

Assessment of Airway Hyperresponsiveness in Mouse Models of Allergic Lung Disease Using Detailed Measurements of Respiratory Mechanics

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Abstract

This chapter provides an outline of the procedures necessary to measure airway hyperresponsiveness to inhaled methacholine in mouse models of allergic lung disease. We present a method for acquiring detailed measurements of respiratory mechanics using broadband low-frequency oscillatory waveforms applied at the subject's airway opening and analyzed using the constant phase model of the lung. We acknowledge that there are other methods of measuring airway responsiveness in allergic rodent models. However, a discussion of the merits and or detriments of these various methods have been vigorously debated in the primary literature and are beyond the scope of this chapter. The goal of this chapter is to provide a guide in how to begin these types of assays in laboratories which have little to no experience with these particular types of assessments.

Key words Airway hyperresponsiveness, Respiratory mechanics, Allergic airway disease, Asthma, Forced oscillation technique

1 Introduction

One of the cardinal characteristics of asthma is airway hyperresponsiveness (AHR) [1]. It is defined as an exaggerated bronchoconstrictor response to a given stimuli [1]. Initially, direct measurement of lung responsiveness in mice was performed by a handful of labs that had developed custom equipment which was sensitive enough to detect changes in the mouse lung [2, 3]. More recently, commercial systems have become available allowing a wide range of researchers to measure changes in the mechanics of the respiratory system. This chapter attempts to provide an outline of how to assess responsiveness to inhaled methacholine in the mouse lung for laboratories with little or no experience in invasive measurements using forced oscillations, a technique allowing partitioning of the respiratory response into airway and parenchymal

Table 1
Constant phase model parameters [4, 8, 9]

Parameters	Name	Layman's term definition
R_N	Newtonian resistance	Term reflecting the opposition of the airways to a flow of air. It is inversely related to the caliber of the large conducting airways.
I	Inertance	Term related to the amount of air in the airways. It has a negligible value in mice below 20 Hz and is not reported.
G	Tissue damping	Term describing the loss of energy to heat in the lung tissue during a forced oscillation maneuver. It is closely related to tissue resistance and will increase with contraction of the airway smooth muscle.
H	Tissue elastance	Term describing the storage of energy in the lung tissue during a forced oscillation maneuver. It reflects the elastic recoil of the lung (or tissue stiffness) that permits its return towards an initial form after a deformation.
η (eta)	Hysteresivity	It is the ratio of G/H . A comparative change in both parameters was described as reflecting airway closure or collapse while a larger change in G relative to H was associated to heterogeneity in ventilation [8].

lung tissue mechanics through the use of advanced mathematical models. The results presented in this chapter were generated using broadband low-frequency forced oscillations (1–20 Hz) delivered at the subject's airways by a piston-ventilator. The resulting respiratory input impedance was then fit to the constant phase model [4] of the lung by the operating software in order to extract physiological significance (Table 1).

2 Materials

2.1 Equipment for Aerosol Generation and Measurement of Respiratory Mechanics

The authors will describe procedures that are specific to the *flexi-Vent* (SCIREQ Inc, Montreal, QC, Canada) system, the only commercial system currently employing the forced oscillation technique in small rodents. To generate the results presented here, the system was equipped with a standard particle size (4–6 μm MMAD) Aeroneb Lab nebulizer (Aerogen Ltd, Ireland) for aerosol generation. The surgical procedures and pharmacological principles described apply to any type of invasive measurement of airway responsiveness.

2.2 Surgical Equipment

1. Two sets of small curved forceps.
2. Blunt lexor-baby scissors.
3. Fine scissors, sharply angled.

4. Non-sterile size three braided silk suture thread.
5. Stainless steel tracheal cannula with Luer-adapter (20 mm long). Experiments were done using three different sizes of outer diameter (1.0, 1.2, or 1.3 mm) for different strains or ages of mice.
6. Homeothermic heating pad and rectal temperature probe.
7. Scale to weigh live mice.

2.3 Reagents

1. Anesthetic agents (e.g., sodium pentobarbital:nembutal sodium solution 50 mg/ml).
2. Muscle relaxant (e.g., pancuronium bromide: 1 mg/ml).
3. Acetyl- β -methyl choline chloride (methacholine).
4. Phosphate-buffered saline, 1 \times .
5. 70 % Ethanol.

