Chapter 13

Induction of Allergic Airway Disease Using House Dust Mite Allergen

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Abstract

Mouse models of allergic airway inflammation have proven essential in understanding the mechanisms and pathophysiology underling human asthma. There is a diverse range of mouse models described in the literature that typically vary slightly by allergen, duration of exposure, and route of sensitization. In general, each of these models has proven to be acceptable surrogates for studying specific aspects of the human disease, including airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling. Here, we describe a highly versatile model based on nasal sensitization with house dust mite antigen (DMA). Mice receive multiple intranasal inoculations with DMA each week for a period of 4–16 weeks, which results in increased Th2-mediated airway inflammation and AHR. However, an added feature of the long-term exposures described here is the ability to more accurately evaluate the impact of chronic inflammation on airway remodeling and lung pathophysiology in response to a clinically relevant allergen.

Key words Asthma, House dust mite, HDM, DerP, DerF, Airway inflammation, Airway hyperresponsiveness, AHR, Airway remodeling, Eosinophil, Th2

1 Introduction

Asthma is a complex genetic disease that is influenced by a diverse repertoire of environmental stimuli. The complex nature and broad spectrum of symptoms in humans have led experts to propose that asthma is not a single disease, but is actually a syndrome of related diseases [1]. Together, these issues can significantly hinder the development of animal models. While the underlying cause and severity of asthma vary greatly between patients, the disease can be characterized by the presence of three cardinal symptoms: chronic airway inflammation; airway hyperresponsiveness (AHR); and reversible airflow obstruction. These symptoms distinguish asthma from other forms of obstructive airway disease, such as cystic fibrosis, emphysema, and chronic obstructive airway disease [2]. The chronic airway inflammation associated with asthma is characterized by an influx of eosinophils into the lungs and airway and is

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predominately driven by the production of proinflammatory cytokines, such as IL-13, IL-4, and IL-5. This chronic inflammation is thought to underlie the airway remodeling that is often observed in patients and is characterized by mucus hyperproduction, airway smooth muscle hypertrophy, and collagen deposition [3]. In addition to airway inflammation, asthma is also characterized by AHR and airway smooth muscle (ASM) constriction, which both contribute to the reversible airflow obstruction that defines the disease. In the case of AHR, the presence and severity of the increased sensitivity to aerosolized stimuli is typically utilized as a surrogate marker for asthma disease progression [4]. Likewise, ASM hyperplasia and hypertrophy have both been shown to contribute to characteristics associated with asthma, including airway inflammation, airway wall remodeling, and airflow obstruction [5].

Due to the complex nature of asthma in humans, laboratory animal models have played pivotal roles in characterizing the pathophysiological mechanisms associated with this disease. Specifically, mouse models have proven to be highly relevant in deciphering the underlying genetic and environmental factors that are associated with airway inflammation and AHR. Mice are an ideal model organism for the study of simple physiological processes associated with allergic airway inflammation due to the ease of genetic manipulation and the availability of ample resources and novel techniques that have been optimized in the mouse that allow for accurate in vivo assessments of airflow obstruction and hyperresponsiveness. However, there are several disadvantages associated with the utilization of mice in asthma research. For example, the human airway has several structural, physiological, and neuronal changes that are associated with AHR, which are not fully recapitulated in mice [6]. Likewise, unlike humans, mice do not spontaneously develop asthma or any other asthma-like disease [7–9]. Thus, all mouse models require artificial induction of allergic airway disease using an exogenous allergen.

The vast majority of allergic airway disease models utilize short-term, acute exposures to simple protein antigens (such as ovalbumin) or complex microorganisms (such as *Aspergillus*). These models commonly evaluate antigen-specific IgE levels, increased T-helper cell 2 (Th2) cytokine production (including IL-4, IL-5, and IL-13), eosinophilic mediated lung inflammation, goblet cell metaplasia, and AHR [10]. However, while these models have been incredibly useful in understanding disease progression, the acute nature of these models does not fully recapitulate several distinct characteristics of human asthma. For example, the inflammation characteristics between these mouse models and human asthma are significantly different. In mice, the inflammation is characterized by acute peribronchiolar and perivascular inflammation in the lung parenchyma, rather than airway wall inflammation in humans [11]. Likewise, allergic airway disease in

