



Chapter 8

Induction of Airway Hypersensitivity to Ovalbumin and Dust Mite Allergens as Mouse Models of Allergic Asthma

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Abstract

Mouse models of allergic asthma have been utilized to establish the role of T helper type 2 (Th2) cells in driving lung inflammation, airway hyperresponsiveness, and obstruction. Here, we present the allergic asthma models, in which mice are hypersensitized to ovalbumin (OVA) and house dust mite (HDM). These models mimic the major characteristics of human asthma including the eosinophilic inflammation and hyperactivity of the airway, overproduction of Th2 cytokines in the lung, and elevated total and allergen-specific immunoglobulin E (IgE) in serum.

Key words Airway hypersensitivity, Allergic asthma, Pulmonary eosinophilia, House dust mite, Immunoglobulin E, Mouse model, Ovalbumin, T helper type 2 cytokines

1 Introduction

Allergic asthma is a chronic inflammatory disease of the airways associated with enhanced T helper type 2 (Th2) responses to inhaled environmental allergens, such as cat dander, pollen, and house dust mite proteins [1–3]. Such Th2 responses lead to bronchial eosinophil infiltration, airway hypersensitivity, mucus hypersecretion, and elevated serum immunoglobulin E (IgE) levels [4]. Some Th2 cytokines, such as interleukin (IL)-5, promote the differentiation and activation of eosinophils, while IL-4 and IL-13 enhance IgE production and further enhance the severity of asthma [5, 6]. Mouse models have been developed to mimic the features of human allergic asthma and are essential to better understand the pathophysiologic mechanisms in asthma development [7, 8]. Here, we describe the mouse models of allergic asthma induced by the experimental allergen chicken egg ovalbumin (OVA) and naturally occurring allergen house dust mite (HDM) [9, 10]. Moreover, the detailed information to determine the severity of asthma responses will be also included, such as pulmonary function test, eosinophil count in bronchoalveolar lavage (BAL) fluid, lung histology, lung

Th2 cytokine measurement by quantitative reverse transcription-polymerase chain reaction (RT-PCR) or flow cytometry, and serum IgE measurement.

2 Materials

2.1 Allergic Asthma Mouse Models

1. 10× Phosphate-buffered saline (PBS) stock solution: pH 7.4. Dissolve 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, and 2.4 g KH₂PO₄ in 800 mL of distilled water. Adjust pH to 7.4 and make up to 1 L with distilled water. Sterilize by autoclave. Dilute 10 mL of 10× PBS stock solution in 90 mL of distilled water to make PBS (1×).
2. Imject™ Alum adjuvant: 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide with inactive stabilizers.
3. OVA solution: 1 mg/mL ovalbumin (OVA) in sterile PBS. Weigh 100 mg of OVA (*see Note 1*) in 100 mL of PBS, filter through a 0.22-μm filter for sterilization. Aliquot 1 mL in sterile 1.5-mL microcentrifuge tubes and store at −80 °C.
4. HDM extract solution: Dissolve 2 mg/mL HDM extract (*Dermatophagoides pteronyssinus*) in PBS. Aliquot 1 mL in sterile 1.5-mL microcentrifuge tubes and store at −80 °C.
5. Rotator: used to mix OVA and adjuvant.
6. 1-mL syringe with a 25-gauge needle.
7. Isoflurane and isoflurane vaporizer connected with oxygen gas.
8. Pipettors and tips.

2.2 Pulmonary Function Test

1. Equipment for pulmonary function test with ventilator: Scireq flexiVent or equivalent.
2. Anesthetic agents: Freshly prepare xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight) by diluting in sterile PBS.
3. 70% Ethanol: Dilute 700 mL of absolute ethanol (100%) with 300 mL of distilled water.
4. Methacholine solutions: Dissolve methacholine in PBS at different concentrations (0, 3, 24, and 48 mg/mL) for pulmonary function test.

2.3 Examination of Bronchoalveolar Lavage (BAL) Fluid Cells

1. Lavage solution: 0.1 mM ethylenediaminetetraacetic acid (EDTA) prepared in PBS.
2. Enzyme-linked immunosorbent assay (ELISA) kits for Th2 cytokines: commercially available.
3. Trypan blue solution: Prepare a 0.4% solution in PBS to stain bronchoalveolar lavage (BAL) cells.

