

Chapter 31

Mouse Models of Allergic Asthma

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Abstract

In the last 20 years, the development of murine models of allergic asthma has provided researchers with a means to explore the mechanisms of this T-helper type 2 (Th2)-driven inflammatory disease. While systemic sensitization and airway challenge with ovalbumin has been the most widely used model, recent emphasis has been placed on the development of models using more naturally occurring antigens. However, the diversity of models currently available makes it hard for investigators new to this field to choose to use the most effective and appropriate model to test their hypothesis. Here we describe three different mouse models of allergic asthma, including the classical ovalbumin model, a modified ovalbumin model that has been shown to be mast-cell dependent, as well as a house dust mite antigen-induced model. We also discuss briefly their characterization and differences, in the aim to facilitate the choice of the appropriate model when working on this intricate Th2 inflammatory disease.

Key words Asthma, Mouse, Ovalbumin, House dust mite, Bronchoalveolar lavage, Inflammation, Intranasal

1 Introduction

Asthma is an inflammatory disease characterized by airway inflammation, airway hyperresponsiveness, and remodelling (reviewed in Barnes [1]). The complex pathophysiology of this disease (including important contributions from various inflammatory cell types such as eosinophils [2–5], lymphocytes [6], and mast cells [7, 8]) leads to an urgent need in the development of mouse models of allergic asthma. In the early 1990s, the first attempts to mimic the allergic reaction leading to airway inflammation and responsiveness were made using IgE receptor cross-linking techniques [9, 10], quickly followed by models using allergens which are still used nowadays, such as the ovalbumin (OVA) mouse model of asthma [11]. In these models, mice are actively immunized against various proteins (such as ovalbumin) using the parallel administration of adjuvant such as alum or $\text{Al}(\text{OH})_3$.

As our understanding of the pathogenesis of asthma improved, it became obvious that there was a need to alter these models to allow their use in the study of the complex mechanisms leading to the development of asthma. Therefore, various versions of these models made their appearance in the literature. Here, we discuss the two most used mouse models of asthma: the OVA model of asthma and the house dust mite (HDM) model of asthma. We will try to elucidate the differences between these models, and discuss the role of mast cells in their development.

1.1 Ovalbumin-Induced Allergic Asthma Model

In the mouse model of OVA-induced asthma, active sensitization is achieved either by concurrent administration of $\text{Al}(\text{OH})_3$ as an adjuvant or via repetitive exposure to low doses of ovalbumin [8]. In the case of the use of $\text{Al}(\text{OH})_3$, a second administration of OVA coupled to the adjuvant is needed to achieve a degree of sensitization which will ensure the development of the inflammatory reaction in response to the airway challenges with OVA. When trying to induce an asthmatic-like airway inflammatory response without adjuvant, the sensitization period comprises repetitive exposure to small doses of OVA (either intraperitoneally or via the airways), followed by a period of time where mice are not exposed to the protein. Then, independently of the use of adjuvant, mice are challenged via the airways using either aerosolized or intranasal administration of ovalbumin to develop pulmonary eosinophilia, a major hallmark of the inflammatory asthmatic response. Of interest is the fact that as long as the sensitization and challenge steps are respected and that 7 days are allowed for the formation of antibodies against ovalbumin (sensitization period), the timing of the challenge period can vary [12, 13].

However, it is important to note here that the use of adjuvant has been reported to induce a mast cell-independent form of airway inflammation in the mouse model of asthma, led mostly by a strong lymphocyte-driven induced Th2 inflammation and eosinophilia. Models not using an adjuvant, such as $\text{Al}(\text{OH})_3$, are reportedly dependant on the presence of mast cells for the development of airway inflammation [8, 14–16]. Of importance is the fact that independently of the use of adjuvant, airway hyper-responsiveness was shown to be strongly dependant on the presence of mast cells.

However, these two models (with and without adjuvant) are not recognized to induce airway remodelling, which is an important hallmark of asthma and is closely related to the development of airway hyper-responsiveness in humans (reviewed in Barnes [1]). In order to induce remodelling of the airways, mice need chronic post-sensitization low-intensity exposure to OVA (over 8–12 weeks) [17, 18]. Although the level of airway inflammation after 12 weeks is lower than what is observed with the acute models, collagen deposition, basement membrane thickening, as well as alterations in the pulmonary function are observed in these mice.