mice appears to occur through a mast cell-independent mechanism, whereas the mast cells are a critical component of the human disease [12]. The airway eosinophilia is also significantly different in mouse models. In mice, the eosinophils appear to lack activation, degranulation, and intraepithelial accumulation, which are all observed in humans [11]. Finally, the short-term, acute nature of the majority of allergic airway disease models in mice does not allow a thorough evaluation of the structural changes associated with airway remodeling that is characteristic of the human condition. While each of these differences between allergic airway inflammation in mice and asthma in humans represents a limitation of the current models, we believe that many of these limitations can be overcome by redesigning the models to focus on long-term, chronic inflammation rather than acute inflammation.

Here, we describe a model of allergic airway inflammation that is based on chronic house dust mite exposure. In our hands, this model successfully recapitulates many physiologically relevant aspects of human asthma and is preferred over the acute OVA models typically utilized for these types of studies. The protocols presented here are designed to maximize the data generated from individual mice and minimize the number of animals required to complete studies. In addition, we also present alternative protocols to evaluate specific aspects of allergic airway disease that are often overlooked by typical studies.

2 Materials

2.1 Mice

1. Adult female mice (*see* **Note 1**), 6–12 weeks old (*see* **Note 2**), that have been bred and housed under specific pathogen-free conditions (*see* **Note 3**).
2. Mice should be acclimated to the housing facility for at least 5 days prior to the beginning of the experiment.

2.2 Reagents and Solutions

1. Dust Mite Extract (Stock Solution of 5,000 AU/ml DerP and 5,000 AU/ml DerF mixed 50:50) (Greer Laboratories, Lenoir, NC) (*see* **Note 4**).
2. 1× Hank's buffered saline solution (HBSS).
3. 10× Phosphate-buffered saline (PBS).
4. Sterile water.
5. Isoflurane (Baxter Healthcare Corporation) (*see* **Note 5**).
6. Evans blue dye (EBD).
7. 10× Buffered formalin.
8. Trypan Blue.
9. Formamide.
10. Diff-Quick Staining Kit (Solutions 1, 2, and 3).

11. Permount.
12. ELISA Kits for IgE and IL-13.
13. O.C.T. Compound (Tissue-Tek).
14. Dry ice.
15. Liquid nitrogen.
16. Study-specific and/or standard reagents for RNA extraction, cDNA amplification, and real-time PCR analysis.
17. Study-specific and/or standard reagents for protein extraction and Western blot analysis.

2.3 Materials and Equipment

1. 1 ml Syringe (with 27 gage needle).
2. 1.5 ml microcentrifuge tubes.
3. Microcentrifuge.
4. p1000, p200, and p20 pipettes.
5. 10 ml Syringe (with 27 gage needle).
6. 1 ml Syringes (without needles).
7. 15 ml Conical tubes.
8. Tracheal Cannula (*see* **Note 6**) (Harvard Apparatus).
9. 4-0, Silk Surgical Suture.
10. Refrigerated benchtop centrifuge (with rotor to accommodate 15 ml conical tubes).
11. Hemacytometer.
12. Microscope (10× and 20× objectives).
13. Cytospin.
14. Microscope slides.
15. Coverslips.
16. Coplin jars.
17. 20 ml Disposable glass scintillation vials with lids.
18. 500 ml Beaker.
19. Clear plastic or glass plate (~7 in. × 7 in.).
20. Absorbent paper towels.
21. Tissue-embedding molds (at least 22 mm × 22 mm × 20 mm deep).
22. Ice bucket.
23. Cryostat.
24. Portable liquid nitrogen container or bucket.
25. Fine-tipped indelible marker.
26. 2 ml Screw cap cryo tubes.

27. Mouse necropsy tools: One pair of large blunt scissors to open the chest; one pair of straight forceps; one pair of blunt 90°-angled forceps; one pair of sharp 90°-angled scissors; one pair of slightly curved blunt scissors.