4. Hemocytometer for counting cell number.
5. Shandon Cytospin® for depositing monolayer cells on slides.
6. Precleaned microscope slides.
7. Wright–Giemsa staining kit: commercially available.
8. Mounting medium.
9. Coverslips.
10. Microscope.

2.4 Lung Histology

1. 10% Formalin solution: prepare in PBS.
2. Ethanol solutions for tissue dehydration/rehydration steps: prepare 70%, 80%, 95%, and 100% solutions.
3. Xylene for tissue dehydration.
4. Paraffin wax: Heat to 58 °C for tissue embedding.
5. Paraffin embedding molds.
6. Microtome.
7. Hematoxylin and eosin (H&E) stain.
8. Perform periodic acid-Schiff (PAS) stain.

2.5 Quantitative RT-PCR of Lung Tissue

1. TRIzol RNA isolation reagent or equivalent.
2. Homogenizer or 1-mL Luer Lock Syringe with a 20-gauge needle.
3. NanoDrop or spectrophotometer to determine RNA concentration.
4. Complementary DNA (cDNA) synthesis kit.
5. PCR tubes and caps.
6. Pipettors and tips: PCR grade.
7. SYBR™ Green real-time PCR reagents master mixes.
8. Primer pairs for IL-4, IL-5, IL-13, IFN- γ , and glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (*see* Table 1).
9. Real-time thermal cycler.

Table 1

Forward and reverse primers for the detection of Th2 cytokine and GAPDH reference gene expression using quantitative RT-PCR

Gene	Forward primer	Reverse primer
IL-4	5'-TTGAACGAGGTCACAGGAGA-3'	5'-AAATATGCGAAGCACCTTGG-3'
IL-5	5'-GCAATGAGACGATGAGGCTT-3'	5'-CATTTCCACAGTACCCCCAC-3'
IL-13	5'-TGCCAAGATCTGTGTCTCTCC-3'	5'-CCAGGTCCACACTCCATACC-3'
IFN- γ	5'-GCGTCATTGAATCACACCTG-3'	5'-GAGCTCATTGAATGCTTGGC-3'
GAPDH	5'-CGTCCCGTAGACAAAATGGT-3'	5'-TCAATGAAGGGGTCGTTGAT-3'

2.6 Lung Phenotyping by Flow Cytometry

1. 1.5-mL microcentrifuge tubes.
2. Surgical scissors.
3. Conical tubes: 15-mL and 50-mL tubes.
4. Liberase™ TL: 25 mg/mL. Add 200 μ L of distilled water into 1 vial of 5 mg Liberase.
5. DNase I: 10 mg/mL. Dissolve 100 mg DNase I in 10 mL of distilled water. Aliquot in 1.5-mL microcentrifuge tubes and store at -20°C .
6. Digestion medium: RPMI 1640 medium supplemented with $1\times$ penicillin/streptomycin and 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES).
7. Orbital shaker.
8. Complete RPMI 1640 medium: Supplement with 5% fetal bovine serum (FBS), $1\times$ penicillin/streptomycin, 2 mM L-Glutamine, $1\times$ nonessential amino acid (NEAA), 1 mM sodium pyruvate, 55 μ M β -mercaptoethanol, and 10 mM HEPES.
9. Sterile 40- μ m cell strainers.
10. 100-mm Petri dishes.
11. Sterile plungers from 1-mL syringes.
12. 47% Percoll gradient solution: Mix 47 mL of 100% Percoll solution with 53 mL of complete RPMI 1640 medium.
13. Refrigerated centrifuge.
14. Microplate centrifuge.
15. 96-well V-bottom plate.
16. Lymphocyte stimulating reagents: 200 μ g/mL phorbol 12-myristate 13-acetate (PMA), 250 μ g/mL ionomycin, 1 mg/mL Brefeldin A, all prepared in dimethyl sulfoxide (DMSO). Store at -20°C .
17. CO_2 incubator: set to 37°C .
18. Fluorescence-conjugated anti-mouse antibodies: anti-CD4, anti-Foxp3, anti-IL-4, anti-IL-5, anti-IL-13, anti-IFN- γ , and cell viability dye.
19. Fixation and permeabilization buffer set for flow cytometry: commercially available.
20. 2% paraformaldehyde.
21. Fluorescence-activated cell sorting (FACS) buffer: 0.5% bovine serum albumin (BSA) prepared in PBS.
22. Multicolor flow cytometer.