1.2 House Dust Mite Mouse Model of Asthma

As the OVA mouse model of asthma grew in popularity, so did the criticizing of its strengths and limits. The main criticism of the OVA mouse model of asthma is the use of a normally nonantigenic protein, and the development of an artificial immune response to this protein via the use of adjuvant. The need for a model using a “natural antigen” (i.e., found in the environment of asthmatic patients), which would cause an asthmatic-like response in mice without the use of adjuvant, became quickly obvious. Also, there was a lot of pressure towards developing a model that would use a natural route of exposure to the allergens (i.e., via the airways, compared to intraperitoneal sensitization in the mouse model of OVA).

As a result, the house dust mite (HDM) model of asthma was originally characterized in 1996 [19], and well described recently by Cates et al. [20]. The latest studies report that administration of HDM extract once a day for 10 days causes an asthmatic response characterized by increased IgE, airway and parenchymal eosinophilia, a Th2 cytokine response, as well as the development of an increased response to methylcholine as a measure of airway hyper-responsiveness.

The exact antigenic epitopes of the HDM extract are yet to be fully characterized; however, the fact that sensitization to HDM can be achieved via a natural route of delivery is likely due to the intrinsic protease activity of the antigenic preparation [21, 22], creating a breach in the epithelial barrier and the subsequent activation of airway antigen-presenting cells. So far, there is no solid proof in the literature of the involvement of mast cells in the development of the asthmatic response in this model; however, the high levels of IgE, histamine, and airway hyper-responsiveness (which is known to be dependant of mast cells independently of the use of adjuvant) all indicate that mast cells are likely to play a major role in the development of the asthma-like response in this disease.

In this chapter, we describe the material and methods necessary to develop these two major mouse models of asthma (OVA and HDM), and the methods used to assess airway inflammation in both models.

2 Materials

2.1 Ovalbumin (OVA)-Induced Allergic Asthma

1. C57BL/6J mice (minimum of 6 weeks old at the start of experiment and age and sex matched): All animal experiments must be conducted according to your institution guidelines (*see* **Notes 1** and **2**).
2. Albumin from chicken egg white, grade III or higher (*see* **Note 3**).
3. Imject Alum adjuvant (Thermo Scientific, 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide).

4. Phosphate-buffered saline (PBS).
5. 1 mL tuberculin syringes.
6. 26–28G needles.
7. Isoflurane (Abbott Laboratories).

2.2 House Dust Mite (HDM)-Induced Allergic Asthma

1. House dust mite lyophilized extract, *Dermatophagoides pteronyssinus* (Greer Labs), resuspended to 2.5 mg/mL in sterile PBS (*see Note 4*).

2.3 Assessment of Airway Inflammation

1. Avertin: Dissolve 10 g of 2,2,2-tribromoethanol with 10 mL of *tert*-amyl alcohol for a stock solution. Prior to use, dilute stock solution 1:40 with PBS, adding stock solution dropwise in PBS that is at 37 °C with constant stirring to avoid formation of a precipitate. Store both solutions at 4 °C protected from light (*see Note 5*).
2. Surgical scissors and forceps.
3. 70 % ethanol (EtOH).
4. Catheters, 22G (BD Insyte) (*see Note 6*).
5. Suture thread.
6. 1 mL tuberculin syringe.
7. PBS.
8. 15 mL conical tubes.
9. 10 % neutral buffered formalin.
10. Red cell lysis buffer: 150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA (pH 7.3).
11. Hemocytometer.
12. 0.4 % Trypan blue solution in PBS.
13. Microscope slides.
14. Shandon Filter Cards (Thermo Scientific).
15. Cytospin 4 Cyto centrifuge (Thermo Scientific).
16. Wright-Giemsa stain:
 - (a) Fixative (100 % MeOH).
 - (b) Solution I: PROTOCOL HEMA-3 (Fisher Scientific).
 - (c) Solution II: PROTOCOL HEMA-3 (Fisher Scientific).
17. Permout mounting medium.

2.4 Enumeration and Differentiation of BALF Leukocytes

1. Flow cytometry buffer: 2 % FBS, 2 mM EDTA, 0.05 % sodium azide in PBS.
2. 96-Well V-bottom plates.
3. Latex counting beads (Invitrogen, cat #: C37259, 10 µm, approximately 1 × 10⁸ beads/mL).