3 Methods

3.1 Induction of Allergic Airway Inflammation (See Notes 7 and 8)

1. Determine the required volume of HDM extract and generate working solutions. The stock solution is supplied as a 5,000 AU/ml mixture of both DerP and DerF extract. Dilute stock to a working concentration of 0.05 AU/ml. The animals will receive 50 μl of the 0.05 AU/ml solution per day.
2. Anesthetize mice using drop method isoflurane in the 500 ml beaker with a glass cover (*see* **Notes 9** and **10**).
3. Sensitization will occur for 5 consecutive days with 2 days of recovery per week for 4–16 weeks (Fig. 1) (*see* **Note 11**).

3.2 Tissue Collection

1. Twenty-four hours following the last DMA exposure, euthanize the mice following appropriate institutional guidelines (*see* **Note 12**).
2. For systemic assessments of circulating cytokines and immunoglobulins, whole blood should be collected utilizing cardiac puncture immediately following euthanasia (*see* **Note 13**). The whole blood should be allowed to coagulate at room temperature for at least 30 min prior to serum isolation.
3. The animals should be perfused using 1× HBSS. Carefully open the peritoneal cavity and cut the portal vein leading to the kidney (either side). This will allow the remaining blood to drain from the animal during the perfusion. Without opening

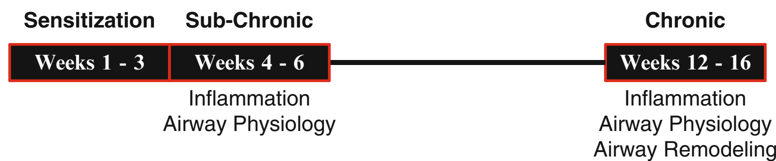

Fig. 1 Schematic depicting typical time courses associated with the induction of allergic airway inflammation in mice. Most models utilize a sensitization phase that lasts 1–3 weeks, based on multiple i.p. or i.n. administrations of a specific allergen. Acute and sub-chronic models typically induce allergic airway inflammation via multiple i.n. exposures to the allergen during weeks 4–6. Common assessments for these short-term models include the evaluation of airway inflammation and airway physiology. Chronic models typically induce allergic airway inflammation via multiple weekly i.n. exposures to the allergen during weeks 4 through 16. The chronic nature of these long-term models improves the evaluation of features associated with airway remodeling

the chest, carefully move the liver to expose the diaphragm. The lungs and heart should be visible behind the translucent diaphragm. Carefully clip the diaphragm at the point of contact with the sternum, making a small nick to access the chest. Once the nick is generated, the lungs and tissues should resend into the chest cavity. The bottom of the heart should now be visible. Using a 10 ml syringe with 27 gage needle attached, slowly and carefully inject the heart and gently perfuse 1–3 ml of HBSS. The lungs should begin to change color from red to pinkish/white and the liquid flowing from the excised kidney should change from red to clear. Caution: If too much pressure is applied to the syringe, saline can be forced into the airways and compromise additional data collection.

4. Once the animal has been perfused, the chest cavity can be exposed. Using a pair of blunt scissors, carefully open the chest cavity and remove each side of the rib cage as completely as possible and without damaging the lungs. Next, carefully remove the collar bones, taking care not to damage the underlying trachea. Using blunt-tipped forceps, separate the salivary glands and remove the thin layer of muscle that lies overtop of the trachea in the mouse's neck. The trachea should now be exposed from the lungs to the larynx.
5. Using the 90°-angled sharp scissors, make a small incision in the trachea 1–3 tracheal rings below the larynx. The incision should be just large enough to insert and secure the tracheal cannula. Caution: Do not sever the trachea as this will cause the trachea to retract into the chest cavity. Insert the tracheal cannula into the incision. Brace the trachea with the straight blunt forceps. Using the 90°-angled blunt forceps, thread the suture directly under the trachea and securely tie the cannula into place.
6. To collect the BALF, fill three 1 ml syringes (without needles) with 1 ml of HBSS. Ensure that no air bubbles are present in the syringe and that the HBSS is flush with the end of the syringe. Gently attach the hub of the syringe to the tracheal cannula and slowly inject 900 μl of HBSS into the mouse lungs in one continuous motion. The lungs should visibly inflate with no obvious leaks. Immediately withdraw the fluid in one slow and continuous motion. Deposit BALF into a 15 ml conical tube on ice. Repeat this process with the other two syringes. However, subsequent lavages should utilize the full 1 ml of HBSS per lavage. Record the final volume of BALF collected for each animal (this volume should be approximately 3 ml total). Keep the BALF on ice until ready to count.
7. To inflate and fix the lungs for histopathology, fill a 1 ml syringe with 10 % buffered formalin. Brace the trachea with the straight blunt forceps. Using the 90°-angled blunt forceps, thread a