2.7 Total and OVA-Specific IgE Measurement

1. 1-mL Syringe with a 25-gauge needle.
2. 1.5-mL Microcentrifuge tubes.
3. Refrigerated centrifuge.
4. ELISA kit for total IgE and OVA-specific IgE: commercially available.

3 Methods

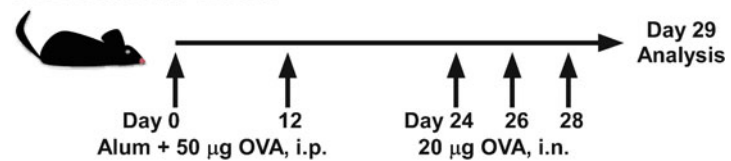
3.1 Allergic Asthma Mouse Model

Mouse models of allergic asthma induced by experimental allergen OVA or clinically relevant allergen HDM are described here (*see Note 2*). In the OVA-induced asthma protocol as shown in Fig. 1, 8-week-old mice, housed under specific-pathogen-free conditions, are sensitized intraperitoneally with 50 μ g OVA emulsified with adjuvant aluminum hydroxide on Days 0 and 12 and then challenged intranasally with 20 μ g OVA on Days 24, 26, and 28 [9]. In the HDM-induced asthma protocol (Fig. 1), mice are exposed intranasally to 100 μ g HDM extract once a week for total four doses of challenge [9]. Prepare all reagents and perform all procedures using sterile technique.

3.1.1 Sensitization and Challenge Protocol for OVA-Induced Asthma Model

1. Before sensitization, freshly prepare OVA emulsified with Imject™ Alum adjuvant. Thaw an aliquot of 1 mg/mL OVA solution prepared in PBS. Mix 250 μ L of the OVA solution, 100 μ L of Imject, and 650 μ L of PBS in a 1.5-mL microcentrifuge tube, and emulsify the mixture on a rotator for 1 h at 4 °C.

OVA-induced model



HDM-induced model

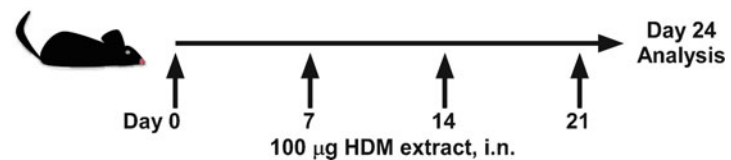


Fig. 1 Schematic diagram depicting the protocols for OVA- and HDM-induced allergic asthma models as described in Subheading 3. *i.p.* intraperitoneal, *i.n.* intranasal

2. Inject each mouse on Days 0 and 12 intraperitoneally with 200 μ L of the OVA/Imject mixture using a 1-mL syringe with a 25-gauge needle. Each mouse will receive 50 μ g OVA and 0.8 mg of aluminum hydroxide per dose. Inject control mice with 200 μ L of PBS/Imject solution (*see Note 3*).
3. Challenge mice on Days 24, 26, and 28. Before challenge, thaw 1 tube of 1 mg/mL OVA solution and dilute with PBS to make 400 μ g/mL OVA solution.
4. Transfer a previously sensitized mouse to the induction chamber for inhalational anesthesia by vaporizing isoflurane and oxygen gas mixture. Remove the mouse from the induction chamber once the respiratory rate slows down (*see Note 4*).
5. Holding the mouse with one hand, pipette 50 μ L of 400 μ g/mL OVA intranasally (*see Note 5*). Total of 20 μ g OVA is given per challenge per mouse. Inject control mice intranasally with 50 μ L of PBS.
6. Sacrifice the mouse for further assessment of asthma phenotype on Day 29, 1 day after the last challenge.