4. Normal goat serum.
5. Purified anti-CD16/32 antibody (clone 2.4G2).
6. Fluorochrome-conjugated antibodies:
 - (a) FITC anti-7/4.
 - (b) PE anti-Siglec-F.
 - (c) PECy7 anti-CD3e.
 - (d) APC anti-CD11c.
 - (e) Pacific Blue anti-CD45.
 - (f) APC-Cy7 anti-B220/CD45R.

2.5 Lung Cell Isolation for Leukocyte Differentiation

1. Dulbecco's modified eagle medium (DMEM).
2. Fetal bovine serum (FBS).
3. 100× penicillin/streptomycin.
4. 12-Well tissue-culture plate.
5. Collagenase, type IV.
6. 70 µm cell strainer.
7. 10 mL pipette.
8. 3 mL syringe.
9. 50 mL conical tube.
10. Red cell lysis buffer: 150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.3.
11. Percoll.
12. 10× Hanks' balanced salt solution (HBSS).

2.6 Lung Inflation and Fixation for Histology

1. 30 mL Luer-Lok syringe.
2. 18 gauge (18G) needle.
3. 250 mL volume Erlenmeyer Filter Flask (Büchner flask).
4. 10 mL polystyrene serological pipet.
5. Male Luers (Bio-Rad).
6. Female-to-Female Luer (Bio-Rad).
7. Tubing, 1.6 mm internal diameter (ID) (Bio-Rad).

3 Methods

3.1 OVA/Alum Model Disease Induction

1. Dilute OVA in PBS to make a 4 mg/mL solution.
2. Shake the inject alum solution well before use. Dilute the 4 mg/mL OVA solution 1:1 with the alum for a final OVA concentration of 2 mg/mL.
3. Mix the OVA/alum mixture for 30 min at RT in order for the alum to effectively adsorb the OVA antigen.

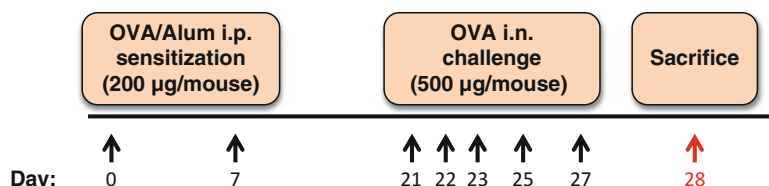


Fig. 1 Mast cell-independent OVA/alum model of allergic asthma. The “traditional” allergic asthma model requires systemic sensitization by intraperitoneal (i.p.) administration of 200 µg ovalbumin (OVA) complexed with alum adjuvant (per mouse) at day 0 and day 7. Two weeks later, mice are lightly anesthetized and then challenged with OVA (500 µg/mouse) delivered in a small volume (~40–50 µL) through the nares (intranasal, i.n.) using the dose schedule shown. On day 28, 1 day after the final i.n. OVA challenge, mice are sacrificed for analysis or otherwise treated experimentally

4. Immunize mice intraperitoneally on days 0 and 7 with 100 µL of the OVA/alum mixture using a 1 mL syringe and 26G needle (Fig. 1).
5. For intranasal challenges on days 21, 22, 23, 25, and 27, dilute OVA in PBS to make a 10 mg/mL solution (*see Note 7*) (Fig. 1).
6. Anesthetize mice until breathing rate reduces to approximately 1 per second (oxygen 1.5 L/min with 3.5 % isoflurane).
7. Once mice are appropriately sedated, intranasally administer 50 µL of 10 mg/mL OVA solution, adding dropwise through the nares (*see Notes 8 and 9*).
8. Monitor the mice until they recover from anesthesia (approximately 5 min).

3.2 Adjuvant-Free OVA Model Disease Induction

1. Dilute OVA in PBS to make a 100 µg/mL solution.
2. Immunize mice intraperitoneally on days 0, 2, 4, 6, 8, 10, and 12 with 100 µL of OVA solution (10 µg OVA/mouse) (Fig. 2).
3. For intranasal challenges on days 40, 43, and 46, dilute OVA in PBS to make a 4 mg/mL solution (Fig. 2).
4. Anesthetize mice until breathing rate reduces to approximately 1 per second (oxygen 1.5 L/min with 3.5 % isoflurane).
5. Once mice are appropriately sedated, intranasally administer 50 µL of 4 mg/mL OVA solution, adding dropwise through the nares.
6. Monitor the mice until they recover from anesthesia (approximately 5 min).