second suture directly under the trachea and below the end of the cannula. Loop the suture in a half-tightened knot. Do not completely tie the second suture. Insert the 1 ml syringe into the cannula. Gently inflate the lungs with approximately 1 ml of 10 % buffered formalin. Do not overinflate the lungs as this will result in distortions in the lung histopathology. Once the lungs are inflated, secure the knot on the half-tied suture.

8. To remove the fixed and inflated lungs, remove the syringe and cannula from the trachea. Grasp the excess suture thread with the forceps and gently lift the trachea. Using the curved blunt scissors, slowly sever the trachea while lifting the inflated lungs out of the chest cavity. Carefully excise the lungs (with the heart still attached) without cutting them. Gently remove the inflated lungs from the mouse. Place the inflated lungs in a 20 ml disposable glass scintillation vial containing approximately 10 ml of 10 % buffered formalin. Place a lid on the vial and label with an indelible pen.
9. Properly dispose of the remaining mouse carcass.
10. For many applications, formalin fixation may yield suboptimal results or is incompatible with subsequent procedures (such as IHC or ISH). In these cases, it is preferable to freeze the lungs to generate frozen lung sections for subsequent histology. To generate frozen lung sections, fill a 1 ml syringe with O.C.T. compound. Fill an ice bucket with dry ice. Label a tissue-embedding mold using an indelible pen and place the mold in the dry ice, taking care to maximize contact with the dry ice. Harvest the whole blood, cannulate the animal, and collect the BALF as previously described. Insert the syringe containing the O.C.T. compound into the cannula. Apply gentle pressure to the syringe plunger and inflate the lungs with O.C.T. (*see* **Note 14**). Tie off the lungs as described above for the formalin fixation protocol.
11. To embed the O.C.T. inflated lungs for histology, place a small amount of O.C.T. compound in the bottom of the tissue mold. This initial layer of O.C.T. should completely cover the bottom of the mold. Carefully remove the lungs from the chest cavity, as described above for the formalin fixation protocol. Place the lungs in the tissue mold and carefully hold in place until the initial layer of O.C.T. thickens enough to secure the bottom of the lungs to the tissue mold. The excess suture thread should not be inserted in the mold. Immediately begin filling the remaining tissue mold with O.C.T. by adding the compound in a circular motion while gently balancing the top of the lungs with the forceps to ensure that they remain vertical and centered in the mold. Once the tissue mold is filled, cut the excess suture thread with scissors. The O.C.T. compound

should completely freeze within 10 min and samples can be stored at −80 °C until ready for use.

12. Histology sections should be prepared using a cryostat. It is also important that the lungs be prepared in either a dorsal or a ventral orientation to maximize visualization of the airway.
13. For studies evaluating gene expression and/or protein levels, it may be preferable to harvest the lungs for RNA or protein extraction rather than for histology. Fill an ice bucket, or other approved container, with liquid nitrogen. For each sample, label a cryotube with an indelible pen. Harvest the whole blood and BALF as described above. Remove the cannula and sutures. Remove the lungs, one lobe at a time, with the curved blunted scissors without inflating. Special care should be taken to remove any additional material from the chest cavity to avoid contaminating material (i.e., ensure that the lung sections do not also include pieces of heart, thymus, lymph node, or esophagus). Place each lung lobe into the cryotube and drop the tube in the liquid nitrogen to flash freeze the tissue. Store the tissue in liquid nitrogen until ready for homogenization.

