



Experimental Mouse Models of Asthma and Analysis of CD4 T Cells

William J. Branchett, Simone A. Walker, and Clare M. Lloyd

Abstract

Asthma is a highly prevalent lung disease, characterized by airway dysfunction and chronic inflammation. Asthma occurs in both children and adults, but frequently originates in early life. Heterogeneous asthma phenotypes exist, but Th2 cells are key players in a large proportion of cases, while other CD4⁺ T cell subsets are also implicated in driving and limiting pathology. In this chapter, we describe methods for establishing allergic airway disease to model asthma in adult and neonatal mice, along with protocols for measuring key disease parameters and quantifying and phenotyping CD4⁺ T cell subtypes.

Key words Th2, Asthma, Allergic airway inflammation, Lung, Flow cytometry, Mouse models

1 Introduction

Asthma is a chronic lung disease characterized by drug-reversible bronchoconstriction, airway inflammation, and structural remodeling of the airway wall. The majority of asthmatics display a type 2^{hi} allergic asthma phenotype, which originates in childhood and is typified by eosinophilic inflammation, raised immunoglobulin E (IgE) to one or more aeroallergens, and production of the hallmark type 2 cytokines IL-4, IL-5, and IL-13 from Th2 cells and group 2 innate lymphoid cells (ILC2s) [1, 2]. However, there is considerable heterogeneity within clinical phenotypes and underlying pathobiological endotypes of asthma, with elevated type 1 and type 17 immunity and neutrophilic or paucigranulocytic inflammation observed, particularly in severe disease [2–5].

CD4 T cells are central to the pathogenesis of both type 2^{hi} and non-type 2 asthma phenotypes [2, 5–7], while regulatory CD4⁺ T cells (Tregs) are also induced by allergen exposure and are associated with protection from asthma symptoms [7]. T follicular helper cells (Tfh), key players in the germinal center reaction, are generated by chronic allergen exposure in the lungs and lung-draining mediastinal lymph nodes (mLN) and contribute to

allergic airway disease (AAD) [8, 9]. Moreover, several newly licensed and emerging biologic therapies for severe asthma are directed at T helper cell-derived cytokines [10, 11]. Enumeration of CD4 T cell subsets and characterization of their phenotypes are therefore highly pertinent to the study of asthma.

Mechanistic studies of asthma are limited by the availability of human samples. Peripheral blood is relatively easily obtained, but does not faithfully represent the immunology of the lung tissue and airway lumen. Bronchoalveolar lavage (BAL) and bronchial wall biopsies can be obtained from asthma patients, typically as part of diagnostic investigations. However, these procedures are not performed on all patients and samples are often not obtained serially. Animal models, principally using the laboratory mouse, are therefore important tools for mechanistic studies of the immunology and pathology of asthma.

Early mouse models of asthma were based on peripheral sensitization to a model allergen such as ovalbumin, by immunization with the Th2-biasing allergen aluminum hydroxide (alum). A pulmonary type 2 immune response is then elicited by repeated airway exposures to ovalbumin [12, 13]. These models generate a strong Th2 immune response in the lung, accompanied by raised ovalbumin-specific IgE and eosinophilic airway inflammation, but bypass the antigen-presenting cell network of the lungs and so represent a nonphysiological method of sensitization [12, 13]. More recently, protocols of repeated exposure to clinically relevant aeroallergens, such as house dust mite (HDM) or the fungus *Alternaria alternata* (ALT), via the airways by intranasal or intratracheal administration have been developed [13]. Airway allergen exposures can be performed intermittently or repeatedly over a number of weeks [13] to better mimic the constant exposure to allergens in the environment. Prolonged airway allergen exposures also drive airway wall remodelling [14, 15]. These protocols can be performed in neonatal mice, with intranasal dosing beginning in the first week of life, to study allergic immune responses in early life and model childhood asthma [15]. This is important since both the immune system and pulmonary system continue development in the postnatal period and there are important distinctions that mark pediatric and adult asthma phenotypes [16]. Airway sensitization protocols rely on pulmonary epithelium and dendritic cells for initiation of allergic immunity [17, 18] and thus better model the natural route of exposure in humans.

Differences in murine AAD protocols are not trivial and can drastically influence the outcomes of in vivo interventions. For example, therapeutic antibody blockade of the three transforming growth factor β (TGF- β) isoforms ameliorated airway remodelling elicited by peripheral sensitization and prolonged airway challenge with ovalbumin [19], whereas the same antibody did not affect remodelling, but increased pulmonary inflammation, in an HDM

inhalation model [20]. Similarly, in a Th2^{hi} model of ovalbumin sensitization and short-term airway challenge, disruption of IL-10 function augmented the Th2 immune response and airway hyper-responsiveness [21], whereas in a model of airway HDM exposure that drives a mixed Th2/Th17 response, the major effect of IL-10 signalling disruption was an increased IFN- γ response to HDM and more severe epithelial pathology [6]. Given these differences and the importance of lung-resident leukocytes and stromal cells during allergic sensitization, we recommend that airway exposure models of AAD are used for experimental asthma research in mice.

In this chapter, we describe methods for generation of AAD in mice by repeated intranasal exposure to aeroallergen, quantifying key disease parameters and characterizing CD4⁺ T cells. We also discuss the potential impacts of different allergens and mouse strains on results and describe modifications to the protocol for application to neonatal mice.

2 Materials

2.1 Mice

1. For adult experiments, specific pathogen-free mice at 6–8 weeks of age. Male or female mice can be used, but proportions of males and females should be controlled between experimental groups and stated in all reports and publications. Various strains of mouse can be used according to user preference, with the BALB/c strain preferred for standard experiments. *See Note 1 on mouse strain choice.*
2. For neonatal experiments, pups derived from timed mating of specified pathogen-free parents as above. Neonates should be used for experiments at *either 3 or 7* days of life as the efficacy of allergic sensitization is reduced when initial exposures are performed later in the neonatal period [22].

2.2 Allergen Administration

See Note 2 on allergen choice.

1. Sterile cell culture grade phosphate-buffered saline (PBS).
2. HDM extract (Greer Laboratories, Lenoir, NC, USA), reconstituted at the required concentration in PBS under aseptic conditions.
3. *Alternaria alternata* extract (Greer Laboratories, Lenoir, NC, USA), reconstituted at the required concentration in PBS under aseptic conditions.
4. Isoflurane solution and anesthetic chamber.

2.3 Measurement of Lung Function

1. Tracheal cannulas appropriate for the size of the mouse. Typically a 19G needle is an appropriate size for adult mice and 21G for mice up to 4 weeks of age.