3.3 House Dust Mite Model Disease Induction

1. Reconstitute lyophilized house dust mite extract to a stock concentration of 2.5 mg/mL in PBS.
2. For HDM challenges on days 0, 1, and 2, dilute stock HDM solution to 2 mg/mL in PBS, and intranasally administer 50 µL dropwise through the nares (Fig. 3).

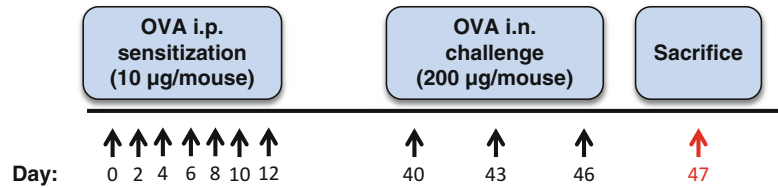


Fig. 2 Mast cell-*dependent* OVA model of allergic asthma. By excluding the alum adjuvant and altering the OVA-sensitization schedule, the OVA allergic asthma model can be rendered mast cell dependent. In this experimental mode, mice are systemically sensitized by intraperitoneal (i.p.) administration of 10 µg ovalbumin (OVA) every 2 days for a total of seven treatments as shown. Four weeks later (day 40), mice are lightly anesthetized and then challenged with OVA (200 µg/mouse) delivered in a small volume (~40–50 µL) through the nares (intranasal, i.n.) using the dose schedule shown. On day 47, 1 day after the final i.n. OVA challenge, mice are sacrificed for analysis or otherwise treated experimentally. The OVA sensitization and challenge doses and the depicted administration schedule were adapted from Nakae et al. [8]



Fig. 3 House dust mite (HDM) model of allergic asthma. House dust mite (HDM) antigen (*Dermatophagoides pteronyssinus*, Der p) prepared in PBS is administered HDM (100 µg/mouse) through the nares (i.n.) of lightly anesthetized mice daily for the first 3 days. Starting on day 13, mice are treated daily with 25 µg/mouse HDM antigen (i.n.). One day after the fifth and final HDM dose, mice are sacrificed for analysis or otherwise treated experimentally. The HDM sensitization and challenge doses and the depicted administration schedule were adapted from Phipps et al. [23]

- For HDM challenges on days 13–17, dilute stock HDM solution to 500 µg/mL in PBS and intranasally administer 50 µL dropwise through the nares (Fig. 3).

3.4 Collection of Bronchoalveolar Lavage Fluid (BALF) and Lung Specimens

- Anesthetize mice with avertin (500 µL injected intraperitoneally per 20 g body weight).
- Place mice ventral side up and pin down forepaws and hindpaws.
- Using scissors and forceps, remove skin above the neck area. Carefully pull the parotid and submaxillary gland laterally, taking care not to rupture the jugular vein.
- Upon visualization of the trachea, remove the muscle layer surrounding it, and then make a pinhole partial cut of the trachea just inferior of the larynx and thyroid. Carefully insert

the flexible 22G catheter into the trachea, using the suture thread to secure the catheter.

5. Prepare three 1 mL slip-tip syringes and fill them each with 1 mL of PBS. Lavage the lungs three times with 1 mL of PBS, slowly advancing and recovering the PBS lavage liquid. Pool the collected lavages into a 15 mL conical tube and store on ice until further use (*see* **Notes 10** and **11**).
6. *Important:* For improved lung histology morphometry, *skip steps 6* and *7* and continue with Subheading 3.5 for lung inflation and fixation.
7. After collecting the BAL, dissect open the thoracic cavity, exposing the left lung and the four lobes of the right lung (superior, middle, inferior, and postcaval).
8. Dissect out some lung specimens for histology, and place in 10 volumes of 10 % buffered formalin. Store specimens at 4 °C for 24 h, and then replace the formalin with 70 % EtOH for long-term storage at 4 °C before paraffin embedding and histology.