3.3 Sample Preparation for Analysis

1. Collect the serum from the whole blood. After allowing the whole blood to coagulate at room temperature for at least 30 min, spin the samples in a microcentrifuge at maximum speed (~17,000 × *g*) for 5 min. Label a 1.5 ml microcentrifuge tube for serum collection with the indelible pen, one tube for each serum sample. Carefully remove the tubes containing the now separated whole blood from the centrifuge. Note the separation of the blood into two distinct phases. The serum is isolated in the top layer. Carefully remove the serum from the tube using a p1000 pipette and transfer the serum to the newly labeled microcentrifuge tube. Keep the tubes on ice until ready for storage. The recovered volume of serum should be approximately equivalent to 20 % of the total volume of whole blood. Store the serum at −80 °C until ready for use.
2. For cytokine and immunoglobulin analysis by ELISA, the serum should be diluted 1:5–1:20 depending on the assay. These dilutions should be empirically determined prior to running the bulk of the samples. Due to the low volume of serum collected, most sample volumes can be reduced by half for loading on the ELISA plate. For example, most commercial ELISAs utilize 100 μl volumes of standards and samples; for serum, load 50 μl of standards and diluted samples. Common ELISAs for serum include IL-13, IgE, and antigen-specific IgE.
3. Collect cell-free BALF from the BAL for cytokine analysis. Spin the BALF that was collected in the 15 ml conical tubes in a refrigerated tabletop centrifuge at 1,530 × *g* for 5 min to

pellet the cells. Label two 1.5 ml microcentrifuge tubes with the indelible pen. Carefully remove the 15 ml tubes from the centrifuge without disturbing the cell pellet. Carefully transfer the BALF supernatant to the 1.5 ml microcentrifuge tubes and keep on ice. Store the BALF at −80 °C until ready for use.

4. For cytokine and immunoglobulin analysis by ELISA, the BALF should be used neat or diluted 1:5 depending on the assay. These dilutions should be empirically determined prior to running the bulk of the samples. Unlike the serum, the BALF should yield ample volume for ELISA and western blot analysis. However, most sample volumes can also be reduced by half for loading on the ELISA plate, as discussed for the serum. Common ELISAs for the BALF include IL-13, IL-4, and IL-5.
5. Collect the cells from the BALF for cellular composition analysis. Lyse the red blood cells by hypotonic saline (*see* **Note 15**). Resuspend the cells in 900 μl of distilled water. Immediately add 100 μl of 10× PBS. Samples should be lysed one at a time. If samples contain excessive amounts of red blood cells, the cells can be spun down in the tabletop centrifuge at 2,040 × *g* for 5 min and repeat the lysis procedures described above.
6. Determine the total BALF cellularity in the 1 ml suspension using a hemacytometer under 10–20× magnification with Trypan Blue staining. These data can be evaluated by either showing as cells/ml or multiplying with the volume of BALF collected and shown as cells/mouse.
7. Collect cells for differential staining (*see* **Note 16**). Label standard microscope slides using a pencil or a solvent-resistant pen. Secure the slides into the holder and funnel for the cytospin. Remove 150 μl of BALF and cytospin at 1,020 × *g* for 5 min. Allow the slides to air-dry overnight. Differential stain the slides following the manufacturer's protocols. Allow the slides to air-dry overnight. Coverslip the slides using permount. Evaluate the slides using a microscope equipped with a 20× and 40× objective.
8. Harvest the remaining cells for subsequent analysis, such as FACS, electron microscopy, confocal microscopy, RNA extraction for gene expression evaluation, and/or protein extraction for Western Blot. In general, these subsequent assays, such as flow cytometry, will be limited by the number of cells collected by the lavage. For most protocols, the cells can be collected by centrifugation at 1,530 × *g* for 5 min, the supernatant removed, and samples stored at −80 °C until ready for use.
9. Prepare the lungs for histopathology evaluation. After 24–48 h of formalin fixation, the whole inflated lungs should be ventrally orientated and embedded in paraffin. The resultant blocks

should be cut to expose the main airway. Increased scoring accuracy can be achieved by orientating the lungs in the same position and cut to the same depth. Five micron serial sections of the lungs should be cut and stained with hematoxylin and eosin (H&E), Masson's Trichrome, and Alcian-blue/periodic acid-schiff reaction (AB/PAS). Additional sections can be cut and prepared for in situ hybridization using standard protocols.

