

Chapter 2

Mouse Models of Acute Lung Injury

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Introduction

Acute Respiratory Distress Syndrome or ARDS is a diffuse inflammatory lung process that frequently manifests in critically ill patients, with an estimated incidence of 190,000 cases and 74,500 deaths per year in the United States alone [1]. Clinical ARDS is associated with specific risk factors that can be broadly divided into intra-pulmonary conditions, including pneumonia, aspiration, and blunt trauma; and extra-pulmonary risk factors, including extra-pulmonary sepsis, trauma, significant blood product resuscitation, and pancreatitis [2]. Interestingly, ARDS frequently develops up to 72 h after hospital presentation and frequently in the setting of mechanical ventilation, suggesting that mechanical ventilation may play a role in the initiation of lung injury [3, 4]. Clinically, ARDS is manifested by bilateral or diffuse radiographic infiltrates, hypoxemia, decreased lung compliance, and increased ventilatory dead space [5, 6]. The histological manifestation of ARDS is diffuse alveolar damage as defined by epithelial injury, hyaline membrane formation and alveolar flooding with proteinaceous fluid, formation of microthrombi and frequently neutrophilic inflammation.

The animal model correlate to ARDS is acute lung injury (ALI). Models are employed to test potential new therapeutic interventions and to investigate underlying mechanistic pathways that lead to diffuse lung injury. Animal models cannot completely recapitulate all of the complex components of ARDS development and

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manifestation; however, an American Thoracic Society workshop concluded that animal models of ALI should at a minimum manifest histological evidence of tissue injury, alveolar capillary breakdown, inflammation, and physiological evidence of dysfunction with the first two components being more important than the last two [7]. The goals of this chapter are to discuss practical considerations when planning to utilize mouse models of acute lung injury and review some of the primary issues surrounding specific model systems.

Initial Considerations When Planning to Use Animal Models of Acute Lung Injury

Choice of Species/Strain/Sex

No animal model of acute lung injury can completely recapitulate clinical ARDS; therefore, when planning an experiment, the most important goal is determining which model system is most appropriate to address the underlying hypothesis being tested. The first consideration is choice of species. Mice have several well-defined advantages and disadvantages. The primary benefits of mice include the availability of genetic models to test specific mechanisms, the short reproductive cycle that allows rapid expansion of well-defined mouse populations, a large number of reagents available for mice, and lower cost as compared with other species. The primary disadvantages of mice include small size, which significantly increases the complexity of physiological monitoring and any surgical preparations, some immune system differences from humans, notably the lack of IL-8, and a general over-reliance on a limited number of inbred mouse strains when a specific genetic background is not required for testing the experimental hypothesis. Because the focus of this chapter is on mouse models of lung injury, the remainder of the discussion will be focused on mice; however, investigators are advised to consider larger animals, which allow more complete physiological monitoring and assessment, if the unique advantages of mice are not required for the planned study.

Genetic background is an important determinant of host response to both infectious lung injury [8, 9] and sterile lung injury [10–15]. Therefore, findings in a specific strain of inbred mouse, e.g., C57BL/6 or BALB/c, may not be applicable to other mouse strains much less other mammalian species. One common approach to overcome the potential confounding effect of limited genetic variation in experiments that do not require a specific genetic background (e.g., pharmacological or toxicological studies) is to employ outbred mouse stocks, e.g., Swiss outbred mice (stock refers to colonies of outbred mice; whereas, strains are used to define colonies of inbred mice). However, a criticism of this approach is that the genetic variability in these stocks can vary widely depending on the source and prior breeding strategies and are not well-defined [16]. Additionally, genetic lability in these populations may make it difficult to accurately estimate sample sizes based on previously published experimental results. Therefore, experiments using outbred

stock typically require significantly larger sample sizes, which need to be empirically determined in preliminary studies. An alternative approach to using outbred stock is a factorial experimental design, which uses smaller sample sizes in multiple inbred lines, allowing evaluation of the impact of genetic variability on the measured variables [17].