3.5 Lung Inflation and Fixation for Histology

1. Before sacrificing mice, assemble apparatus for lung inflations.
 - (a) In a 250 mL Erlenmeyer filter flask, add distilled H₂O until it is about two-thirds full.
 - (b) Take a 10 mL pipette, remove the cotton plug, using a black rubber plug on top of the filter flask, and insert the pipette into the flask until the tip is a few centimeters below the water line.
 - (c) Using a ruler, measure 20 and 25 cm from the top of the water line towards the top of the pipette. Mark the level with a felt pen.
 - (d) Pierce a 30 mL syringe with an 18G needle at around the 10 mL mark. Bond the needle to the syringe with an epoxy or other adhesive, making sure to produce an airtight seal.
 - (e) Connect the hose barb on the side of the flask to the 1.6 mm ID tubing and then add a male-luer adaptor to the end of the tubing in order to connect it to the syringe side port. It will be necessary to step down from the wide tubing connected to the hose barb protruding from the flask to the thinner tubing. Use a female-to-female luer connected to the original tubing and then use a male luer with a 1.6 mm barb to connect to the small-diameter tubing.
2. Once apparatus is assembled, sacrifice mice as mentioned in Subheading 3.5.
3. After collecting the BAL, fill up the 30 mL syringe with 10 % formalin (there should be a fair amount of air in the syringe).

Connect the tip of the syringe to the catheter in the trachea, and connect the tubing from the filter flask to the side port of the syringe.

4. Instill formalin into the lungs until the water inside the filter flask reaches the 25 cm mark on the pipette (indicating a pressure of 25 cm H₂O).
5. Tie off the trachea with the suture thread and remove the catheter.
6. Dissect out the lung and trachea en bloc being careful not to puncture the lung, and place in a 50 mL tube with more 10 % formalin to fix overnight at 4 °C.

3.6 Enumeration and Differentiation of BALF Leukocytes

1. Record collected BALF volume and then spin down at $453\times g$ for 5 min.
2. Resuspend cell pellet in 1 mL of red cell lysis buffer, incubate for 3–5 min, then dilute with PBS, and spin down at $453\times g$ for 5 min.
3. Resuspend cell pellet in 1 mL of PBS or media. Collect aliquot of cells for dilution with trypan blue and enumeration on hemocytometer.
4. Prepare aliquots of cells for cytopspins. Dilute aliquots to $5.0\text{--}7.5\times 10^5$ cells/mL, and cytospin 100 μL of cell dilutions to each slide for 3 min at $55\text{--}60\times g$ (approximately 50,000–75,000 cells/slide, *see* **Note 12**).
5. Allow slides to dry for 2 h to overnight, before staining.
6. Insert slides for 30 s each into 100 % MeOH, solution I and solution II of the PROTOCOL HEMA-3 staining set. Allow excess MeOH/stain to drip down in between solutions.
7. Allow slides to air-dry, and then, if desired mount with cover slip using Permount.
8. Perform differential cell counts from a minimum of 400 cells/slide using standard morphological criteria of macrophages, lymphocytes, neutrophils, and eosinophils (*see* Table 1 and **Note 13**).

3.7 BALF Leukocyte Cell Population Analysis by Flow Cytometry

1. Record collected BALF volume. Spin down at $453\times g$ for 5 min.
2. Resuspend cell pellet in 1 mL of FACS buffer, and aliquot 200 μL into a well of a 96-well v-bottom plate. Pool some of the remaining cells and aliquot into seven individual wells for the single-stained controls needed for FACS setup.
3. Spin down cells for 3 min at $453\times g$, aspirate supernatant, and resuspend cells in 50 μL of blocking buffer (FACS buffer with 10 % goat serum and 5 $\mu\text{g/mL}$ anti-CD16/32 to block Fc receptors, *see* **Note 14**).