3.1.2 Challenge Protocol for HDM-Induced Asthma Model

1. Before challenge, thaw an aliquot of 2 mg/mL HDM extract solution.
2. Transfer a mouse to the anesthetic chamber with vaporizing isoflurane and oxygen gas mixture. Monitor respiratory rate for anesthetic depth. Proceed to the next step once respiration slows.
3. Pipette 50 μ L of 2 mg/mL HDM extract solution (100 μ g per dose per mouse) to the mouse intranasally as described in the OVA-induced protocol in Subheading 3.1.1. Inject control mice intranasally with 50 μ L of PBS. Challenge mice on Days 0, 7, 14, and 21.
4. Sacrifice mice for further evaluation on Day 24, 3 days after the last challenge (*see Note 6*).

3.2 Pulmonary Function Test

Airway hypersensitivity is one of the characteristic features of asthma [11]. One day after the last challenge for the OVA-induced model, or 3 days after the last challenge for the HDM-induced model, the airway resistance is measured in intubated and ventilated mice by stimulating with increasing concentrations of methacholine (0, 3, 24, and 48 mg/mL in PBS) using a computer-controlled ventilator.

1. Anesthetize the mouse with the anesthetic reagent (xylazine and ketamine mixture) intraperitoneally.
2. Verify that the mouse shows no reaction to a toe pinch and reaches a surgical level of anesthesia.

3. Disinfect the throat area with 70% ethanol. Cut the skin and gently remove the submaxillary gland to expose the trachea. Then, perform a tracheotomy to insert a 20-gauge metal cannula inside the trachea and use a surgical stitch to fix the cannula in place.
4. Connect the mouse to the ventilator (*see Note 7*). Expose the mouse to nebulized PBS for baseline measurement, and subsequently to increasing concentrations (3, 24, and 48 mg/mL) of nebulized methacholine in PBS. Record the resistance index (RI) at each dosage for 3 min. The dynamic airway resistance can be determined by using Scireq software or equivalent.
5. Euthanize the intubated mouse by gradually filling the induction chamber with carbon dioxide (*see Note 8*). Mice euthanized after the pulmonary function test are used to collect BAL fluid as described in Subheading 3.3.

3.3 Examination of BAL Fluid Cells

Eosinophils are the key inflammatory cells involved in asthma pathophysiology [12]. In asthma mouse models, allergic airway inflammation can be assessed by counting the total number of cells and eosinophils in BAL fluid.

1. Mice euthanized after the pulmonary function test in Subheading 3.2 are used to collect BAL fluid.
2. Lavage the lung with 0.7 mL of PBS containing 0.1 mM EDTA by connecting a 1-mL syringe to the metal cannula intubated in Subheading 3.2. Then, transfer the BAL fluid to a 1.5-mL microcentrifuge tube on ice.
3. Centrifuge the cells in BAL fluid for 5 min at $800 \times g$ at 4 °C. Collect the supernatants of BAL fluid in a new 1.5-mL microcentrifuge tube, and store at –80 °C for airway cytokine measurements. Determine Th2 cytokine levels in the BAL fluid by using commercial ELISA kits according to the manufacturer's instructions.
4. Resuspend BAL cells in 1 mL of PBS and count the absolute number of BAL cells using trypan blue dye exclusion under a microscope.
5. Centrifuge the BAL cells onto slides at 1000 rpm for 3 min in a Shandon Cytospin (*see Note 9*). Air-dry the slides before proceeding with staining.
6. Stain the BAL cells with Wright–Giemsa stain in a staining jar for 3 min, and then rinse with distilled water three times and air-dry (*see Note 10*).
7. Mount the slide with mounting medium and coverslip. Characterize and count the cell types, that is, eosinophils, neutrophils, lymphocytes, and macrophages based on staining morphology profiles (Fig. 2a) under a microscope at $1000\times$ magnification for a total of 200 cells per slide.