2. 50 mg/ml ketamine and 10 mg/ml pentobarbital anesthetic solutions.
3. FlexiVent apparatus and software (Scireq, Montreal, Quebec, Canada).
4. Size 5-0 Suture thread (Harvard apparatus).
5. Various doses of methacholine solutions made up in PBS. *See Note 3 on methacholine dose range.*

2.4 Tissue Processing and Cell Isolation

1. Microtainer 0.6 ml Serum Separator Tubes (SST; BD).
2. 70µm cell strainers.
3. Complete RPMI media (cRPMI): RPMI supplemented with fetal calf serum (10%), penicillin/streptomycin (100 U/ml) and L-glutamine (2 mM).
4. Lung digest media: cRPMI as above, plus collagenase D (0.15 mg/ml) and DNase I (25 mg/ml).
5. Erythrocyte lysis buffer: 0.155 M ammonium chloride, 10 mM potassium bicarbonate, and 0.11 mM disodium EDTA in deionized water at pH 7.2–7.4.

2.5 Histology

1. Tissue cassettes.
2. 10% buffered neutral formalin.
3. Superfrost microscope slides.
4. Standard histological staining reagents for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining.

2.6 Differential Cell Counts

1. Standard Fisherbrand microscope slides.
2. Shandon Cytospin 3 cytocentrifuge (Thermo Fisher).
3. Shandon Kwik-Diff Stains (Thermo Fisher).
4. DPX mounting media.
5. 22 × 22 mm 0.13–0.17 mm thick glass coverslips.
6. Microscope with a 40-times objective.

2.7 Total and Allergen-Specific Immunoglobulin Enzyme-Linked Immunosorbent Substrate Assays (ELISAs)

1. High-binding ELISA-grade 96-well microplates.
2. Coating buffer: 0.1 M sodium bicarbonate in deionized water at pH 9.6.
3. ELISA wash buffer: PBS containing 0.05% Tween-20.
4. Blocking buffer: PBS supplemented with 3% bovine serum albumin (BSA).
5. ELISA buffer: PBS supplemented with 1% BSA and 0.05% Tween-20.
6. Capture and biotinylated detection antibodies as described in Table 1.

Table 1
Suggested antibody clones for total and allergen-specific immunoglobulin ELISAs

Antibody (clone)	Purpose
Purified anti-mouse IgE (R35-72)	Total IgE capture
Purified anti-mouse IgG1 (A85-1)	Total IgG1 capture
Purified mouse IgE (C38-2)	Total IgE standard
Purified mouse IgG1 (MOPC-31C)	Total IgG1 standard
Biotinylated anti-mouse IgE (R35-118)	Total/specific IgE detection
Biotinylated anti-mouse IgG1 (A85-1)	Total/specific IgG1 detection

- 7. Mouse IgE and IgG1 standards as described in Table 1.
- 8. Streptavidin-HRP solution.
- 9. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate.
- 10. 0.18 M sulfuric acid (ELISA stop solution).
- 11. Microplate reader capable of reading absorbance at 450 nm.

2.8 Cell Stimulations and Flow Cytometry

- 1. Intracellular cytokine stimulation media: cRPMI as under Sub-heading 2.4, supplemented with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml), ionomycin-free acid (1.5µg/ml) from Streptomyces globatus, and Brefeldin A (5µg/ml).
- 2. Flow cytometry-grade PBS.
- 3. Flow cytometry staining buffer: flow cytometry grade PBS, as above, supplemented with 1% BSA and 0.05% sodium azide.
- 4. eBioscience IC fixation buffer (Thermo Fisher; for intracellular flow cytometry).
- 5. FIX & PERM™ Cell Permeabilization Kit (Thermo Fisher; for intranuclear flow cytometry).
- 6. eBioscience™ Permeabilization Buffer (10×), diluted to 1× in deionized water (Thermo Fisher).
- 7. LIVE/DEAD™ Fixable Dead Cell Stain (Thermo Fisher; various fluorescent excitation/emission options are available).
- 8. Flow cytometry antibodies as detailed in Table 2.

3 Methods

3.1 Induction of Allergic Airway Disease (AAD)

Allergen is stored in sterile aliquots at −20 °C and equilibrated to room temperature prior to administration. Allergen, or PBS vehicle control, is administered three times over a 7-day period with 1–2 day intervals (Fig. 1). Researchers may want to administer on

Table 2
Suggested antibody panels for mouse T cell flow cytometry

Intracellular cytokine staining antibodies (clone)	Surface/transcription factor phenotype antibodies (clone)
Live/dead fixable dead cell stain (viability dye)	Live/dead fixable dead cell stain (viability dye)
CD45 (30-F11)	CD45 (30-F11)
CD3e (145-2C11)	CD3e (145-2C11)
CD4 (RM4-5)	CD4 (RM4-5)
NKp46 (29A1.4)	NKp46 (29A1.4)
<i>IFN-γ</i> (XMG1.2)	CD44 (IM7)
<i>IL-5</i> (TRFK5)	CD62L (MEL-14)
<i>IL-13</i> (eBio13A)	CXCR5 (L138D7)
<i>IL-17A</i> (TC11-18H10.1)	PD-1 (RPM1-40)
<i>IL-10</i> (JES-16E3)	<i>FoxP3</i> (FJK-16s)
Anti-CD16/32 for blocking (2.4G2)	Anti-CD16/32 for blocking (2.4G2)

Italics, intracellular antigen; bold italics, intranuclear antigen. Specific viability dye to be selected by the user based on available channels on flow cytometer

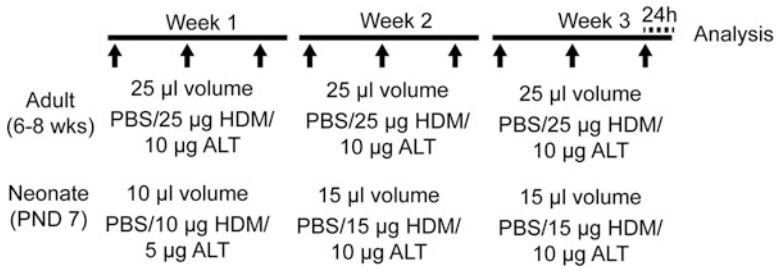


Fig. 1 Schematic of mouse allergic airway disease models. PBS, phosphate-buffered saline (control); HDM, house dust mite; ALT, *Alternaria alternata*; PND, post-natal day. Neonatal model can also be initiated at PND 3, adjusting the change in allergen volume/dose accordingly to begin at PND 14. See Subheading 3.1.2 for full details

Mondays, Wednesdays, and Fridays to avoid administrations on weekends. The time of analysis post the final allergen administration must be controlled. 24 h post-final challenge is recommended for general analysis of AAD parameters (Fig. 1), but maximal detection of specific parameters is variable at different time-points following final challenge so can be adjusted if necessary for experimental aims [23].

