



Chapter 1

A Fungal Protease Model to Interrogate Allergic Lung Immunity

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Abstract

Allergic airway diseases (asthma and chronic rhinosinusitis) are among the most common of all human diseases in heavily industrialized societies. Animal models of asthma have provided remarkable insight into allergic disease pathogenesis and will continue to drive the discovery of new therapeutic insights. We provide in this chapter a detailed protocol for inducing allergic immunity in the lungs of mice using a purified fungal protease and include related protocols for assessing immune endpoints.

Key words Allergic immunity, Asthma, Airway hyperresponsiveness, T_H2 cell, Protease, Fungi

1 Introduction

Allergic diseases of the respiratory tract, comprised largely of asthma (lower airway) and chronic rhinosinusitis (upper airway), are among the most common of all human diseases in heavily industrialized societies. The incidence and prevalence of these conditions continue to rise in the United States at the same time that therapy remains inadequate [1]. Although fundamental insight into the pathogenesis of allergic lung diseases has been made and has resulted in the development of novel therapeutic approaches [2], much additional study through experimental models will be required before salutary changes in allergic disease epidemiology ensue.

All allergic disorders share a distinctive pattern of inflammation dominated by characteristic immune cells (e.g., T helper cells type 2 (T_H2), eosinophils, innate lymphoid cells type 2 (ILC2)), cytokines (e.g., interleukin 4 (IL-4), IL-5, IL-13), chemokines (CCL7, CCL17, CCL26), and antibody responses (e.g., immunoglobulin E (IgE)) that often mediate common physiological alterations

(e.g., airway and gut hypermotility or hyperresponsiveness and mucus secretion) [3]. The fundamental importance of protease activity for allergic inflammation was a transformative discovery in the pathogenesis of allergic lung disease [4]. Regardless of whether the allergen contains proteases or not, endogenous proteases such as thrombin are the likely danger signals that mediate allergic inflammation [5]. Nonetheless, data increasingly suggest that exogenous proteases, and particularly protease-secreting pathogens such as fungi, are of fundamental importance to human allergic airway diseases [6–8].

We provide in this chapter a detailed protocol for inducing allergic immunity in the lungs of mice using a purified fungal protease. In addition to describing the method for preparing and challenging mice with fungal protease, we include additional protocols for measuring lung mechanics (airway responsiveness), collecting airway immune cells via bronchoalveolar lavage, and processing blood and lung for immune studies.

2 Materials

2.1 Protease/ Allergen Model

1. Age-matched (ideally 4–12 weeks old), sex-matched mice of suitable strain for proposed study: C57BL/6, Balb/c, FVB/N, knockout strain wild type, etc. (*see Note 1*).
2. Phosphate-buffered saline (PBS).
3. Protease solution: 1.8 mg/mL protease from *Aspergillus oryzae* (Sigma: P6110), stored in 55 μ L aliquots at -80°C . Aliquots are resuspended to 550 μ L with PBS for treatment of mice with 50 μ L of protease (*see Note 2*).
4. Basic tabletop anesthesia machine and chamber
5. ISOTHEsia – isoflurane inhalant
6. Oxygen supply

2.2 Plethysmography

1. Assess airway responsiveness with custom built or commercially available plethysmography equipment (Buxco or FlexiVent).
2. Small animal ventilator.
3. PROTECTIV[®] Safety IV angi catheters, radiopaque, 20-gauge $\times$ 1 1/4", FEP polymer.
4. 26-gauge needle for establishing tail IV or suitable equivalent as defined by lab equipment.
5. NERL[™] Blood Bank Saline, buffered to a pH of 7.0–7.2 (Thermo Fisher Scientific).
6. Acetylcholine stock solution: 1.6 mg/mL acetylcholine (ACh) in PBS and stored in 10 mL aliquots at -80°C .

7. Sterile 1 mL syringes.
8. Amidate (etomidate).

2.3 Tissue Collection and Processing

1. 1 mL syringes.
2. 1.5 mL microcentrifuge tubes.
3. Neutral buffered saline.
4. Hemacytometer or cell counting equipment.
5. Cytospin.
6. Superfrost Plus Microscope Slides.
7. Formalin fixative solution: 10% formalin.
8. Hematoxylin and eosin (H&E) stain.
9. Complete media (DMEM or RPMI): DMEM or RPMI containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL).
10. 6-well tissue culture plates.
11. Falcon cell strainer, 40 micron.
12. 50 mL conical tubes.
13. 3 mL syringes.
14. RNeasy lysis buffer.
15. TRIzol.
16. Surgical thread.
17. Necropsy equipment.

