol levels, euthanasia is performed by decapitation [20, 29]. In contrast, our mice were woken up in the middle of their sleep cycle, anesthetized, subjected to forced oscillations of methacholine, and then harvested. In a study on golden hamsters, cortisol levels were positively correlated with the amount of time that it took to capture the hamsters before the administration of anesthesia and decapitation [30]. Since our mice were handled much more, subjected to flexivent, and the method of euthanasia is slower than instant death by decapitation, all of these could have contributed to the increased cortisol levels. Additionally, the anesthetic that was used may have influenced the cortisol levels. The cortisol levels from this model were compared to those from a traditional intranasal model and they were not statistically different. However, cortisol levels rise in response to acute severe stressors and since the animals are harvested in the same manner in both models, this could account for the similarity in the measured cortisol levels. In order to test whether the attempted model causes less stress on the animals, they would need to be anesthetized and immediately decapitated for the purpose of measuring cortisol rather than going through the flexivent process and subsequent harvest of organs.

Leukocytes, commonly referred to as “immune cells” play an important role in the pathology of different asthma endotypes. Increased levels of eosinophils are

characteristic in Th2 driven [25] disease and increased levels of neutrophils are more characteristic of Th17 driven disease [26]. T cells are a type of lymphocyte that plays a major role in the development and pathophysiology of allergic asthma. In particular the CD4+ Th2 lymphocytes help to induce some of the inflammation by promoting the activation and recruitment of eosinophils [24, 31]. Macrophages are primarily associated with the innate immune response and are responsible for the phagocytosis of foreign materials and inducing inflammation. Th2 cytokines, which are overexpressed in Th2 driven asthmatics, promote macrophages to induce inflammation. The inflammatory factors produced by macrophages are meant to maintain pulmonary homeostasis; however, when they are overexpressed they cause tissue damage [32]. In our model, there were no differences in the amount of total cells, macrophages, neutrophils, eosinophils, or lymphocytes that were present in the bronchoalveolar lavage (BAL) fluid of mice exposed to the four different doses of house dust mite. This indicates that the HDM inoculated into the bedding nestlets did not influence the production and recruitment of immune cells.

In these studies, we determined that the approach for this model was not successful in inducing an asthmatic physiologic and immunologic phenotype. In traditional models of sensitization and challenge to house dust mite used by our lab mice develop airway hyperresponsiveness in response to methacholine, have elevated levels of eosinophils and lymphocytes in the BAL fluid, produce HDM immunoglobulins, produce cytokines associated with the Th2 response, and display immunologic memory. Since there was no display of airway hyperresponsiveness or house dust mite specific immune responses, future studies will determine whether allergen sensitization and asthma-like

changes in the lung can be elicited through a single intranasal instillation of 1 µg HDM extract, followed by the protracted 6-week challenge of persistent exposure to varying concentrations of HDM inoculated into the bedding nestlets of mice. It will also be determined whether or not the changes that occur are consistent with those found in human allergic asthmatics. Previous studies have found that a single intranasal instillation of 1 µg HDM extract and subsequent challenge with 10 µg HDM one week later on five consecutive days has been successful in inducing allergic sensitization and asthma-like changes in the lung [33]. Once the model has been optimized, it will be used to test for the exacerbation of disease in response to challenge with relevant triggers: a higher dose of allergen exposure, influenza virus infection, nitrogen dioxide, and simultaneous subjection to a high fat diet. If successful, this model could be useful for creating new treatments for asthma due to the natural route for allergen delivery, the persistent exposure, and physiologically relevant doses of HDM based on Derp1 content which more accurately reflect how humans develop asthma.

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