**3.1.1 Allergen
Administration to Adult
Mice**

1. Dilute allergen to allow for a quantity of 25µg (HDM) or 10µg (ALT) in a 25µl administration volume.
2. Place mice, 2–3 at a time, into an anesthetic chamber and switch on isoflurane flow.
3. Once under light anesthesia (immobilized with regular slow breathing) remove mice one at a time, hold upright, and gradually drop 25µl of allergen solution or PBS onto nostrils with a pipette, allowing for inhalation.
4. Place mice in a cage or container for recovery before returning to the home cage.

**3.1.2 Allergen
Administration to Neonatal
Mice (Postnatal Day 3–13)**

Intranasal allergen exposures in this age window are performed in reduced volumes and without anesthetic, before switching to the method described under Subheading 3.1.3.

1. Dilute allergen to allow for a quantity of 10µg (HDM) or 5µg (ALT) in a 10µl administration volume.
2. Temporarily place mother in a separate cage to minimize distress caused by handling of pups.
3. Take pups one at a time and hold upright with thumb and forefinger to stabilize head. Gradually drop 10µl of allergen or PBS onto nostrils and allow for inhalation.
4. Place pup in a separate container or cage for recovery. When finished with all pups, return pups and mother to the home cage.

**3.1.3 Allergen
Administration to Juvenile
Mice (Postnatal Day 14–28)**

Once pups reach 14 days of age, the remainder of the neonatal allergen exposure protocol is performed using isoflurane anesthesia as described under Subheading 3.1.1. However, the administration volume is lower at 15µl. Allergen is diluted to allow for a quantity of 15µg (HDM) or 10µg (ALT) in 15µl volume.

**3.2 Measurement
of Lung Function**

Lung function is measured to determine airway responsiveness to inhaled methacholine, with allergen-treated mice displaying airway hyperresponsiveness compared to controls. Resistance measurements indicate how easily air flows through the lungs, while compliance is a measure of elastic recoil.

1. Weigh and record weight of mouse.
2. Anesthetise mouse with pentobarbital (50 mg/kg, intraperitoneally) and ketamine (100 mg/kg, intramuscularly). Surgical anesthesia should be reached between 5 and 15 min.
3. Calibrate the FlexiVent machine using the recorded weight of the mouse and the cannula that will be used for cannulation.
4. Surgically expose the trachea and insert cannula and secure with suture thread.

- 5. Start FlexiVent ventilator before attaching mouse via its canula to machine.
- 6. Record parameters of lung function at baseline and in response to increasing concentrations of methacholine using the following script or similar.

3 × TLC	
Snapshot 150 (Resistance and Compliance)	3 s
13 s normal ventilation	13 s
Prime 3 (other lung function parameters)	3 s
13 s normal ventilation	13 s
Prime 3	3 s
13 s normal ventilation	13 s
Repeat steps 2–7 five more times	
When prompt for next dose select yes and nebulize 10µl methacoline for 10 s	
Repeat steps 2–9 for all doses of methacholine	

- 7. Plot the dose–response graph using the mean of the values obtained at baseline and each dose of methacoline measured (Fig. 2).

3.3 Tissue Processing and Cell Isolation

All performed following a terminal intraperitoneal overdose of anesthetic, e.g., pentobarbital. Carbon dioxide inhalation and cervical dislocation methods are not recommended as these methods cause damage to the lungs.

3.3.1 Peripheral Blood/Serum

- 1. Following anesthetic overdose, make incisions starting at the base of the sternum to open the ribcage and expose the heart.
- 2. Using a 1 ml syringe and 25G needle, slowly draw blood from a single chamber of the heart and transfer immediately to a 0.6 ml SST tube.
- 3. Centrifuge at 10,000 × g for 5 min. Serum will separate to form a top layer in which cytokines and immunoglobulins can be measured.

3.3.2 Bronchoalveolar Lavage (BAL)

- 1. Using 1 ml syringes, dispense PBS into lungs via age-appropriate tracheal cannulas as described under Subheadings 2.3 and 3.2 and recover. It is easiest to perform BAL while the mouse is flat on the bench. Use 3 × 0.3 ml PBS for mice up to 3 weeks of age and 3 × 0.4 ml for mice of 4 weeks or older.
- 2. Lavage lungs a total of three times and pool recovered fluid in a single 15 ml centrifuge tube.

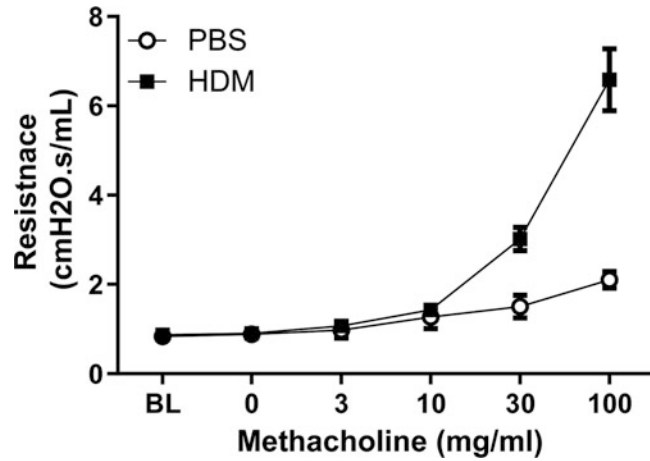


Fig. 2 Assessment of lung function. Mice exposed to HDM for 3 weeks (squares) showed increased resistance to inhaled methacholine in comparison to PBS exposed controls (circles) demonstrating airway hyperresponsiveness

3. Centrifuge at $300 \times g$ for 5 min at 4 °C, supernatant can be stored at –80 °C for subsequent analysis (e.g., cytokine measurements), resuspend cells in cRPMI.

3.3.3 Mediastinal Lymph Nodes (mLNs)

1. Dissect away ribcage on left side of the thorax, remove individual mLNs from beneath the trachea with forceps, and place in cRPMI on ice.
2. Pass mLNs through 70µm cell strainers into 50 ml centrifuge tubes by gently pressing with a 2 ml syringe plunger and flushing with cRPMI.
3. Centrifuge at $300 \times g$ for 5 min at 4 °C and resuspend cells in cRPMI.