3 Method

3.1 Murine Challenge with Protease

1. Adequate numbers of mice should be acquired to allow for 5–6 mice per treatment group (*see Note 1*). Naïve vehicle control (PBS) and positive control groups (protease challenged) should be assessed in every experiment, along with the desired test groups (drug treatment, adoptive transfer, knockout mice, etc.) to ensure changes in airway responsiveness and inflammation.
2. Defrost and bring protease solution to a final volume of 550 µL, a standard volume for treatment of ten mice (five/group) and one extra dose for error (*see Note 2*). A tube of equivalent volume of PBS/diluent/vehicle should be prepared for treatment of naïve control groups (*see Note 3*).
3. Mice should be anesthetized in accordance to approved animal protocol, to sufficient depth for reliable intranasal (*i.n.*) treatment (tip: titrate anesthesia plane to one breath every 1–2 s (*see Note 4*)). Once sufficient sedation is achieved, apply 50 µL dose of protease/vehicle to the nares using a handheld pipette

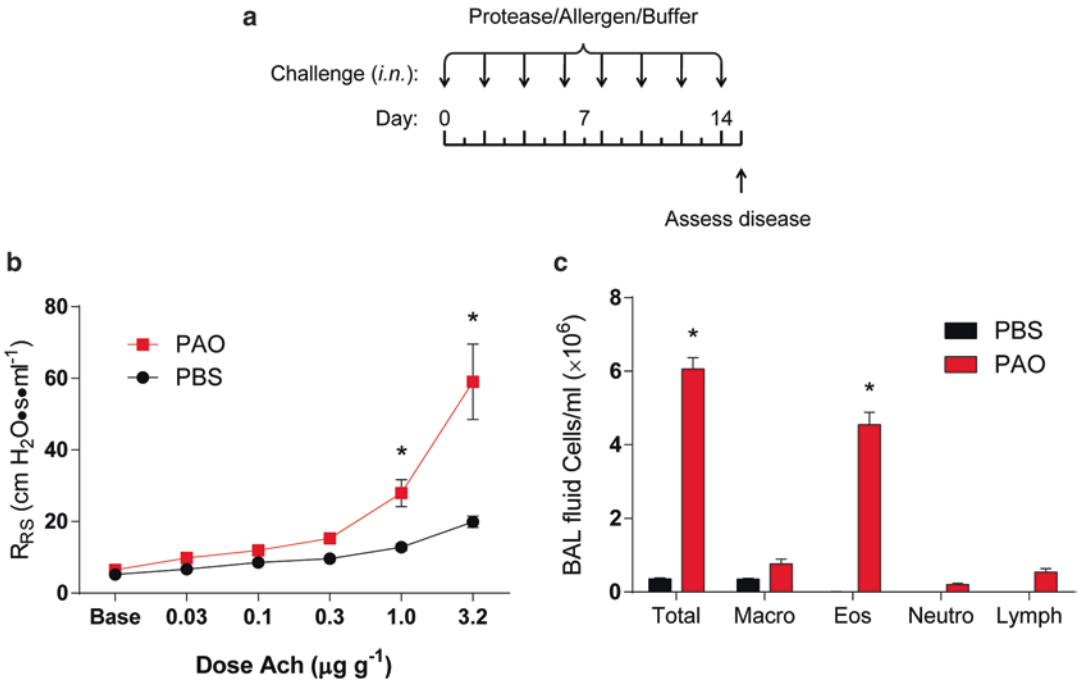


Fig. 1 Protease model. **(a)** Timeline for protease/allergen challenge in which mice are intranasally (i.n.) challenged every other day for seven challenges. **(b)** Exemplary graph of respiratory system resistance measurements (R_{RS}) plotted against increasing concentrations of acetylcholine chloride (ACh) injected intravenously to C57BL/6 mice challenged with *Aspergillus oryzae* protease (PAO) or PBS. **(c)** Bronchoalveolar lavage (BAL) fluid total and differential inflammatory cell counts from the same mice undergoing airway mechanic testing **(b)** including total cells and total macrophages (Macro), eosinophils (Eos), neutrophils (Neutro), and lymphocytes (Lymph). $N = 5$ mice/group. * $P < 0.05$ Mann-Whitney test

(100 μL maximum volume), and allow the mouse to inhale the solution completely (*see Note 4*). Return the treated mice to their cage and monitor for complete recovery.

4. Allow mice to rest for a day, and repeat challenges for a total of seven challenges every other day (Fig. 1a). Following the final challenge, allow the mice to rest overnight before assessing disease.

3.2 Airway Plethysmography

1. Defrost acetylcholine (ACh) stock solution. Dilute 3.2 mL of ACh stock (fifth working dilution) into 6.8 mL of PBS in a 15 mL conical tube, and repeat to make four serial dilutions, corresponding to the fourth, third, second, and first working ACh solutions used to assess changes in airway resistance (*see Note 5*). Prepare an additional 50 mL conical tube with PBS, and prime a 1 mL syringe for each of the doses of acetylcholine and saline tubes.
2. Prepare all 6-well plates and tubes for sample collection (*see Note 6*).