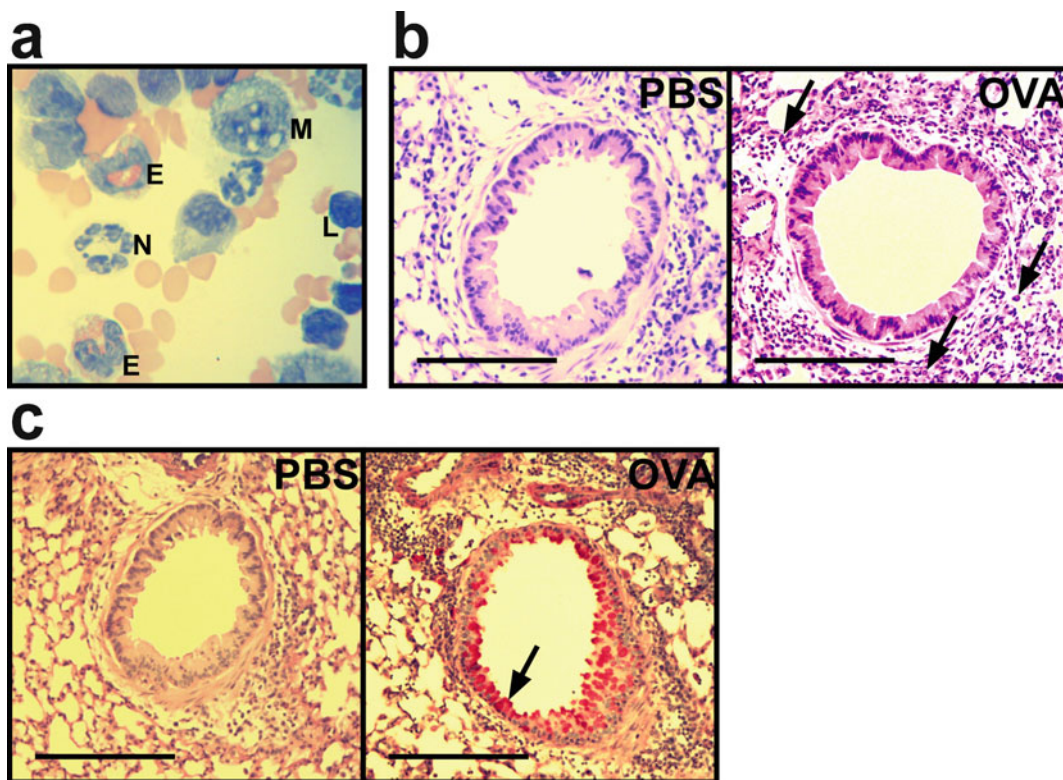


Fig. 2 (a) Wright–Giemsa staining of the BAL from sensitized mouse airway. *M* macrophages, *L* lymphocytes, *N* neutrophils, *E* eosinophils. Magnification, $\times 1000$. (b) H&E-stained lung sections from unsensitized (PBS) and sensitized (OVA) mice are shown. Cells are largely infiltrated in the perivascular and peribronchial spaces in sensitized lung tissue. Scale bar, 200 μm . (c) PAS staining of lung sections from unsensitized (PBS) and sensitized (OVA) mice is indicated. The arrow indicates a PAS⁺ mucus-secreting cell in a bronchiole. Scale bar, 200 μm

3.4 Lung Histology

The lung inflammation can also be assessed by lung histology. Hematoxylin and eosin (H&E) staining is used to stain cell and tissue structures [13]. Periodic acid-Schiff (PAS) stain is performed to detect mucus hypersecretion by goblet cells [14].

1. Harvest the lung tissue, and immediately fix it in 10% formalin solution.
2. After fixation, dehydrate the lung tissues with the following steps to embed the lung tissue in paraffin wax for section:
 - (a) 70% Ethanol: 2 $\times$ 1 h each.
 - (b) 80% Ethanol: 1 $\times$ 1 h.
 - (c) 95% Ethanol: 1 $\times$ 1 h.
 - (d) 100% Ethanol: 3 $\times$ 1 h each.
 - (e) Xylene: 3 $\times$ 1 h each.
 - (f) Paraffin wax at 58 $^{\circ}\text{C}$: 2 $\times$ 2 h each.
3. Embed tissues in paraffin blocks.