3.3.4 Lung Tissue

1. Remove lungs and heart en bloc with forceps and scissors, remove heart and separate the left lung and 4 right lung lobes.
2. Inflate the right super lobe with around 50µl PBS using a 27G needle, place in a tissue cassette, and fix for 24 h in 10% BNF for histology (*see* Subheading 3.4).
3. The remaining right lung lobes can be flash frozen in liquid nitrogen for protein and RNA analysis, as required.
4. Mechanically chop whole left lungs with scissors and place in 1 ml of cRPMI in 15 ml centrifuge tubes.
5. Add 4 ml of lung digest medium to tubes and incubate for 1 h in a shaking water bath at 37 °C.
6. Pass digested tissue through 70µm cell strainers into 50 ml centrifuge tubes by gently pressing with a 2 ml syringe plunger and flushing with cRPMI.

7. Transfer to 15 ml centrifuge tubes, centrifuge at $300 \times g$ for 5 min at 4 °C and resuspend cells in 3 ml of erythrocyte lysis buffer, before incubating for 5 min at room temperature.
8. Add 3 ml of cRPMI to stop lysis and centrifuge at $300 \times g$ for 5 min at 4 °C.
9. Suspend cells in cRPMI.

3.4 Histology

1. After fixation in 10% BNF, transfer cassettes to 70% ethanol and store at 4 °C until processing.
2. Perform wax embedding and sectioning of tissue as per local protocols, preparing 4µm sections on Superfrost microscope slides.
3. Perform H&E and PAS staining as per standard histological protocols, counterstaining PAS sections with hematoxylin.

3.4.1 H&E Inflammation Scoring

1. Randomly allocate identifiers to each section to allow blinded scoring.
2. Examine inflammatory infiltrates around blood vessels and airways and in lung parenchyma (Fig. 3) and score each section 0–5 based on the following criteria:
 0. No inflammatory infiltrates visible around any airways/vessels.
 1. A single inflammatory infiltrate up to 3 cells deep around an airway or vessel.
 2. Inflammatory infiltrates up to 3 cells deep around more than one airway/vessel.
 3. Large inflammatory infiltrates >3 cells deep in up to 50% of airways/vessels.
 4. Large inflammatory infiltrates (>3 cells) in >50% of airways/vessels.
 5. As for 4, but with parenchymal inflammation in alveolar beds.

3.4.2 PAS Mucus Scoring

1. Randomly allocate identifiers to each section to allow blinded scoring.
2. For each airway, score from 0 to 4 as below based on the proportion of epithelial cells stained pink for mucus (Fig. 3):
 0. No positive epithelial cells.
 1. 1–25% of epithelial cells positive
 2. 26–50% of epithelial cells positive
 3. 51–75% of epithelial cells positive
 4. 76–100% of epithelial cells positive.
3. Report mean score of all airways per sample.

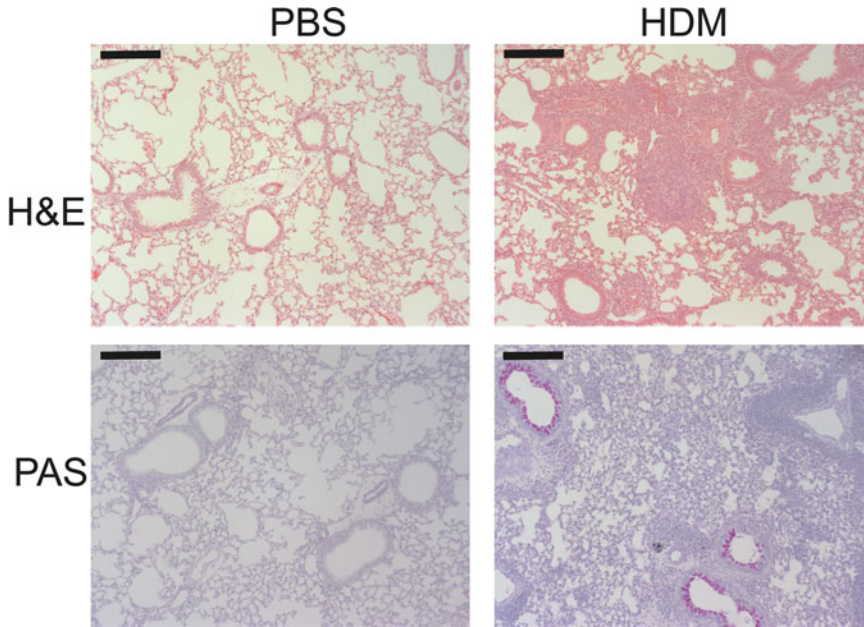


Fig. 3 Lung pathophysiology. Mice exposed to HDM for 3 weeks show increased inflammatory infiltrates around blood vessels and airways and in lung parenchyma by H&E staining (top) and mucus producing epithelial cells (pink) by PAS staining (bottom), when compared to PBS exposed control mice. Scale bar, 200 μ m

3.5 Differential Cell Counts

1. Using isolated cells prepared as described under Subheadings 3.3.2 and 3.3.4 cytocentrifuge 50,000 cells in 100 μ l cRPMI on to standard microscope slides at $18.06 \times g$ for 4 min using a Cytospin.
2. Air dry slides for 5 min.
3. Fix cells in methanol for 5 min. Slides can be stained immediately or later.
4. Using Shandon Kwik-Diff Stains, stain slides using a modified protocol from those supplied by the manufacturer (steps 5–11).
5. Incubate slides in reagent 1 for 1 min in. Allow excess to drain into jar and blot edge on absorbent paper.
6. Air dry slides for 1 min.
7. Dip slides five times (1 s per dip) into reagent 2. Allow excess to drain into jar and blot edge on absorbent paper.
8. Dip slides 2–3 times (1 s per dip) into reagent 3. Allow excess to drain into jar and blot edge on absorbent paper.
9. Rinse slides in distilled or deionized water by dipping or swishing.
10. Air dry until completely dry.