3 Methods

3.1 Preparation

1. Prepare the anesthetic agent. Results presented in this protocol were generated under sodium pentobarbital-induced anesthesia. Thus, procedures described here are specific to that regimen. Dilute the stock solution based on the desired dose and the injection volume (*see* **Notes 1, 4, and 5** and **Table 2**).
2. Prepare the muscle relaxant. Pancuronium bromide is typically used in this assay. Prepare a dilution based on the desired dose and the injection volume (*see* **Notes 1, 5, 8, and 9** and **Table 2**).
3. Prepare the methacholine. Typically a stock solution of 50 mg/ml is made in PBS and serial dilutions are performed to generate concentrations of 25, 12.5, 6.25, and 3.125 mg/ml or less, if needed (*see* **Notes 5, 9–11**).

Table 2
Preparation of surgical reagents

Agent	Desired dose (mg/kg ip)	Injection volume (ml/kg)	Dilute to	Administer
Sodium pentobarbital (stock solution at 50 mg/ml)	70	10	7 mg/ml (i.e., for 5 ml: 0.7 ml of stock + 4.3 ml of saline)	0.2 ml for 20 g mice
Pancuronium bromide (stock solution at 1 mg/ml)	0.8	10	0.08 mg/ml (i.e., for 5 ml: 0.4 ml of stock + 4.6 ml of saline)	0.2 ml for 20 g mice

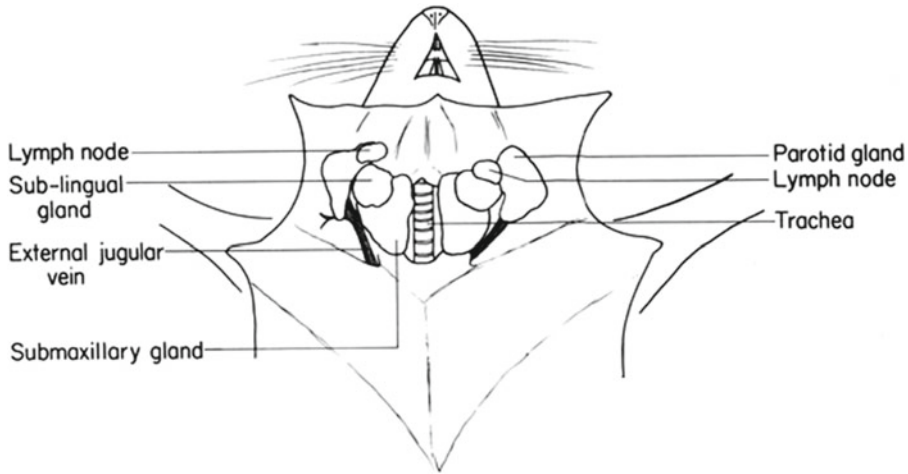


Fig. 1 Drawing of ventral dissection of neck region in mouse. Reproduced from Cook [11]. Copyright holder, Elsevier. Reproduced with permission

4. Calibrate the *flexiVent* system. Weigh the animal, enter its weight and an identifier in the *flexiVent* operating software, and proceed with the calibration of the system using the appropriate endotracheal cannula (*see Note 2*).
5. Anesthetize the mouse. Inject the anesthetic agent intraperitoneally based on the animal's weight and the desired volume of injection (*see Table 2*). Place the animal back in its cage and allow a period of 5–10 min for the drug to reach effect. The animal should become sedated and then lose righting reflex.
6. Verify that a surgical plane of anesthesia has been reached. This can be done by evaluating the animal's toe pinch reflex. A lack of any observable response indicates that a surgical plane of anesthesia has been reached. Prior to advancing to additional steps, verify that the animal's breathing is regular and relaxed.

3.2 Tracheostomy and Cannulation

1. After having established that a suitable plane of anesthesia was reached, place the mouse in supine position typically on a heating pad secured to a moveable board. At this point, the means of measuring and regulating animal's body temperature should also be established (*see Note 6*).
2. Wipe down the fur on the neck area with 70 % ethanol. Using blunt-tipped scissors make a centrally located vertical incision starting between the forelimbs and proceeding almost to the chin (Fig. 1). The submaxillary gland should now be visible.
3. Gently tease apart the two lobes of the submaxillary gland. The trachea will now become visible. A sheath of muscular tissue which surrounds the trachea should now be exposed.