4. Cut the paraffin-embedded lung tissues at 10 μm by a microtome and transfer the sections onto slides.
5. Deparaffinize and rehydrate the lung sections with the following wash sequence:
 - (a) Xylene: 3×3 min each.
 - (b) 100% Ethanol: 3×3 min each.
 - (c) 95% Ethanol: 1×3 min.
 - (d) 70% Ethanol: 1×3 min.
 - (e) Wash slides in distilled water.
6. Stain lung paraffin sections with hematoxylin and eosin stain according to the manufacturer's instructions for examining lung pathology due to airway allergic reaction. Eosinophilic inflammation can be detected in the perivascular and peribronchial spaces in sensitized lung tissue (Fig. 2b).
7. Perform periodic acid-Schiff (PAS) stain following the manufacturer's instructions to detect goblet cell hyperplasia (Fig. 2c). Quantitate the level of goblet cell hyperplasia in the airway by counting PAS⁺ epithelial cells in individual bronchioles. Assess at least 10 bronchioles.

3.5 Th2 Cytokine Determination in the Sensitized Lung

Th2 cells produce cytokines IL-4, IL-5, and IL-13, which have been shown to drive disease pathology in patients with asthma [15]. For detecting cytokine levels in sensitized lungs, Th2 cytokines can be detected in BAL fluid from sensitized airway by commercial ELISA kits (*see* Subheading 3.3, steps 2 and 3 for collection of BAL fluid). Alternatively, the gene expression level of Th2 cytokines can be determined by quantitative RT-PCR of lung tissue or by analyzing cytokine-producing T cells via flow cytometry. Both approaches are described here.

3.5.1 RNA Isolation and Quantitative RT-PCR of Lung Tissue

1. Harvest one lobe of lung (upper right) and store in 1 mL of TRIzol reagent at -80°C freezer before RNA extraction.
2. Thaw the lung tissue sample at room temperature and homogenize the tissue in TRIzol reagent using a homogenizer (*see* Note 11).
3. Extract total lung RNA by using TRIzol reagent following the manufacturer's instructions. Determine the RNA concentration by a NanoDrop or spectrophotometer.
4. Generate cDNAs according to the instruction provided by the kit's manufacturer.
5. Perform quantitative real-time PCR using SYBR Green PCR kits. The thermal cycling conditions used for all genes are 95°C for 10 min, and 40 repeats at 95°C for 15 s and 60°C for 1 min. Calculate the relative expression of each cytokine gene by normalizing with housekeeping gene GAPDH expression.

3.5.2 *Ex Vivo Lung
Phenotyping by Flow
Cytometry*

1. Dissect remaining lobes of the lung, wash in plain RPMI 1640, and transfer to 1.5-mL microcentrifuge tubes (*see Note 12*).
2. Chop the lung tissues into small pieces by small surgical scissors.
3. Enzymatically digest the lung tissue in 10 mL of the digestion medium containing Liberase™ (0.16 U/mL, Liberase TL) and 0.05% DNase I in a 50-mL conical tube for 30 min at 37 °C on an orbital shaker at 190 rpm (*see Note 13*).
4. Inactivate the enzyme reaction by adding 10 mL of cold complete RPMI 1640 medium.
5. Pass the digested tissue mixture through a 40- μ m strainer on a 100-mm Petri dish. Mash up the tissue on the strainer with the plunger flange of a 1-mL syringe. Collect the filtered cell suspension in a 50-mL conical tube.
6. Add additional 10 mL of cold complete RPMI 1640 medium to the 40- μ m strainer and repeat tissue mashing.
7. Collect the filtered cell suspension into the same collection tube.
Repeat **steps 6** and **7** one more time for a total volume of 40 mL.
8. Centrifuge the cell suspension for 5 min at $400 \times g$ at 4 °C and discard the supernatant.
9. Resuspend the cell pellet with 10 mL of 47% Percoll gradient solution, transfer to a 15-mL conical tube, and centrifuge for 10 min at $400 \times g$ at 4 °C to enrich lymphocytes.
10. Wash cell pellet with 10 mL of complete RPMI 1640 medium and count the cell number.
11. Centrifuge the cell suspension, discard the supernatant, and adjust the cell concentration to 1×10^6 cells/mL with complete RPMI 1640 medium.
12. To detect cytokine production, place 200 μ L of the lung suspension from **step 12** into a 96-well V-bottom plate (2×10^5 cells per well).
13. Centrifuge the plate, discard the supernatant, and resuspend the cells with 200 μ L of complete RPMI 1640 medium containing a working concentration of each of the lymphocyte stimulating reagents (50 ng/mL PMA, 0.5 μ g/mL ionomycin, and 1 μ g/mL Brefeldin A) to stimulate cytokine production. Incubate for 4 h at 37 °C in a CO₂ incubator.
14. Centrifuge the plate, discard the supernatant, and then wash the cells with 200 μ L of FACS buffer twice.
15. Resuspend the cells with 50 μ L FACS buffer containing Fixable Viability Dye and a fluorescence-conjugated anti-mouse CD4 antibody for CD4 T-cell staining. Incubate the cells at 4 °C for 30 min in the dark.