Table 1
Morphological criteria of HEMA-3-stained leukocyte lineages from BALF

Cell type	Color of nucleus	Color of cytoplasm	Color of granules	Nucleus morphometry
Macrophages	Violet	Sky blue	No granules	Round, indented, or kidney shaped
Lymphocytes	Violet	Sky blue	No granules	Round, occupies majority of cells with narrow ring of cytoplasm
Neutrophils	Dark blue	Pale pink	Reddish-lilac	Multilobed connected by thin strands
Eosinophils	Blue	Blue	Red/red-orange	Segmented, usually two lobes

4. Incubate cells for 20 min at 4 °C, then spin down cells at $453\times g$ for 5 min, and aspirate supernatant.
5. In FACS buffer, make up antibody mix containing fluorochrome-conjugated antibodies to 7/4, Siglec-F, CD3e, CD11c, CD45, and B220/CD45R. In addition, make up dilutions of each antibody individually for single-stained controls (*see Note 15*).
6. Add 50 μL of antibody mix or single-stained controls to the appropriate samples. Incubate samples protected from light at 4 °C for 20 min.
7. Add 150 μL of FACS buffer to each well, spin down cells at $453\times g$ for 5 min, and aspirate supernatant.
8. Wash samples one more time with 200 μL of FACS buffer, then spin down cells at $453\times g$ for 5 min, and aspirate supernatant.
9. Dilute latex beads 1:100 in FACS buffer to obtain a bead concentration of 1×10^6 beads/mL (*see Notes 16 and 17*).
10. Resuspend samples in 100 μL of diluted beads and resuspend single-stained controls in 100 μL of FACS buffer. Acquire samples on BD LSRII cytometer using FACS Diva software. Ensure that you can differentiate between leukocyte and bead populations on the FSC vs. SSC graph (*see Note 18*).
11. Analyze results using FlowJo software. On FSC vs. SSC graph, gate the beads and leukocyte populations. Using the count function of the analysis software, enumerate the events in both populations and quantitate the total number of cells using the following formula:
 - (a) Number of leukocytes counted $\times$ (number of beads loaded $\div$ number of beads counted) $\times$ dilution factor: In this example, the dilution factor is 5 (200 μL of BALF cells were used out of a total volume of 1 mL, i.e., 1/5th of total BALF cells were used).

Table 2
Cell surface antigen profile of leukocyte lineages in BALF and lung tissue

Lineage	Cell surface antigen profile
Alveolar macrophages	CD11c ⁺ , Siglec-F ⁺ , auto-fluorescence ^{hi}
Dendritic cells	CD11c ^{+/lo} , Siglec-F ⁻ , auto-fluorescence ^{neg}
Eosinophils	Siglec-F ⁺ , CD11c ⁻
Neutrophils	7/4 ⁺
T-cells	CD3e ⁺ , B220 ⁻
B-cells	B220 ⁺ , CD3e ⁻

- (b) Determine the number of BALF cells/mL by dividing the total number of cells by the recorded BALF volume collected.
12. Using the FlowJo FACS analysis software, determine the different leukocyte populations based on their surface staining markers (*see* Table 2 and Notes 19 and 20).

3.8 Lung Leukocyte Isolation for Flow Cytometry

- Lung lobes can be stored in culture media on ice in a 12-well tissue culture plate during animal harvest.
 - Culture media (DMEM with 2–10 % FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, *see* Note 21).
- After animal harvest, aspirate culture media from each well and mince lungs with scissors into small pieces (roughly 1 mm³).
- Add 2 mL of collagenase solution to each well, and incubate for 30–120 min at 37 °C (200 U/mL of collagenase, type IV, diluted in culture media, *see* Note 22).
- After digestion, collect digested lung tissue and media with a 10 mL pipette and transfer to a 70 µm cell strainer on top of a 50 mL conical tube. Pass tissue pieces through strainer using the plunger end of a 3 mL syringe. Wash cells through strainer using extra culture media.
- Spin down cells for 5 min at 453 × *g*.
- Aspirate supernatant, resuspend cells in 3 mL of red cell lysis buffer, incubate at 37 °C for 5 min, then dilute with 10 mL of PBS or culture media, and spin down cells for 5 min at 453 × *g*.
- Make up 30 % Percoll solution.
 - Dilute stock Percoll 9:1 with 10× HBSS to obtain a 90 % Percoll solution with a balanced salt concentration.
 - Dilute 90 % Percoll solution with culture media to make a 30 % Percoll solution.