4. Dissect a small piece (1 cm) of the ventral portion of this muscular tissue to directly expose the trachea at which point the trachea rings should be clearly visible.
5. Guide a piece of surgical thread underneath the trachea and between the trachea and muscular tissue. Placing a pair of slightly open (0.5 cm) forceps against the trachea will allow the investigator to push a closed pair of forceps, which are holding the end of a 10 cm piece of surgical thread, underneath the trachea and through to the other side.
6. Once the thread is properly positioned, make a small horizontal incision directly in the trachea between the cartilaginous rings. The higher on the trachea the initial incision is made the more space available to repeat this process if a problem occurs.
7. Insert a tracheal cannula into the incision. Slide the cannula down past the incision until there is an adequate length of cannula inside the trachea so that the thread can be tied around the trachea in order to form an airtight seal with the cannula.

3.3 Mechanical Ventilation and Administration of Muscle Relaxant

1. Maneuver the mouse board so that the cannula can be attached to the ventilator system in a manner that allows the cannula to be directly aligned with the natural direction of the trachea.
2. Start the ventilator and attach the cannula to it (*see Note 7*).
3. Administer the muscle relaxant via intraperitoneal injection and allow a period of 3–5 min for the agent to become effective (*see Notes 5, 8, and 9 and Table 2*).

3.4 Measurement of Airway Responsiveness

1. Select a predefined script or create one. Results presented in Figs. 2 and 3 were generated using a predefined script for inhaled methacholine in mice (*see Notes 2 and 3*).
2. Initiate automated measurement sequences by activating the script. The operator is guided by the software through all the steps necessary for AHR assessment (*see Notes 1 and 14*).
3. When required during the course of the experiment, load increasing concentrations of methacholine in the nebulizer. A typical methacholine challenge includes aerosol challenges at 3, 6, 12, 25, and 50 mg/ml concentrations (Fig. 2).

3.5 Post-methacholine Challenge

1. Upon completion of the last methacholine challenge, stop the ventilator and detach the subject.
2. Remove any leftover solution of methacholine in the nebulizer. Detach the nebulizer from its mount, rinse it with water, and blot dry it.
3. Disconnect the Y tubing, rinse it with water, and dry it using compressed air before reassembling the system.