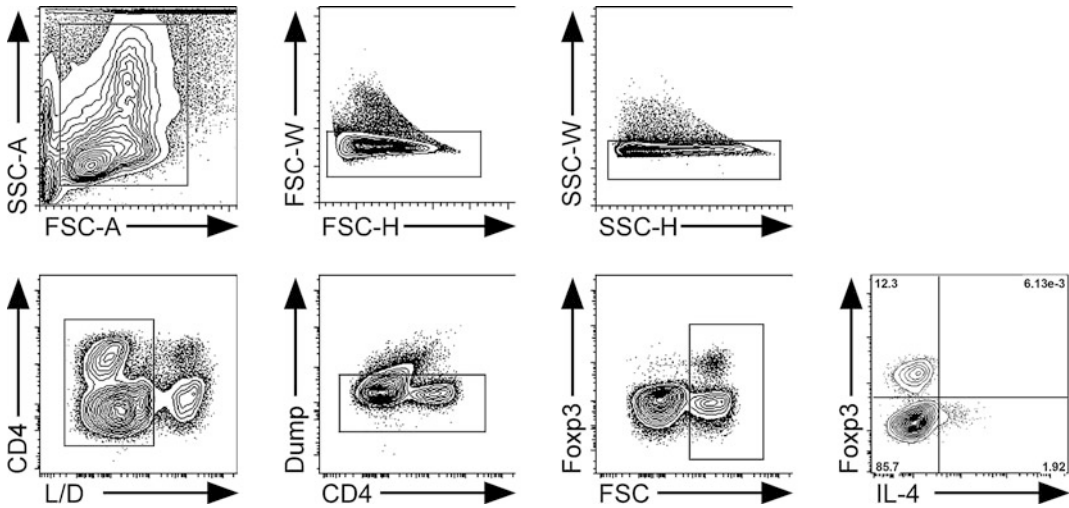


Fig. 3 Flow cytometry analysis gating strategy to identify cytokine-secreting T effector cells. Gating sequence: upper panel, from left to right; and then bottom panel, from left to right

16. To the existing FACS buffer, add 150 μ L of FACS buffer to each well, centrifuge the plate, and discard the supernatant. Wash the cells with 200 μ L of FACS buffer.
17. Fix the cells with 200 μ L of fixation and permeabilization buffer and incubate at 4 $^{\circ}$ C for 30 min or overnight, according to the manufacturer's instructions.
18. Add 150 μ L of the permeabilization buffer to each well, centrifuge the plate, and discard the supernatant. Wash the cells with 200 μ L permeabilization buffer.
19. Resuspend the cells with 50- μ L permeabilization buffer containing anti-mouse Foxp3, IL-4, IL-5, IL-13, and IFN- γ antibodies for intracellular staining for 45 min at room temperature in the dark.
20. Add 150- μ L permeabilization buffer to each well, centrifuge the plate and discard the supernatant. Resuspend the cells with 200 μ L of 2% paraformaldehyde.
21. Run the stained samples on a flow cytometer, acquiring 2×10^5 cell events to analyze data. Figure 3 shows the gating strategy to identify cytokine-positive T effector cells. Determine the relative frequencies for cytokine-positive T effector cells.

3.6 Total and OVA-Specific IgE Measurement

IgE plays a central role in the pathogenesis of allergic asthma. Elevated serum IgE level is one of the main features of human asthma [16]. Total and OVA-specific IgE levels can be determined by enzyme-linked immunosorbent assay (ELISA).