10. Utilize H&E staining and scoring to evaluate overall lung inflammation. The most efficient technique to evaluate H&E staining in these types of assays is through semiquantitative inflammation scoring of the left lung lobe. Sections of the left lobe should be cut to yield the maximum longitudinal visualization of the intrapulmonary main axial airway. Histopathology can then be evaluated by the following inflammatory parameters, which are scored between 0 (absent) and 3 (severe): mononuclear and polymorphonuclear cell infiltration; airway epithelial cell hyperplasia and injury; extravasation; perivascular and peribroncheolar cuffing; and percent of the lung involved with inflammation. These parameter scores can then be averaged for a total histology score or used individually to quantify specific aspects of disease progression. Scoring should always be conducted in a double-blind fashion, with reviewers blinded to both genotype and treatment. This scoring system has been previously described [13–17].
11. Evaluate collagen and pre-collagen deposition. Collagen and pre-collagen deposition is often a feature of long-term, chronic models of allergic airway inflammation. To evaluate collagen deposition in the lungs, histology sections can be prepared as described above and stained with Masson's Trichrome. Masson's Trichrome results should be assessed and scored by an experienced reviewer who is blinded to genotype and treatment, as previously described [17]. This technique generates a qualitative or a semiquantitative dataset. However, collagen levels in the lungs can be accurately quantified using biochemical assays, such as the hydroxyproline assay.
12. Evaluate goblet cell hyperplasia. Goblet cell hyperplasia is also a characteristic feature of allergic airway disease and can be assessed in the disease models using AB/PAS staining. Sections of the left lung lobes should be sectioned, as described above, and stained with AB/PAS. For proper evaluation of mucus production and in an effort to avoid bias, the identical area from all lungs should be evaluated. A 2 mm length of airway located midway along the length of the main axial airway should be marked and digitally imaged at 10× and 20× magnification. Using ImageJ software (NIH, National Technical Information Service, Springfield, VA), the length and area of the AB/PAS-stained region in the lung sections can be imaged

and measured (see user guide for software use). The resultant data is expressed as the mean volume density (Vs = nl/mm^2 basal lamina + SEM of AB/PAS-stained material within the epithelium), as previously described [18].

4 Notes

1. Female mice are preferred in these assays due to their more docile nature. There is an increased probability that adult male animals will become aggressive during the course of this type of long-term experiment. If male mice are to be utilized, consider individual housing.
2. We have successfully utilized 6–12-week-old C57Bl/6, 129SvEv, and BALB/c mice in these assays. If strain is not a limiting factor, BALB/c mice are preferred due to their Th2 skewing and robust response. It is possible that some aspects of this protocol may need to be adjusted and further optimized when using mice from different genetic backgrounds.
3. All studies should be conducted in accordance with the local and institutional animal care and use guidelines and in accord with the prevailing national regulations.
4. Mice were exposed i.n. to 0.05 AU/ml of purified 50:50 DerP and DerF whole-body extract. There are a variety of sensitization protocols and dosing parameters reported in the literature for house dust mite exposure. It is also common practice to use either DerP or DerF unmixed. In our experience, all of these procedures appear to work equally well under the conditions described in this protocol.
5. 2,2,2 Tribromoethanol (Avertin) is a common substitute for drop method isoflurane anesthesia in allergic airway inflam mation protocols that require fewer rounds of sensitization (i.e., many ovalbumin models). However, in our experience, the deep plain of anesthesia induced by avertin can actually reduce the effectiveness of the intranasal administrations. Likewise, for the dust mite protocol, the frequency of i.n. administrations (5/week) make i.p. anesthesia impractical and likely to induce significant pain and distress in the animals.
6. We recommend the use of specialized, commercially available tracheal cannulas. However, 16 gage needles can be used as substitutes. In our experience, this alternative works best when the needles are ground down to a blunt end.
7. There are many allergens that could be substituted for DMA using this protocol, including *Aspergillus* sp. and cockroach antigen. However, the sensitization protocols for each allergen should be empirically determined. We have found that the use

of DMA is preferable to ovalbumin in chronic models due to the following: (a) repeated challenges of OVA will eventually result in tolerance; (b) HDM, *Aspergillus* sp., and cockroach antigen are clinically relevant to the human disease; and (c) the robust nature of OVA-induced inflammation typically obscures subtle, yet highly relevant, aspects of disease pathogenesis.