8. Aspirate supernatant from cells, resuspend cells in 3–5 mL of 30 % Percoll, and spin down cells for 10 min at $290\times g$ with the brake turns off (*see Note 23*).
9. Aspirate supernatant, resuspend purified leukocytes in 1 mL of culture media, and proceed for any downstream application.
 - (a) Isolated cells can be diluted with trypan blue and counted on a hemocytometer to obtain total lung leukocyte counts and cytopun for differentials, following the same protocol for BALF leukocytes in Subheading 3.6.
 - (b) Isolated cells can be enumerated and differentiated by FACS as in Subheading 3.7.
 - (c) Isolated cells can be utilized for in vitro antigen re-stimulation cultures. Plate 1×10^6 to 8×10^6 cells/mL of isolated lung cells in culture media into tissue-culture-treated plates containing various amounts of antigen (1–100 $\mu\text{g/mL}$ of OVA or HDM) and place in 37 °C TC-incubator for 24–96 h. Collect cell-free supernatant after incubation for cytokine analysis by ELISA (*see Note 24*).

3.9 Lung Specimen Histopathology

1. Paraffin-embed lung specimens, section 3–6 μm thick, and then stain with H&E.
2. Under microscopic examination, score disease severity based on the following criteria (Table 3A–C). Score four to five airway sections per mouse for each criterion and take the average of the sum for a final score. Therefore maximum possible score is 12.

4 Notes

1. Most methods mentioned above are tailored for mice on the C57Bl/6J background. Due to the increased Th2 bias of the immune response in other inbred strains (Balb/cJ or 129S1/SvImJ for example) it would be advisable to titrate down the antigen concentration for both the OVA and HDM asthma models.
2. It is important to use only age- and sex-matched mice for all asthma experiments due to the reported sex differences in asthma susceptibility in mice [24, 25].
3. It is important to keep the stock OVA and aluminum hydroxide sterile; only open inside a biosafety cabinet. We also notice that the aluminum hydroxide tends to lose potency after a while and we discard unused adjuvant 1 year after opening.
4. Reconstituted house dust mite extract should be kept at 4 °C for use within 1 month. HDM extract can be reconstituted

Table 3
Infiltration

Score	Criteria (appearance of tissue section, H&E stain)
A. Perivascular infiltration	
0	No cells
1	A few cells
2	Entire encirclement of vessel with infiltrate 1–2 cells deep
3	Entire encirclement of vessel with infiltrate 3–5 cells deep
4	Entire encirclement of vessel with infiltrate >5 cells deep
B. Peribronchiolar infiltration	
0	No cells
1	A few cells
2	Entire encirclement of bronchiole with infiltrate 1–2 cells deep, slight loss of uniformity of airway epithelium
3	Entire encirclement of bronchiole with infiltrate 3–5 cells deep, with a more pronounced hyperplasia and metaplasia of the airway epithelium
4	Entire encirclement of vessel with infiltrate >5 cells deep, complete loss of airway epithelial structure including tissue damage leading to rupture of the bronchiole
C. Parenchymal infiltration	
0	No cells
1	A few scattered cells throughout the lung
2	One quadrant of the lung with a high degree of infiltration
3	Two quadrants of the lung with a high degree of infiltration
4	More than two quadrants of the lung with a high degree of infiltration

and aliquoted for long-term storage at -20°C , but thawed aliquots should not be refrozen. Make note of the Certificate of Analysis values for Derp1 and endotoxin as there is sometimes considerable batch-to-batch variation. Endotoxin should be less than 100 EU per mg of protein; significantly higher endotoxin contamination will result in a model more similar to LPS-induced acute lung injury and will not induce a Th2 response.

5. Use only anesthetics approved by your local animal care committees. Anesthetics such as a combination of ketamine and xylazine can be used in place of avertin.
6. It is important to have a tight seal with the catheter inside the trachea. For 6–8-week-old C57Bl/6 mice a 22G catheter

works well; for older and larger mice a lower gauge catheter, such as an 18G, would be recommended.