Similar to genetic background, sex can have a significant impact on experimental response to injury and, presumably, to any interventions. For example, sex likely impacts response to prolonged hyperoxia. In our unpublished experience, female C57BL/6 mice are more susceptible to hyperoxia-induced lung injury than male mice. This is consistent with a recent publication, demonstrating a modest increase in mortality with prolonged hyperoxia in female C57BL/6 mice and female mice from the F1 cross of C57BL/6J mice (susceptible to hyperoxia) and 129X1/SvJ mice (resistant to hyperoxia) [18]. In contrast, other groups have found greater susceptibility to hyperoxia-induced lung injury in male mice [19, 20], suggesting that sex effects are complex and may interact with multiple other factors, including strain and environment. Another example of the effect of sex on lung injury models is the finding that C57BL/6 male mice are reported to be more susceptible to bleomycin-induced lung injury [21, 22], which is consistent with unpublished findings from our lab. However, female Fisher rats are reported to be more susceptible to bleomycin-induced fibrosis than male rats [23]. The etiology of sex-based differences in lung injury response is often attributed to sex hormones, and several studies have evaluated this but yielded limited mechanistic insight. Given these results, it is tempting to identify the more responsive sex for the particular model of interest and then use only this sex in the experimental design to reduce overall number of animals required. However, the NIH has recently emphasized the importance of including both sexes in preclinical studies unless a strong rationale can be provided for only studying one sex [24].

In summary, the primary advantages of using mice to model acute lung injury are the ability to utilize genetic systems to isolate and evaluate specific mechanistic hypotheses, the wide availability of reagents, and low cost. If a specific genetic strain is not required for the experiments, there may be advantages to evaluating a limited number of inbred mouse strains to both evaluate the potential impact of genetics on the response to injury and to decrease the likelihood that a specific genotype may be associated with hyporesponsiveness to any tested interventions. Similarly, both sexes should be included in experimental design unless a compelling reason exists to exclude one sex. However, data analysis should include evaluating the potential of a sex model interaction.

Choice of Lung Injury Model

ARDS is associated with a variety of pre-disposing conditions, which can be categorized as either direct injury (e.g., pneumonia, aspiration, pulmonary contusion) or indirect (sepsis, trauma, transfusion) or indirect injury. Accordingly, there are models that utilize direct injury (e.g., bacterial, viral, lipopolysaccharide (LPS), or

acid instillation) and models that utilize indirect injury (e.g., cecal ligation and puncture (CLP), intraperitoneal LPS injection, transfusion associated lung injury). However, generally models utilizing an extra-pulmonary trigger for lung injury result in at most mild lung injury in the absence of a second insult such as mechanical ventilation. For example, CLP, a model of severe sepsis that is associated with significant mortality, results in minimal lung injury in mice [25]. In contrast, models of lung injury that rely on a direct pulmonary insult cause reproducible injury that can be readily titrated by exposure dose/duration. The addition of mechanical ventilation increases lung injury to most if not all direct models of lung injury, including instillation of LPS and other pathogen-associated molecular patterns (PAMP) [26–28], bacterial pneumonia [29], acid instillation [30, 31], and hyperoxia [32]. Because the majority of lung injury models utilize a direct exposure, the remainder of this chapter will review the more common model systems. The focus will be on practical issues necessary to initiate different model systems in a lab. Individuals interested in further reading regarding mechanisms of lung injury with different models are referred to a previously published review as a starting reference [33].

Instillation of Pathogen-Associated Molecular Patterns

Instillation of natural or synthetic pathogen-associated molecular patterns (PAMPs), which are recognized by specific germ-line encoded pattern recognition receptors (e.g., Toll-like receptors or TLRs) causes reproducible sterile lung inflammation and injury. The most common PAMP used is lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria. LPS binds to its cognate receptor, TLR4 and the co-receptor, CD14. However, LPS is available in a variety of preparations, which may contain varying contamination with other pathogen-associated molecular patterns. Thus, ultra-pure LPS, available from select companies (e.g., Invivogen or List Biological Laboratories), will not cause inflammation in the absence of TLR4. In contrast, many sources of LPS will contain impurities that signal via additional TLRs. In our experience, the potency in terms of inflammation and injury is typically higher for lower purity preparations, presumably due to parallel signaling by multiple pattern recognition receptors. We routinely use phenol-extracted LPS from *Escherichia coli* 0111:B4, purchased from Sigma-Aldrich. We resuspend LPS at 5 mg/mL in sterile saline. Heating to 37 °C and/or sonication can facilitate resuspension of LPS. Aliquots of stock solution can then be stored at –20 °C indefinitely.