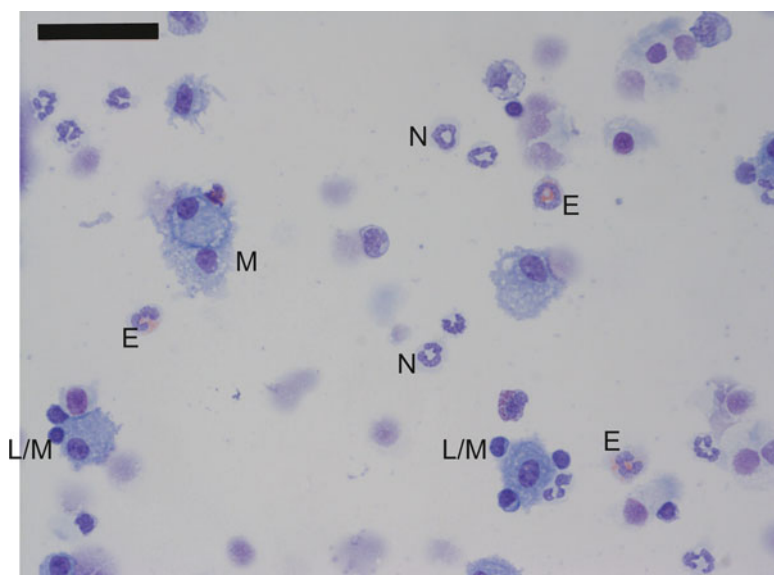


Fig. 4 Differential cell counts from cytocentrifuge preparation. Representative image of lung cells from a HDM-treated mouse, stained with Shandon Kwik-Diff Stains. Example cell type annotations are shown. Eosinophils (E), neutrophils (N), macrophages (M), and lympho/mononucleocytes (L/M). Scale bar, 200 μ m

11. Mount slides with DPX mounting media and coverslips. Air dry overnight.
12. Using a microscope with a 40-times objective, count a total of 400 cells to accurately enumerate the proportion of eosinophils, neutrophils, macrophages, and lympho/mononucleocytes. Lymphocytes and monocytes are counted as one group due to their similar morphology and variation in staining intensity (Fig. 4).

3.6 Immunoglobulin E and G1 ELISA

Immunoglobulins E and G₁ are detected in serum (see Subheading 3.3.1) using either paired capture and biotinylated detection antibodies (for total immunoglobulin) or biotinylated detection antibodies and allergen-coated plates (for allergen-specific immunoglobulin). Standard or half-area plates can be used, with a working assay volume of 100 μ l and 50 μ l, respectively. Plates are washed 4–5 times between steps with ELISA wash buffer.

3.6.1 Total Immunoglobulin

1. Coat 96-well microplates with 2 μ g/ml capture antibody in 0.1 M sodium bicarbonate by incubating at 4 °C overnight.
2. Wash plates and block for 1 h at room temperature in blocking buffer.
3. Wash plates and incubate overnight at 4 °C with immunoglobulin standards in twofold serial dilutions in ELISA buffer (top standard concentrations 400 ng/ml for IgE and 10 ng/ml for

IgG1) and test serum at an assay-dependent dilution in ELISA buffer (at least 1 in 20 for IgE and 1 in 10^5 for IgG1). Duplicate assay wells are recommended for samples and standards. Include blank wells (ELISA buffer only) for subtraction of background.

4. Wash plates and incubate with 2 µg/ml biotinylated detection antibodies for 1 h at room temperature.
5. Wash plates and incubate with streptavidin-HRP at the lot-specific dilution for 30 min at room temperature in the dark.
6. Wash plates and develop with TMB substrate, stopping with ELISA stop solution once a full standard curve has developed.
7. Read absorbance at 450 nm and determine concentrations in test serum using an interpolation method appropriate for the standard curve.

3.6.2 Allergen-Specific Immunoglobulin

1. Coat 96-well microplates with 50 µg/ml HDM or ALT allergen in 0.1 M sodium bicarbonate by incubating at 4 °C overnight.
2. Wash plates and block for 1 h at room temperature in blocking buffer.
3. Wash plates and incubate overnight at 4 °C with test serum serially diluted in ELISA buffer in fourfold (IgE) or fivefold (IgG1) steps down the plate (8 dilutions total). Exact dilutions must be determined by the user depending on the likely amount of immunoglobulin present in samples, but 1 in 10 and 1 in 100 are recommended starting dilutions for IgE and IgG1, respectively. Duplicate assay wells are recommended. Include blank wells (ELISA buffer only) for subtraction of background.
4. Wash plates and incubate with 2 µg/ml biotinylated detection antibodies for 1 h at room temperature.
5. Wash plates and incubate with streptavidin-HRP at the lot-specific dilution for 30 min at room temperature in the dark.
6. Wash plates and develop with TMB substrate, stopping with ELISA stop solution once a full standard curve has developed.
7. Read absorbance at 450 nm and plot curves of absorbance against dilution factor. Area under curve or endpoint titer analysis can be performed on curves, if desired.

3.7 Cell Stimulation to Measure HDM-Specific Cytokine Response

See Note 4 on aseptic technique ahead of this method.

1. Count live cells in lung and mLN cell suspensions from HDM- and PBS-treated mice by trypan blue exclusion.

2. Seed 2×10^5 cells in either cRPMI (control) or cRPMI containing 50 μ g/ml HDM into wells of sterile round bottom 96-well cluster plates in a volume of 200 μ l. Avoid outer wells due to greater risk of evaporation—fill these with medium without cells.
3. Incubate for 96 h in a standard humidified tissue culture incubator at 37 °C and 5% carbon dioxide.
4. After stimulation, centrifuge plates at $300 \times g$ for 5 min to pellet cells and harvest supernatants for storage at –80 °C until analysis of cytokines (e.g., by ELISA).

3.8 Cell Stimulation for Intracellular Cytokine Staining

1. Add up to 2×10^6 cells in suspension to wells of round bottom 96-well plates. Centrifuge at $300 \times g$ for 5 min, discard supernatants, and resuspend pellets in 200 μ l of intracellular cytokine stimulation medium.
2. Incubate for 4 h in a standard humidified tissue culture incubator at 37 °C and 5% carbon dioxide.
3. Proceed immediately to flow cytometry staining.

3.9 Flow Cytometry for T Cell Quantification and Phenotyping

Start with up to 2×10^6 cells in suspension in round bottom 96-well plates, either unstimulated (for standard T cell quantification or phenotyping) or following the stimulation protocol described under Subheading 3.8 (if assessing intracellular cytokine staining). See Note 5 on cell numbers for flow cytometry staining. Washes are performed by centrifuging plates at $300 \times g$ for 5 min at 4 °C, discarding supernatant and replacing with 200 μ l of the indicated solution unless otherwise stated.