7. The OVA dosage for immunizations and challenges has been modified for use with C57Bl/6 mice that are more Th1 biased than other strains. When utilizing Th2-biased strains such as Balb/c or 129/Sv it would be advisable to titre down the dosage of OVA and/or alum. Additionally, we have found that irradiated mice from bone marrow transplantations develop more severe disease symptoms and the amount of OVA and/or adjuvant should be titrated down accordingly.
8. For intranasal challenges, the volume of solution administered can be modified but should not exceed 50 μ L.
9. For intranasal administration, it is imperative to treat the mice at the optimal sedation point. We normally find this to be when the mice have a breathing rate of one breath every second.
10. To avoid the entrance of blood in the BAL, avoid performing cardiac punctures until after the collection of the BAL. After collection of the BAL, a cardiac puncture can be performed with a 1 mL syringe and 25G needle. Collected blood can then be allowed to clot and serum collected for measurement of serum antibodies (i.e., IgE and IgG₁) or cytokines.
11. If interested in measuring cytokines or protein in the BAL fluid (BALF) it is advised to collect the first lavage in a small volume of PBS (i.e., 0.5 mL) and collected into a 1.5 mL microfuge tube. Follow this with two subsequent instillations of 1 mL that are pooled into a 15 mL conical tube. Spin down the microfuge tube and collect the supernatant noting its volume. Resuspend the pellet in an equal volume of PBS and pool with the other two washes. Freeze the BALF immediately for downstream ELISA measurements.
12. It is important to normalize the number of cells cytopspun onto the glass slides. Too high a cell density makes it difficult to accurately differentiate and count the leukocytes, whereas too low a density will slow the counting on 400 individual events.
13. Accurately performing differential cell counts visually from stained cytopspins can take a fair amount of training and cannot identify between different myeloid or lymphoid populations (i.e., macrophages and dendritic cells or T-cells, B-cells, and NK-cells). Performing differentials using flow cytometry, as in Subheading 3.7 or as performed by van Rijjt et al. [26], allows for rapid, accurate, and reproducible differentials between different researchers or research sites.
14. The use of V-bottom plates makes it easier to identify the cell pellet and remove the supernatant. For larger sample sizes, after centrifugation plates can be inverted and carefully “flicked” to remove supernatant rather than aspirating individual

wells. Check for cell pellets before and after to ensure no loss of cells during this procedure.

15. This protocol is optimized for a BD LSRII cytometer equipped with 405, 488, and 633 nm excitation lasers. The fluorochromes and antibodies listed are suggested and can be modified depending on the instrumentation available. For example, CD19 can easily be substituted for B220 in order to mark B-cells depending on antibodies available. If FACS detectors are at a premium, CD45 can be omitted and CD3e and B220 antibodies can be used conjugated to the same fluorochrome, as in the protocol by van Rijjt et al. [26], reducing the number of different fluorochromes to four from six. Conversely, other antibodies can be added, such as NK1.1, to differentiate NK- and NKT-cells from other lymphocytes, and CD4 and CD8 to differentiate the different T-cell subsets, depending on available detectors on the accessible cytometers.
16. After diluting latex beads, remove aliquot and confirm bead concentration using a hemocytometer.
17. We find that the use of a viability marker, such as propidium iodide (PI), helps clean up the FACS results if there are unused detectors. If using PI, dilute latex beads in FACS buffer containing PI and add to cells. Allow cells to sit for 5–10 min protected from light at RT and then analyze on a flow cytometer. The PI stain needs to be with the cells during acquisition; *do not* wash PI out after staining.
18. When acquiring samples, setting FSC to linear scale and SSC to log scale will make it easier to differentiate between cell and bead populations.
19. Alveolar macrophages are highly autofluorescent and can bleed into several FACS channels. It is advised to gate them on their autofluorescent properties and expression of both CD11c and Siglec-F, and then remove them from the population for the analysis of other leukocyte subsets.
20. It should be noted that the staining parameters provide a rough differential. For instance, there are multiple different dendritic cell subsets present in the inflamed alveolar space or lung. Additional markers, such as CD11b and CD103, can also be used to further classify the infiltrating leukocyte cells.
21. IMDM and RPMI can be substituted for DMEM. In addition, media can be supplemented with HEPES (25 mM final concentration) to buffer pH.
22. This isolation protocol is tailored for isolation of leukocyte cells from lung tissue (approximately >95 % CD45⁺). If endothelial or epithelial cells are also desired, dispase should be added to the collagenase solution (to a concentration of 1.5–3.0 mg/mL), and the Percoll step should be omitted.

23. If aiming to isolate both leukocytes, endothelial cells and epithelial cells, omit the Percoll step and instead add dispase and/or DNase into the digestion mixture along with collagenase. Samples may need to be passed through another 40 or 70 μ m cell strainer to obtain a single-cell suspension before downstream applications.
24. If interested in culturing isolated lung cells, perform **steps 4–9** in a biosafety cabinet to maintain a sterile work environment.

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