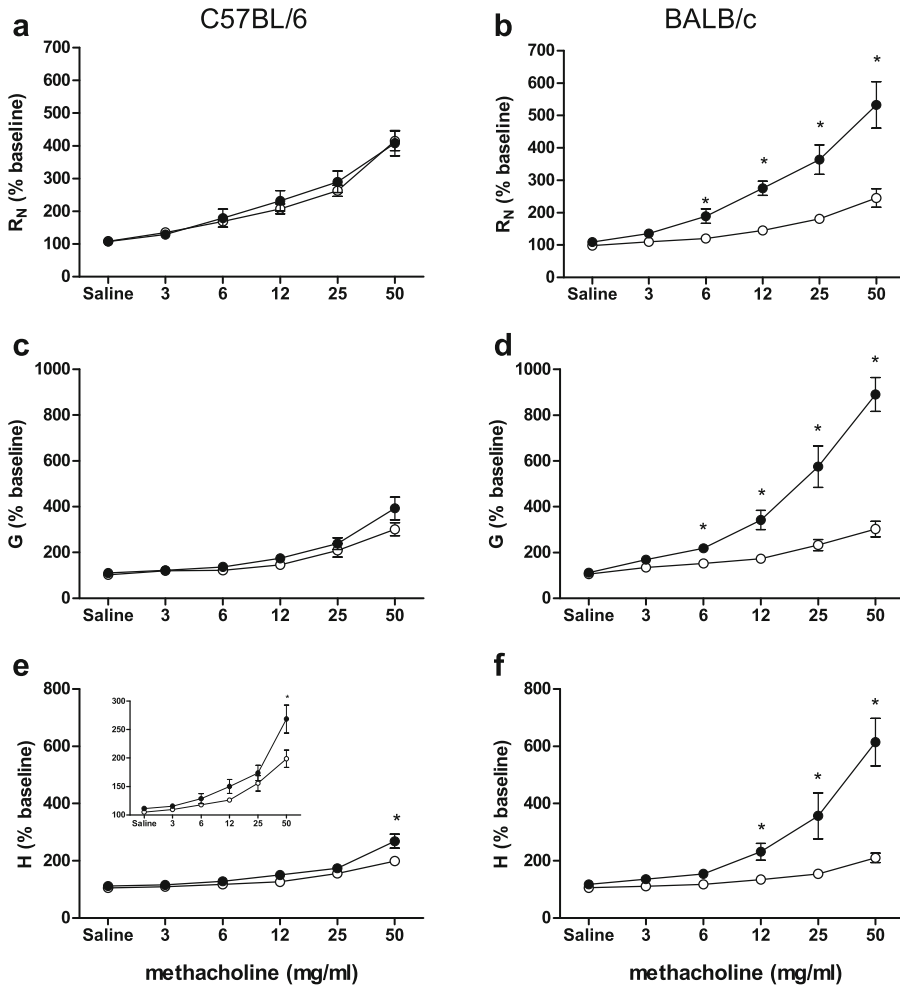


Fig. 2 Assessment of airway hyperresponsiveness in C57BL/6 and BALB/c mice. Allergic pulmonary inflammation was induced using two ovalbumin (OVA) sensitization injections (ip) with alum and three aerosol OVA challenges. Respiratory mechanics were measured using a Prime-4 perturbation with fitting of the respiratory input impedance to the constant phase model of the lung. Individual animal's parameters (R_N , G , and H) were then normalized to percentage of the average baseline measurement for that animal. The average of the group was calculated and all measurements taken were plotted in order to identify the group peak response for each parameter and methacholine concentration. Individual values for each animal at that set time point (i.e., group peak) were then used to generate a dose-response [10]. **(a, c, e)** Changes in respiratory mechanics as measured by percentage of baseline in wild-type C57BL/6 female mice between 3 and 4 months of age. *Open symbols* represent animals challenged with OVA ($n=32$); *solid symbols* represent animals sensitized and challenged with OVA ($n=32$). **(b, d, f)** Changes in respiratory mechanics as measured in percentage of baseline in wild-type BALB/c female mice between 3 and 4 months of age. *Open symbols* represent animals challenged with OVA ($n=11$); *solid symbols* represent animals sensitized and challenged with OVA ($n=12$). Differences between groups were analyzed by analysis of variance (ANOVA) for repeated measures using the logarithm (Log_{10}) of individual responses to ensure homogeneity of variances. This was followed by Bonferroni multiple comparison tests for differences between means. * $p < 0.05$ was considered statistically significant

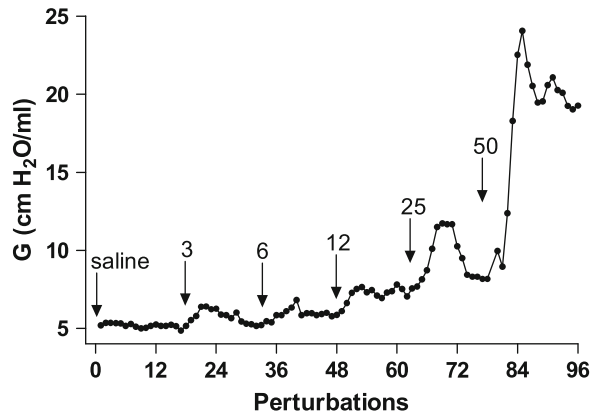


Fig. 3 Plot of automated closely spaced measurements of G . Measurements were recorded following increasing methacholine challenges in sensitized and challenged C57BL/6 female mice ($n=32$) [10]. Arrows denote time point of saline or methacholine challenge (in mg/ml). Prime-4 perturbations were executed every 10 s following aerosol challenge with a total of 16 measurements for each concentration assessed

4. Run the nebulizer briefly with PBS while holding it in your hand to visualize aerosol production (*see Note 12*).
5. Remove any condensation that may have formed at the bottom of the nebulizer or that could have accumulated inside the mount before starting with a new subject.
6. If running a multi-subject experiment, replace the subject with a new one in the *flexiVent* operating software and proceed with the calibration of the system as directed by the software.
7. Repeat all the necessary procedures described above with the following animal.
8. Upon completion of an experimentation session, clean nebulizer and Y tubing as previously described and empty the water PEEP trap (if used). Follow the manufacturer's instructions on module maintenance and expiratory valve cleaning in order to maintain the performance of your system.