1. Wash cells once in PBS.
2. Resuspend cells in 30 μ l of a suitable LIVE/DEAD fixable dead cell stain and incubate in the dark for 20 min at room temperature.
3. Add 150 μ l of PBS to wells, centrifuge at $300 \times g$ for 5 min at 4 °C, and discard supernatant.
4. Wash in PBS.
5. Resuspend cells in a 30 μ l volume of flow staining buffer containing and appropriate combination of fluorochrome-conjugated antibodies to extracellular antigens and unconjugated anti-CD16/CD32 blocking reagent (see Table 2). Incubate at 4 °C for 20 min in the dark (see Note 6 on CXCR5 chemokine receptor staining).
6. Add 150 μ l of flow staining buffer to wells, centrifuge at $300 \times g$ for 5 min at 4 °C, and discard supernatant.
7. Wash in flow staining buffer, resuspending in 50 μ l of either IC fixation buffer (when subsequently intracellular cytokine

staining or surface only staining) or FIX & PERM™ fixation buffer (when subsequently staining for nuclear antigens, e.g., FoxP3, prepared from concentrate as per manufacturer's instructions immediately prior to use). Fix cells at room temperature according to buffer manufacturer's instructions.

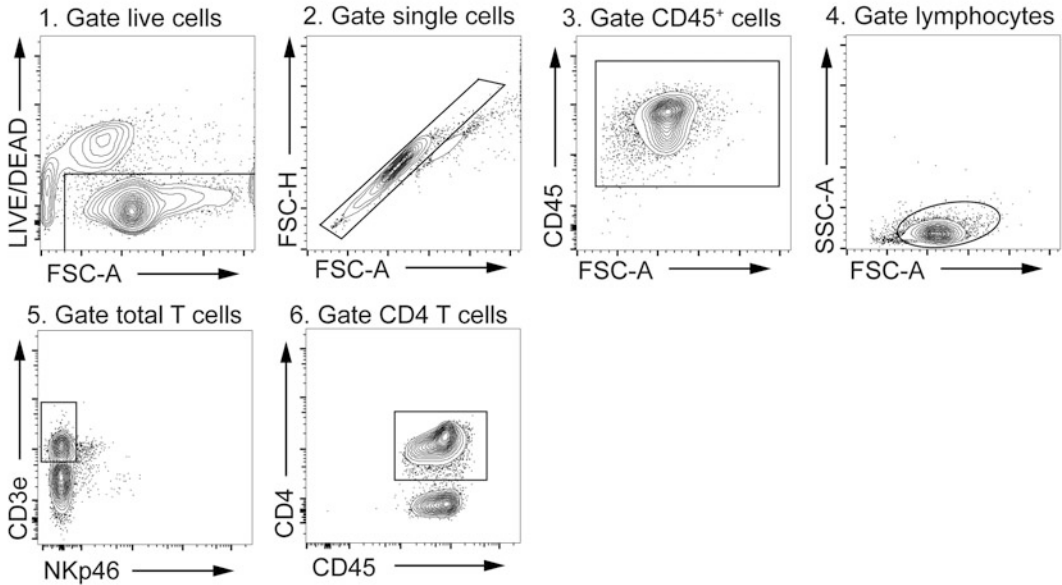
8. Where staining for intracellular/nuclear antigens, centrifuge fixed cells at $300 \times g$ for 5 min at 4 °C and resuspend in 100µl of a 1× solution of Permeabilization Buffer and incubate for 5 min at room temperature in the dark.
9. Centrifuge cells at $300 \times g$ for 5 min at 4 °C and resuspend in a 30µl volume of Permeabilization Buffer containing antibodies to intracellular/nuclear antigens (*see* Table 2). Incubate for 20 min at 4 °C in the dark.
10. Add 150µl of Permeabilization Buffer to wells, centrifuge at $300 \times g$ for 5 min at 4 °C and discard supernatant.
11. Wash in Permeabilization Buffer and resuspend in an appropriate volume of flow staining buffer and in appropriate round bottom tubes for flow cytometry analysis.
12. Analyze samples on a flow cytometer with appropriate and sufficient laser and filter combinations for the antibody and fluorochrome panels used. *See Note 7 on compensation controls.*
13. Analyze .fcs files using appropriate software, suggested gating as follows:
 - (a) Total CD4 T cells: LIVE/DEAD⁻, singlet, CD45⁺, lymphocyte (forward/side scatter^{lo}), CD3e⁺, NKp46⁻, CD4⁺ (Fig. 5a).
 - Effector CD4 subsets (cytokine-producing): IFN-γ/IL-5/IL-10/IL-13/IL-17A⁺ (Fig. 5b).
 - CD4 T cell memory subsets (Fig. 5c).
 - Naïve: CD44⁻, CD62L⁺.
 - Effector/effector memory: CD44⁺, CD62L⁻.
 - Central memory: CD44⁺, CD62L⁺.
 - FoxP3⁺ Tregs (intranuclear staining): FoxP3⁺ (Fig. 5d).
 - T follicular helper cells (intranuclear staining): CD44^{hi}, FoxP3⁻, PD-1⁺, CXCR5⁺ (Fig. 5d).

4 Notes

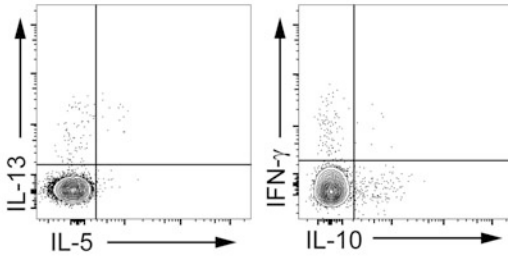
1. Mouse strain choice.

AAD can be induced by repeated intranasal allergen exposure of several inbred laboratory mouse strains, including the common BALB/c and C57BL/6 strains. However, the nature

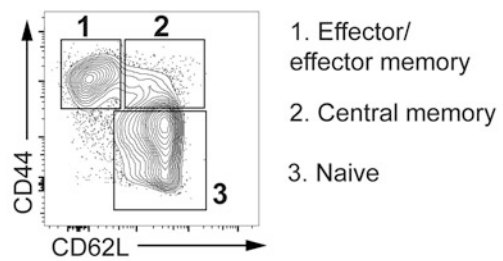
a General gating for total CD4 T cells



b Cytokine gating of CD4 T cells



c Gating memory CD4 T cells



d Gating of Foxp3⁺ Tregs and T follicular helper cells

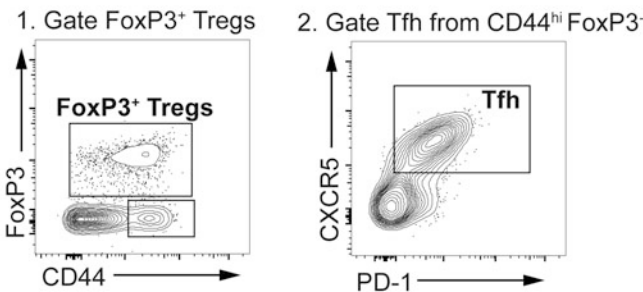


Fig. 5 Flow cytometry gating strategies for CD4⁺ T cells in mouse models of asthma. **(a)** Hierarchical gating scheme to identify total CD4⁺ T cells, upstream of gating in **(b–d)**. **(b)** Gating of CD4 T cells with positive intracellular cytokine staining. **(c)** Gating of memory CD4⁺ T cell populations based on CD44 and CD62L expression. **(d)** Hierarchical gating strategy to identify Foxp3⁺ Tregs and Tfh (T follicular helper) cells. Representative plots from mLNs of mice treated with HDM for 3 weeks are shown