8. Intranasal administration requires extensive practice to achieve proficiency. Improper technique can result in sinus deposition and inefficient sensitization, which result in weak and highly variable inflammatory responses. In our hands, we have found that EBD is an effective training tool. A 1 % solution of EBD in 1× PBS can be generated, filter sterilized, and administered i.n. To quantify the efficiency of the i.n. administration, the lungs can be removed and incubated in formamide for 48 h at room temperature to extract the EBD. The absorption of Evans blue can be measured using a standard plate reader at 620 nm and deposited Evans blue can be calculated against a standard curve to quantify efficiency.
9. Drop method isoflurane induces a low level of anesthesia that is recommended for this procedure. We have found that light anesthesia allows for more effective antigen instillation compared to other techniques, which often suppress breathing volumes and rates. Drop method isoflurane induces anesthesia within 30 s and will lightly anesthetize the mouse for approximately 30 s. Each individual institution will have specific guidelines regarding the use of drop method anesthesia.
10. Note that inhalation anesthetics, such as isoflurane, may result in confounding issues when studying lung physiology. Therefore, ensure that control animals are properly utilized and limit the animal's exposure to the anesthetic as much as possible.
11. We have successfully utilized the described DMA protocols for both short-term (4–6 weeks) and chronic sensitization (12–16 weeks). The short-term models are ideal to evaluate elements associated with inflammation; however, the long-term exposures provide a more physiologically relevant model of the human disease and allow assessments of airway remodeling that is absent in the short-term exposure.
12. All studies should be conducted in accordance with the local and institutional animal care and use guidelines and in accord with the prevailing national regulations.
13. The blood should be harvested by heartstick using the 1 ml syringe with a 27 gage needle attached. There are multiple

approved methods of conducting the heartstick. We have found that it is most effective when performed prior to making any incisions on the animal. Immediately after removal from the CO_2 chamber, ensure proper euthanasia by toe pinch reflex and pin the mouse to a surgical board. Spray the animal with 70 % ethanol and locate the base of the sternum. Insert the needle between the last two ribs slightly to the right of the center. Using a controlled and singular motion, begin withdrawing the blood from the heart. With practice, this procedure can typically recover 500–800 μl of whole blood. Transfer the blood from the syringe to a labeled 1.5 ml microcentrifuge tube. Critical note: Remove the needle from the syringe prior to transferring the blood. Forcing the blood through the needle will induce cell lysis and inhibit serum collection.

14. For optimal results, the lungs must be inflated with O.C.T. As an alternative to utilizing the cannula, the lungs can be inflated through simple injection with O.C.T. using a 27 gage needle. Lung inflation is critical as it allows for observation of the lungs in the most physiologically relevant state.
15. There are many different protocols for red blood cell lysis. The protocol described here is optimized for the subsequent basic morphology assessments by differential staining and total cell counts. However, this procedure results in suboptimal results in higher resolution analyses, such as FACs. Red blood cell lysis via AKT is a viable alternative for procedures requiring less background and higher resolution.
16. Differential staining allows for morphology-based identification of BALF cellularity. To ensure the optimal results, the samples should be cytospun on the same day they were collected and the staining reagents should be prepared fresh prior to each use. DiffQuick-based protocols allow the differentiation of eosinophils (granules stain red) and neutrophils (granules do not stain). Monocytic cells can be easily identified, but are difficult to distinguish. Therefore, these cells should be identified as monocytes, rather than macrophages. Likewise, lymphocytes are also commonly observed in the BALF. However, it is also unlikely that typical researchers can distinguish T-cells from B-cells based on morphology alone. Thus, many investigators have modified these procedures for use with flow cytometry. The only limiting factor is the low number of total cells typically harvested from control animals. Even with flow cytometry, differential staining should be used to confirm the results.

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