3.6 Analysis

1. Export included parameters associated with each perturbation used directly into a spreadsheet (Microsoft® Excel®) (*see Note 13*).
2. Look at results in their raw form. Compile the responses for each experimental group and plot group averages for each parameter and condition. This should generate graphs similar to what is shown in Fig. 3. You may then consider the alternatives listed below to further analyze an experiment.
3. Alternative 1: Perform a detailed analysis of the time course curves to gather information relative to the general profile of the curves, the time to reach peak, or the area under the curve.

4. Alternative 2: Normalize individual animal's parameters (R_N , G , and H) to percentage of the average baseline measurement for that animal.
5. Alternative 3: Generate, using either form of data expression mentioned above (raw form or normalized), dose–response curves for each parameter and experimental groups by looking at the response of each animal at a set point (e.g., peak value for individual parameters) or set time after methacholine challenge (Fig. 2).
6. Alternative 4: Calculate the amount of change from baseline. The following changes in airway resistance (R_N ; expressed as percentage of baseline; Fig. 2b) were observed in naïve BALB/c mice after methacholine challenge: 3 mg/ml = 110 %, 6 mg/ml = 120 %, 12.5 mg/ml = 145 %, 25 mg/ml = 180 %, and 50 mg/ml = 245 %.
7. Alternative 5: Calculate, from the individual dose–response curves, the concentration (or dose) of methacholine required to induce a doubling of baseline (PC₂₀₀ or PD₂₀₀) and report an average value for each parameter and experimental condition.

4 Notes

1. Because procedures are performed in living animals, the investigator needs to obtain the appropriate approvals by the Institutional Animal Care and Use Committee (IACUC) prior to conducting any experiments.
2. Familiarize yourself with the *flexiVent* system and its operating software prior to doing any animal experiments. Learn to start an experiment and to calibrate the system. Best practice recommends that the system be calibrated each day before use. The operator is guided through all the steps necessary for calibration by the software at the opening of an experimentation session and also when required during the course of an experiment (e.g., following a change of subject). The operator can also refer to the owner's manual for additional details.
3. Learn to operate the system using automated tasks (referred to as scripts) in order to standardize procedures during aerosol delivery and measurements following methacholine challenge. This step, which can be done using test loads, will help the investigator plan how much time is necessary each day for equipment preparation as well as for animal assessment. It will also provide an opportunity to make adjustments to the script, if needed. A selection of predefined scripts is provided with each *flexiVent* system. Operators can also create or modify scripts. When creating or modifying a script for automated airway responsiveness assessment, care should be taken to include in the specified order the key steps described in Table 3.

Table 3**Key steps to include in a script for automated assessment in mice of airway responsiveness to inhaled methacholine using broadband low-frequency forced oscillation measurements**

Steps	Description	Parameters
1. Normalization of lung volume and opening of closed areas	This is done by initiating a large amplitude perturbation, e.g.: <ul style="list-style-type: none"> • Deep inflation (or TLC in earlier software versions) • PVs-P or PVs-V (stepwise, pressure- or volume-driven pressure–volume curves) 	<ul style="list-style-type: none"> • Deep inflation: <ul style="list-style-type: none"> IC: Inspiratory capacity • PVs-P or PVs-V: <ul style="list-style-type: none"> <i>A</i>: Salazar–Knowles equation parameter; provides an estimate of inspiratory capacity <i>K</i>: Salazar–Knowles equation parameter; assesses the curvature of the upper portion of expiratory limb of the pressure–volume curve. C_{st}: Quasi-static compliance Area: Area between inspiratory and expiratory branches of pressure–volume curve
2. Baseline measurements	Several broadband low-frequency forced oscillation measurements are taken using perturbation(s) of the Primewave family, e.g.: <ul style="list-style-type: none"> • Quick Prime-3 • Prime-4^a • Prime-8 	Constant phase model parameters (Table 1)
3. Administration of aerosol	Automated activation of the nebulizer is done by adding a command in the script: Output name = “Aeroneb” state = on	
4. Closely spaced measurements	Measurements every 10–15 s for at least 3 min using perturbations of short duration, e.g.: <ul style="list-style-type: none"> • Quick Prime-3 • Prime-4^a 	Constant phase model parameters (Table 1)