and magnitude of allergic inflammatory responses vary between these strains [24, 25]. BALB/c mice are generally [26] more biased towards Th2 immunity than C57BL/6 mice, so are preferred for models of type 2^{hi} allergic asthma. However, many transgenic and knockout mouse lines have a C57BL/6 genetic background, meaning that this strain is used in some studies. Repeated HDM exposure of C57BL/6 mice has been shown to drive a mixed Th2/Th17 pulmonary immune response, with an augmented Th1 response where IL-10 signalling is disrupted [6]. The nature of the immune response of interest, as well as the genetic background of available transgenic and knockout mouse lines must therefore be taken into account when selecting strains for studies. Litter-mate control mice should be used for all experiments due to potential confounding effects of differences in microbiome in separate groups of mice, even within standard laboratory strains [27].

2. Allergen choice.

Both HDM and ALT allergens are well established in our laboratory for generating AAD in adult and neonatal mice [15, 23, 28, 29] and other allergens and combinations thereof have been used via the intranasal route in such models [30]. However, it is important to note that allergens are not directly interchangeable, with key differences in the nature and magnitude of immune response generated and the mechanisms involved. For example, in neonatal mice, ALT drives a greater IL-33, Th2 cell and group 2 innate lymphoid cell response than HDM [28], likely because of the rapid protease-mediated IL-33 release upon inhalation of ALT, but not HDM [31]. Both the clinical relevance and in vivo effects must therefore be considered when selecting allergens for AAD models.

In addition, when purchasing new batches of allergen, direct comparison to the previous effective batch is recommended, even when purchasing from the same supplier, due to the potential for lot-to-lot variability.

3. Methacholine dose range.

Different strains of mice display different levels of sensitivity to changes in lung function which can also be influenced by the allergen used, with C57BL/6 being more resistant than BALB/c and HDM induced changes being lower than those seen with ALT. All of these can also be influenced by genetic manipulations. Therefore, the concentrations of methacholine used to measure lung function are context-dependent. For an HDM exposed wild-type BALB/c mouse a maximal concentration of 100 mg/ml is used with 5 concentrations being examined.

4. Aseptic technique ahead of allergen-specific cytokine assays.

Due to the 4-day culture period used for these assays, it is important that aseptic technique is employed during preparation of cells before and during culture to prevent contamination. Dissections and cell preparation (*as described under Subheading 3.3*) should be performed in a class II microbiological safety cabinet and sterile plasticware and media used throughout.

5. Cell numbers for flow cytometry staining.

The staining volumes stated are optimized for up to 2×10^6 cells. More cells than this can be stained, but staining and analysis volumes should be increased proportionally.

6. CXCR5 chemokine receptor staining.

Owing to the ready internalization of chemokine receptors, when staining for Tfh it is necessary to perform CXCR5 staining for 1 h at room temperature to allow recycling to the surface. This can be performed after staining for other extracellular antigens, washing with flow staining buffer between stains.

7. Compensation controls.

Either cells or antibody-binding beads can be used for single color compensation controls for flow cytometry. However, when using cells, it is important to ensure that enough positive cells for each antibody used will be present to ensure a clear positive signal, so the right source and number of cells must be used, especially for rarer markers. For example, mLNC cells from allergen-treated mice are a good source of cells for compensation using antibodies to T cell markers.

References

1. Lambrecht BN, Hammad H (2015) The immunology of asthma. *Nat Immunol* 16 (1):45–56. <https://doi.org/10.1038/ni.3049>
2. Wenzel SE (2012) Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 18(5):716–725. <https://doi.org/10.1038/nm.2678>
3. Ray A, Raundhal M, Oriss TB, Ray P, Wenzel SE (2016) Current concepts of severe asthma. *J Clin Invest* 126(7):2394–2403. <https://doi.org/10.1172/JCI84144>
4. Modena BD, Bleecker ER, Busse WW, Erzurum SC, Gaston BM, Jarjour NN, Meyers DA, Milosevic J, Tedrow JR, Wu W, Kaminski N, Wenzel SE (2017) Gene expression correlated with severe asthma characteristics reveals heterogeneous mechanisms of severe disease. *Am J Respir Crit Care Med* 195(11):1449–1463. <https://doi.org/10.1164/rccm.201607-1407OC>
5. Raundhal M, Morse C, Khare A, Oriss TB, Milosevic J, Trudeau J, Huff R, Pilewski J, Holguin F, Kolls J, Wenzel S, Ray P, Ray A (2015) High IFN- γ and low SLPI mark severe asthma in mice and humans. *J Clin Invest* 125(8):3037–3050. <https://doi.org/10.1172/JCI80911>
6. Branchett WJ, Stölting H, Oliver RA, Walker SA, Puttur F, Gregory LG, Gabryšová L, Wilson MS, O'Garra A, Lloyd CM (2020) A T cell–myeloid IL-10 axis regulates pathogenic IFN- γ -dependent immunity in a mouse model of type 2–low asthma. *J Allergy Clin Immunol* 145(2):666–678. e669. <https://doi.org/10.1016/j.jaci.2019.08.006>
7. Lloyd CM, Hessel EM (2010) Functions of T cells in asthma: more than just T(H)2 cells. *Nat*