3. Calibrate plethysmography equipment in accordance to lab/manufacturer's protocol, check oxygen supply, and verify the ventilation equipment is properly set to a ventilation rate of 150–160 strokes/min and a volume of ≈ 0.15 mL (*see Note 7*).
4. Sedate the first mouse with a weight-adjusted dose of etomidate (etomidate = weight (g) $\times$ 24 + 100 μ L) (*see Note 8*).
5. Intubate the mouse with a 20-gauge angiocatheter and place on ventilator, establish tail vein IV in accordance with equipment setup, and prepare equipment for analysis.
6. Verify tidal volume is sufficient ($\approx 9 \times$ weight) and baseline is stable before injecting first dose of ACh (*see Note 7*). Inject the first dose (volume = (2 $\times$ weight) + void volume of IV) to induce the first dose response and allow resistance to return to baseline.
7. Challenge mice with increasing doses (volume = (2 $\times$ weight) + void volume of IV) of ACh, and assess/record changes in peak airway resistance, allowing the mouse to return to baseline between challenges (*see Note 9*). Remove tail IV and flush line with saline to remove remaining ACh. Remove mouse from plethysmograph, euthanize, and collect samples to assess inflammatory markers of disease. Data are presented as peak resistance in response to dose ACh (Fig. 1b).
8. A detailed protocol for measuring airway hyperresponsiveness in mice, including video, has previously been published [8].

3.3 Bronchoalveolar Lavage Fluid (BALF) Collection by Catheter Intubation, Differential Counts, and Supernatant

1. Inject 0.8 mL of saline into mouse lungs through catheter, and withdraw and place in 1.5 mL Eppendorf tube on ice. Inject another 0.8 mL of saline, and collect and place in the same tube. Count/record the total number of cells (cells/mL) in each BAL, excluding red blood cells (RBCs). Prepare slides in a cytospin, and spin down approximately 2.0×10^5 cells in 200 μ L per slide and air dry overnight (*see Note 10*). Fix and stain slides according to PROTOCOL HEMA 3 Stain Set, and count 200 cells/slide, recording the number of eosinophils, monocytes, neutrophils, and lymphocytes. Data presented as total number of cells/mL (Fig. 1c).

3.4 Blood Collection

1. Collect blood using standard methods for whole blood collection in accordance to animal protocol and the appropriate institutional office(s). Allow blood to clot overnight at 4 °C, spin down at 3000 RPM in a microcentrifuge for 10 min, and collect serum. Store samples at -20 °C for desired antibody/serum factor analysis.

3.5 Lung Dissection for Histologic, Cell and Cytokine, Gene Expression, and Protein Analysis

1. *Histologic Analysis*: Open the chest cavity to allow clear access to the lungs and upper airway. Mice dedicated for histologic analysis of airway remodeling should remain intubated, while the airway up to the trachea is cleared of dermal tissue and glands (*see Note 6*). Two surgical threads should be made to wrap around the upper airway and catheter now visible in the trachea, tying off the most proximal thread to create a seal between the airway and catheter. Place a 1 mL syringe containing 0.7 mL of formalin fixative solution on the catheter, and inject the total volume to inflate the lungs. Tie the most distal thread tight over the airway and distal to the catheter, sealing the fixative in the inflated lungs. Remove the catheter, cut the airway above the distal thread, and carefully cut down the dorsal side of the conducting airway and lungs to remove the whole, intact lungs and heart. Place the lungs in a tube with sufficient volume of formalin fixative solution to cover the tissue, and store for processing and periodic acid-Schiff/immunohistochemical staining later.
2. *Additional Analyses*: Open the chest cavity to allow access to the lungs. Remove a lobe of the lung, and place in a tube containing RNeasy/TRIzol for gene expression and/or protein analysis (*see Note 11*). Collect the remaining lung, and place in a 6-well plate containing 2 mL of complete media (DMEM or RPMI). All samples must be processed from this point on under sterile conditions. Place lungs in a 40 μ m cell strainer, and pulverize the lung tissue in the strainer within the well with the back of a 3 mL syringe plunger. Place the strainer over a 50 mL conical tube, and pipet the 2 mL cell suspension through the strainer into the 50 mL conical tube. Wash the well with complete media and transfer the wash to the 50 mL conical tube, and label and store on ice until all samples are processed. Spin cell suspensions down at 400 g-force for 5 min, and resuspend as needed for cellular assays. Cell suspensions are ready for standard cell isolation by MACS/cell sorting or quantification of overnight cytokine production (IFN- γ , IL-4, IL-17, etc.) by ELISPOT/ELISA/intracellular staining.