^aThe Prime-4 perturbation was available in earlier *flexiVent* operating software versions. It is mentioned here since results presented in this chapter were generated with it

4. Establish an anesthetic regimen. Sodium pentobarbital is frequently used in this type of experiment. It is administered either alone (by intraperitoneal injections of approximately 70–100 mg/kg body weight) or following preanesthetic medication with agents such as xylazine [5] or a combination of drugs such as ketamine/diazepam [6]. Variations in time to reach a surgical plane of anesthesia can be seen between animals or mouse strains. The addition of a preanesthetic medication can be helpful to standardize this step, minimize any excitation phase associated with anesthesia induction, and reduce the administered

dose of sodium pentobarbital. Under the present protocol of AHR assessment with a graduated methacholine challenge, a typical experiment lasts between 30 and 50 min. Therefore, it would be advisable to establish a regimen providing adequate coverage for this length of time in a few naïve animals that would be mechanically ventilated, but would not undergo AHR assessment. Documenting if and when these animals begin to recover from a surgical level of anesthesia and verifying at the end of that time period that the subjects are still alive would be of particular importance. This step would also allow new operators to familiarize themselves with animal handling, injections, and surgical procedures in a small rodent.

5. Some formulations of sodium pentobarbital can precipitate with time. Prepare a working solution fresh each day. Pancuronium bromide is sensitive to light and oxygen but stable at room temperature. The stock solution should be stored at 4 °C to prolong its shelf life. Methacholine should be stored at -20 °C with desiccant as it is highly hygroscopic.
6. During the surgical preparation, it is helpful to place a loop of thread between the teeth in order to pull the animal's head slightly over the end of the board to fully expose the neck area for the tracheostomy as well as to provide as straight as possible flow of air from the ventilator into the lungs. Ideally the animal should be positioned so as to minimize changes in the direction of the ventilation tubing and tracheal cannula when it is connected to the ventilator. The mouse can be immobilized by taping or restraining the limbs. When using a rectal probe to monitor body temperature, strive to maintain a temperature on the lower end of the acceptable range (35–37 °C) throughout the duration of the assessment. A body temperature over 38 °C can rapidly cause the animal to expire. If necessary, body temperature can be reduced by wiping down part of the animal's fur with 70 % ethanol. If during the surgical process any movement is noted, a supplemental dose of sodium pentobarbital should be administered ($1/4$ – $1/2$ dose).
7. When ready to proceed with mechanical ventilation, start the ventilator before attaching the cannula to assure no interruption in the flow of air into the lungs. If working with an external water trap to establish a positive end-expiratory pressure (PEEP), it would be important to adjust the desired pressure before the start of mechanical ventilation. Typically, ventilation in rodents is performed against a 3 cmH₂O PEEP. It is useful to confirm at the start of mechanical ventilation that the animal is still alive (e.g., using vital sign transducers) and that there is no leak around the cannula. This latter step can be done by initiating a “Deep Inflation” perturbation (also known as “TLC” in earlier *flexiVent* software versions; Table 3). In the absence of

leaks, the system should be able to maintain the set pressure (30 cmH₂O) over a period of 3 s without any significant increase in the volume of air displaced by the piston of the ventilator. Running a “Deep Inflation” perturbation will also standardize lung volume, which contributes to decreasing variability between animals.