- Rev Immunol 10(12):838–848. <https://doi.org/10.1038/nri2870>
8. Ballesteros-Tato A, Randall TD, Lund FE, Spolski R, Leonard WJ, Leon B (2016) T follicular helper cell plasticity shapes pathogenic T helper 2 cell-mediated immunity to inhaled house dust mite. *Immunity* 44(2):259–273. <https://doi.org/10.1016/j.immuni.2015.11.017>
 9. Uwadiae FI, Pyle CJ, Walker SA, Lloyd CM, Harker JA (2019) Targeting the ICOS/ICOS-L pathway in a mouse model of established allergic asthma disrupts T follicular helper cell responses and ameliorates disease. *Allergy* 74(4):650–662. <https://doi.org/10.1111/all.13602>
 10. Eger KA, Bel EH (2019) The emergence of new biologics for severe asthma. *Curr Opin Pharmacol* 46:108–115. <https://doi.org/10.1016/j.coph.2019.05.005>
 11. Chupp GL, Kaur R, Mainardi A (2020) New therapies for emerging Endotypes of asthma. *Annu Rev Med* 71:289–302. <https://doi.org/10.1146/annurev-med-041818-020630>
 12. Lloyd CM, Gutierrez-Ramos JC (2004) Animal models to study chemokine receptor function: in vivo mouse models of allergic airway inflammation. *Methods Mol Biol* 239:199–210. <https://doi.org/10.1385/1-59259-435-2:199>
 13. Aun MV, Bonamichi-Santos R, Arantes-Costa FM, Kalil J, Giavina-Bianchi P (2017) Animal models of asthma: utility and limitations. *J Asthma Allergy* 10:293–301. <https://doi.org/10.2147/JAA.S121092>
 14. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M (2004) Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med* 169(3):378–385. <https://doi.org/10.1164/rccm.200308-1094OC>
 15. Saglani S, Mathie SA, Gregory LG, Bell MJ, Bush A, Lloyd CM (2009) Pathophysiological features of asthma develop in parallel in house dust mite-exposed neonatal mice. *Am J Respir Cell Mol Biol* 41(3):281–289
 16. Lloyd CM, Saglani S (2019) Opening the window of immune opportunity: treating childhood asthma. *Trends Immunol* 40(9):786–798. <https://doi.org/10.1016/j.it.2019.07.004>
 17. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN (2009) House dust mite allergen induces asthma via toll-like receptor 4 triggering of airway structural cells. *Nat Med* 15(4):410–416. <https://doi.org/10.1038/nm.1946>
 18. Plantinga M, Guillems M, Vanheerswynghe M, Deswarte K, Branco-Madeira F, Toussaint W, Vanhoutte L, Neyt K, Killeen N, Malissen B, Hammad H, Lambrecht BN (2013) Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38(2):322–335. <https://doi.org/10.1016/j.immuni.2012.10.016>
 19. McMillan SJ, Xanthou G, Lloyd CM (2005) Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-beta antibody: effect on the Smad signaling pathway. *J Immunol* 174(9):5774–5780. <https://doi.org/10.4049/jimmunol.174.9.5774>
 20. Fattouh R, Midence NG, Arias K, Johnson JR, Walker TD, Goncharova S, Souza KP, Gregory RC Jr, Lonning S, Gauldie J, Jordana M (2008) Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling. *Am J Respir Crit Care Med* 177(6):593–603
 21. Tournoy KG, Kips JC, Pauwels RA (2000) Endogenous interleukin-10 suppresses allergen-induced airway inflammation and nonspecific airway responsiveness. *Clin Exp Allergy* 30(6):775–783. <https://doi.org/10.1046/j.1365-2222.2000.00838.x>
 22. Gollwitzer ES, Saglani S, Trompette A, Yadava K, Sherburn R, McCoy KD, Nicod LP, Lloyd CM, Marsland BJ (2014) Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat Med* 20(6):642–647. <https://doi.org/10.1038/nm.3568>
 23. Gregory LG, Causton B, Murdoch JR, Mathie SA, O'Donnell V, Thomas CP, Priest FM, Quint DJ, Lloyd CM (2009) Inhaled house dust mite induces pulmonary T helper 2 cytokine production. *Clin Exp Allergy* 39(10):1597–1610. <https://doi.org/10.1111/j.1365-2222.2009.03302.x>
 24. Gueders MM, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove C, Tournoy K, Louis R, Foidart JM, Noel A, Cataldo DD (2009) Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflamm Res* 58(12):845–854. <https://doi.org/10.1007/s00011-009-0054-2>
 25. Entwistle LJ, Gregory LG, Oliver RA, Branchett WJ, Puttur F, Lloyd CM (2020) Pulmonary group 2 innate lymphoid cell phenotype is context specific: determining the effect of strain, location, and stimuli. *Front Immunol*

- 10:3114. <https://doi.org/10.3389/fimmu.2019.03114>
26. Gessner A, Blum H, Rollinghoff M (1993) Differential regulation of IL-9-expression after infection with *Leishmania major* in susceptible and resistant mice. *Immunobiology* 189(5):419–435. [https://doi.org/10.1016/S0171-2985\(11\)80414-6](https://doi.org/10.1016/S0171-2985(11)80414-6)
 27. Franklin CL, Ericsson AC (2017) Microbiota and reproducibility of rodent models. *Lab Anim (NY)* 46(4):114–122. <https://doi.org/10.1038/labani.1222>
 28. Castanhinha S, Sherburn R, Walker S, Gupta A, Bossley CJ, Buckley J, Ullmann N, Grychtol R, Campbell G, Maglione M, Koo S, Fleming L, Gregory L, Snelgrove RJ, Bush A, Lloyd CM, Saglani S (2015) Pediatric severe asthma with fungal sensitization is mediated by steroid-resistant IL-33. *J Allergy Clin Immunol* 136(2):312–322. e317. <https://doi.org/10.1016/j.jaci.2015.01.016>
 29. Loser S, Gregory LG, Zhang Y, Schaefer K, Walker SA, Buckley J, Denney L, Dean CH, Cookson WOC, Moffatt MF, Lloyd CM (2017) Pulmonary ORMDL3 is critical for induction of *Alternaria*-induced allergic airways disease. *J Allergy Clin Immunol* 139(5):1496–1507. e1493. <https://doi.org/10.1016/j.jaci.2016.07.033>
 30. Goplen N, Karim MZ, Liang Q, Gorska MM, Rozario S, Guo L, Alam R (2009) Combined sensitization of mice to extracts of dust mite, ragweed, and aspergillus species breaks through tolerance and establishes chronic features of asthma. *J Allergy Clin Immunol* 123(4):925–932. e911. <https://doi.org/10.1016/j.jaci.2009.02.009>
 31. Snelgrove RJ, Gregory LG, Peiro T, Akthar S, Campbell GA, Walker SA, Lloyd CM (2014) *Alternaria*-derived serine protease activity drives IL-33-mediated asthma exacerbations. *J Allergy Clin Immunol* 134(3):583–592. e586. <https://doi.org/10.1016/j.jaci.2014.02.002>