1. Withdraw cardiac blood with a 1-mL syringe with a 25-gauge needle and transfer the blood to 1.5-mL microcentrifuge tubes (*see Note 8*). Allow the blood to coagulate at room temperature for 30 min.
2. Centrifuge the peripheral blood samples for 5 min at $800 \times g$ at 4°C .
3. Transfer the serum samples to new 1.5-mL microcentrifuge tubes. Approximately 200 μL serum can be collected from one mouse. Serum samples can be immediately used in the next step or can be kept in a -80°C freezer for long-term storage. If frozen, ensure that serum samples are thawed on ice prior to use.
4. Make a two- to fivefold dilution of serum samples using sample dilution buffer provided by the selected ELISA kit. Determine total and antigen-specific IgE in serum by commercial ELISA kits according to the manufacturer's instructions.

4 Notes

1. Many commercially available recombinant OVAs have some levels of endotoxin (lipopolysaccharide, LPS) contamination that will largely affect the readouts of asthma responses. We, therefore, recommend that OVA used in asthma models will be carefully screened for endotoxin contamination by an endotoxin quantitation kit.
2. Protocols described here are for the C57BL/6J mouse strain. Protocols used in other mouse strains need to be adjusted accordingly. We have adjusted the asthma protocol in the BALB/c strain by sensitizing mice on Days 1, 2, 3, 14 and challenging the mice on Days 14, 17, 20, 23, and 26 [17]. All mice should be maintained and handled in accordance with the Institutional Animal Care and Use Guidelines and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.
3. Gently invert the tube with OVA/Imject solution between injections to make sure that OVA and the alum adjuvant are evenly distributed in the solution and that all mice are sensitized with the equal amount of OVA/Imject solution.
4. Carefully monitor the anesthetic depth either by a toe pinch or by observing the respiratory rate via movement of the chest wall. Do not wait until the mouse starts hiccoughing, as this will cause the solution to flow out during intranasal administration.

5. Inject the antigen immediately once the respiratory rate slows down. Administer approximately half of the amount in each nostril. Hold the mouse still for a few seconds to allow the mouse to inhale the antigen solution into the lung.
6. To mimic natural asthma induction, mice are challenged directly with HDM extract without sensitization in the HDM-induced asthma model. We observe the highest pulmonary inflammation at 3 days following the last challenge.
7. Align the animal to the ventilator at the same level to avoid a possible tracheal twist. Conscious active breathing by the mouse will largely distort the lung function measurement by the intubated ventilation method. Thus, double check that the respiratory system of the mouse remains passive under deep anesthesia to obtain valid resistance value during measurements.
8. If you want to collect blood to save serum samples, collect the blood right after mouse is euthanized by carbon dioxide to avoid blood clotting. Pull back the syringe plunger slowly to prevent collapsing, which can stop blood flow into the syringe.
9. Alternatively, BAL cell slides can be prepared without using a Cytospin. Concentrate the BAL cell suspension by centrifugation at $800 \times g$ at 4°C for 5 min, resuspend the BAL cell pellet with 0.1 mL of PBS, then spread the concentrated cell suspension onto a slide evenly by using another clean slide. Allow the slide to air-dry before proceeding with the staining protocol.
10. Wright–Giemsa stain can be reused within 1 month. Adjust the staining duration accordingly, for example, prolong staining time for 10 more seconds when Wright–Giemsa stain has been reused after more than 1 week.
11. If a homogenizer is not available, tissue homogenization can also be performed by passing the tissue in TRIzol reagent through 1-mL Luer Lock syringe with a 20-gauge needle up to 10 times.
12. To obtain actual lung tissue-infiltrating lymphocytes, perform whole-body perfusion with PBS before harvesting the lung tissues. Take 10 mL of PBS by a 10-mL syringe with a 25-gauge needle, and then slowly infuse all of the PBS into the right ventricle of the heart, and the lungs should turn white in color.
13. Collagenase is cheaper and can be used as an alternative to digest lung tissue, but Liberase digestion will have better cell viability and recovery rate.

Acknowledgments

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