8. Under the present protocol, a muscle relaxant was administered in addition to the surgical anesthesia during AHR assessment. The purpose of the muscle relaxant is to prevent any breathing efforts during measurements (perturbations) as these efforts would contaminate the oscillatory airflow signal sent by the system and invalidate measurement outcomes. It is important to remember that once the muscle relaxant has been administered, it will be impossible to ascertain the level of anesthesia by skin or paw pinch. If required, vital sign transducers (e.g., heart rate, blood pressure, body temperature) can be integrated into the *flexiVent* system for online monitoring of the subjects [7].
9. Confirmation of adequate muscle relaxation can be achieved by running a perturbation (e.g., PVs-P; Table 3) and ensuring that the recorded pressure signal traces show no downward drops at each plateau. The absence of spontaneous breathing efforts is critical in order to generate valid data sets.
10. Establish a protocol in which inhalation of aerosolized methacholine produces measurable changes in respiratory mechanics with your specific strain of mice using naïve animals. It is critical that the investigator validates that operators can consistently and reliably measure respiratory mechanics with their particular system before investing effort and resources in generating mice with pulmonary allergic inflammation. It is suggested that new operators perform AHR assessment on at least 6–8 naïve mice on two different occasions. Once a consistent data set of naïve animals has been generated, the investigator should confirm that the protocol produces baseline values in relation to previously published results for that mouse strain (Table 4) and that changes in respiratory mechanics reproduced those presented in Figs. 2 and 3. Following success in naïve animals, proceed to establish cohorts of mice with allergic lung disease to assess AHR.
11. C57BL/6 and BALB/c mice are the mouse strains most commonly used in these types of experiments. BALB/c mice have been shown by multiple investigators to have a robust increase in airway responsiveness to inhaled methacholine when pulmonary allergic inflammation is present. However the majority of gene-targeted mice are available on the C57BL/6 background. The C57BL/6 strain, under most protocols for allergic sensitization, produces much lower levels of AHR than the BALB/c strain. The protocol, as outlined below, produces

Table 4
Baseline values in C57BL/6 and BALB/c mice

Strains	Parameters	Average	Standard deviation	Minimal value	Maximal value	Coefficient of variation	Count
C57BL/6	R_N (cmH ₂ O s/ml)	0.29	0.05	0.22	0.38	0.17	12
	G (cmH ₂ O/ml)	5.22	0.45	4.61	6.08	0.09	12
	H (cmH ₂ O/ml)	23.10	1.45	20.21	25.29	0.06	12
BALB/c	R_N (cmH ₂ O s/ml)	0.37	0.08	0.26	0.50	0.23	12
	G (cmH ₂ O/ml)	4.02	0.26	3.64	4.37	0.07	12
	H (cmH ₂ O/ml)	17.93	0.92	16.66	19.63	0.05	12

significant changes in response to methacholine in sensitized and challenged BALB/c mice in all three constant phase model parameters (Table 1), namely, R_N (airway resistance), G (tissue damping, which is closely related to tissue resistance), and H (tissue elastance) (Fig. 2). The same protocol, when applied to the C57BL/6 animals, produces a much more modest change which reaches statistical significance only at the highest concentration of methacholine and in one parameter (tissue elastance or H) (Fig. 2).

12. Aeroneb nebulizers have a limited life span (between 3 and 24 months). The frequency at which a given unit needs to be replaced depends on the operating conditions (e.g., usage, substance nebulized, or cleaning care). We recommend for facilities with multiple investigators using a single system that each investigator purchases its own unit. The volume of aerosol produced by a nebulizer can be determined gravimetrically and different units or types of nebulizers can produce different volumes of aerosol. Monitoring nebulizer performance on a regular basis, keeping accurate records, and modifying, if needed, the nebulization settings to adjust aerosol output rates should ensure reproducibility of results during and between studies.
13. For each data set, a coefficient of determination (COD) indicates how well the mathematical model fits the data. Typically, parameters of any given perturbation will be included provided that the COD is greater or equal to 0.9. Rejected data sets appear as an open symbol in the software "Trend" view.
14. Despite special attention to key steps (system calibration, positioning of animal, leaks, or breathing efforts), it is possible to see data sets being rejected. If baseline perturbations are being excluded, review each of these key steps. Temporary buildup of aerosolized liquid in the subject's airways can account for perturbations being excluded immediately following methacholine challenge. High levels of bronchoconstriction can also

result in data set exclusion around peak response. In either case, reviewing the nebulization part of the protocol including the concentrations of methacholine at which airway reactivity is assessed should improve the situation. Finally, working with a high-resistance cannula relative to the animal's airway resistance can have a negative impact on the signal-to-noise ratio or even lead to negative R_N values. The resistance of the cannula (R_t) is measured during the open dynamic calibration step and can be monitored. Increasing the diameter of the cannula or shortening its length will result in a reduction of the R_t value.

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