4 Notes

1. Mice must be age (± 1 week) and sex matched for reliable cross group comparison. Significant variation in airway responsiveness between young (< 3 months) and older mice (> 6 months) is observed and should be avoided in experimental design. A total of five mice per group is sufficient to allow for significant power at all time points and downstream assays. Greater num-

bers may be required for studies assessing more subtle differences or cell-isolation studies not described here.

2. It is convenient to prepare 55 μL aliquots of protease at a 10 $\times$ concentration (1.8 mg/mL) to allow for quick dilution, by adding 495 μL of PBS to achieve the desired dose (9 $\mu\text{g}/50 \mu\text{L}$ challenge). Resuspension/dilutions can be easily adjusted to accommodate the addition of drugs or additional compounds as required by the study design.
3. Although this method describes protease-induced allergic lung disease, numerous proteases, allergens, and/or pathogens can be used. Purified fungal proteases are very powerful, having significant potential to degrade airway epithelial and lung integrity. More sensitive mechanistic studies may benefit from use of less potent inducers of disease. Alternative sources of protease (i.e., viral, house dust mite, papaya, ragweed pollen, etc.) or live fungal conidia (i.e., $>1.0 \times 10^5$ conidia/treatment prepared from *Aspergillus* spp. and others) are sufficient to induce the hallmarks of allergic disease assessed in this model.
4. Adequate sedation is critical to ensure that the mouse reliably inhales with sufficient force to deliver protease/buffer to the airway and minimizes swallowing. Too deep a sedation (>4 s between breaths) will result in death due to asphyxiation. Additionally, it is important to place a small drop of protease/PBS solution on the nares to assess if solution is absorbed by sinuses or swallowed. If the drop is passively absorbed by the sinuses or swallowed, allow one breath to pass and test again. If the drop remains on the nares and is not slowly absorbed between breaths, then quickly apply the bulk of the dose to be delivered, and allow the mouse to inhale over 1–2 s. Immediately following inhalation, apply the remaining dose, and wait for complete inhalation of the dose to be delivered before returning the mouse to the cage.
5. Once thawed, four serial dilutions of 3.2 mL acetylcholine into 6.8 mL of buffered saline result in five tubes of increasing doses of acetylcholine (first dose, 0.01; second, 0.03; third, 0.32; fourth, 1.0; fifth, 3.2 $\mu\text{g}/\text{g}$).
6. Lung, spleen, blood, and bronchial airway lavage samples can be collected. Lung tissue can be partitioned into sections for creation of a single cell suspension for ELISPOT, ELISA, and/or flow cytometric analysis or process for protein or mRNA. Whole lung inflation or partial lung inflation (i.e., cannulation of the airway prior to hydrostatic inflation with fixative) may be performed to assess goblet cell metaplasia. If mucus plugging is a significant observation, it is recommended that additional mice be included. Ideally, mice dedicated for histologic assessment should not undergo airway mechanic

testing, due to the increased fluid movement/mucus flushing induced by high doses of acetylcholine.

7. An initial tidal volume of 0.15 cc is typically sufficient for most mice. It is best to continuously calibrate tidal volume at the beginning for each mouse, ensuring that observed airway resistance values are not artificially enhanced or reduced due to tidal volume error.
8. A factor of 24 times body weight is an overdose and ensures that the mouse is completely sedated for analysis and harvesting. A factor of 16 times body weight can be used and is sufficient to achieve sedation but allows the mouse to recover from analysis for experiments where survival is desired. For survival studies, mice should be allowed to recover in a cage supplied with oxygen and warmed by a heating pad. Additionally, mice should remain intubated until their respiration rate has increased and regained a strong twitch response. It is critical that the catheter is removed once the mouse has significantly recovered from sedation in order to prevent choking and death.
9. Higher doses may require additional time between injections to allow for sufficient recovery and return to baseline conditions. Repeating doses is acceptable, but void volume must be considered. Priming the IV with one lower dose before repeating the desired dose will ensure the proper dose delivered.
10. Total number of cells can vary significantly across treatment and control groups. As long as a clear monolayer of cells is placed on the slide in sufficient numbers to allow the differential count of at least a 100–200 cells, the number of cells spun down is not critically restricted to 2×10^5 . Additionally, it is important to maintain fresh stocks of H&E stains to ensure proper staining of eosin for eosinophil identification.
11. The lobe selected for RNA or protein analysis should be consistent throughout the experiment. Ideally, a lobe is dedicated to RNA or protein. RNAlater allows sample collection and storage for future RNA isolation by TRIzol. If protein stability is an issue, it is strongly suggested that the lobe be partitioned and snap-frozen in liquid nitrogen or immediate homogenization and cell lysis in the presence of protease and phosphatase inhibitors.

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