LPS is easily administered via oropharyngeal aspiration, and a detailed protocol is provided below. The dose of LPS can be titrated to achieve the desired degree of inflammation and injury. For phenol-extracted LPS from *E. coli* 0111:B4 purchased from Sigma-Aldrich, a dose of 2.5–3.75 mg/kg results in moderate lung injury that peaks in 48–72 h and resolves by 10 days (Fig. 2.1) [34, 35]. Other PAMPs, e.g., poly(I:C), a synthetic analog of dsRNA and TLR3 ligand, and Pam3CSK4 a

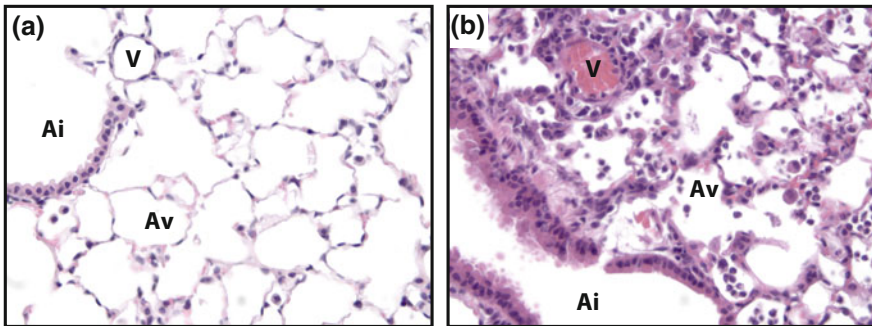


Fig. 2.1 Hematoxylin and Eosin stained, formalin-fixed lung sections from mice exposed by oropharyngeal aspiration 72 h previously to **a** phosphate-buffered saline or **b** 2.5 mg/kg of *E. coli* serotype 0111:B4 LPS. Note damage to the airway wall, thickening of alveolar septae, and inflammatory cell infiltrate, following LPS exposure. *Ai*—airway, *Av*—alveolus, *V*—vasculature. Note the alveolar septal thickening, alveolar inflammatory infiltrate, and airway wall injury in the LPS-exposed mouse lung

synthetic triacetylated lipoprotein and TLR1/2 ligand, can be administered by oropharyngeal aspiration to induce mild to moderate lung injury [28]. Similar to LPS, dose titration and a time course are recommended in preliminary experiments to define optimal experimental parameters.

Bacterial Pneumonia Models

Bacterial infection is a commonly used and clinically relevant model of lung injury. In addition to modeling neutrophilic inflammation and alveolar capillary barrier dysfunction, use of live bacteria allows assessment of additional relevant host response questions, including bacterial clearance and bacterial dissemination. These additional parameters are particularly important when assessing potential new therapeutic interventions, given that pneumonia and sepsis are two of the most common causes of ARDS. Mice have varying susceptibility to different bacteria. C57BL/6 mice will clear *Staphylococcus aureus* and *Pseudomonas aeruginosa* without antibiotics and will survive infections with relatively high bacterial loads of 10^6 – 10^7 [36]. In contrast mice are highly susceptible to *Klebsiella pneumoniae*, and intratracheal inoculation of 700 cfu in CBA/J mice results in ~20 % mortality by 72 h [37].

Bacteria can be aerosolized and delivered via a whole body or nose-only exposure system or given by oropharyngeal aspiration. The advantages of aerosolization include dosing a large number of mice simultaneously and achieving a uniform deposition in the lung, however, the cost for setting up an aerosolization system can be significantly higher. We use the AeroMP aerosol management platform combined with a whole body exposure chamber (Biaera Technologies,

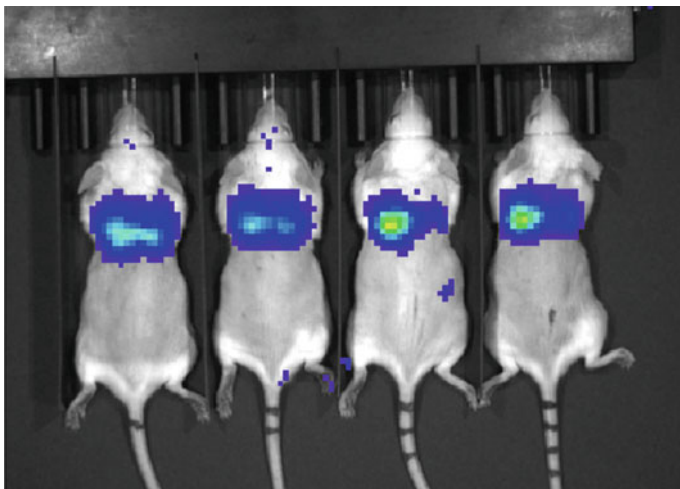


Fig. 2.2 Four mice imaged, using the IVIS in vivo imaging system 6 h after oropharyngeal administration of bioluminescent *P. aeruginosa* (Xen 05, Perkin Elmer). Mice are anesthetized with isoflurane and imaged in the supine posture. Note heterogeneous distribution typical of oropharyngeal aspiration in mouse 3 and mouse 4

Hagerstown, MD), but other systems, including non-commercial set-ups are also effective. Additionally, when delivering bacteria by aerosol, preliminary experiments, in which mice are euthanized immediately after exposure for quantitative cultures of the lungs are necessary to define the relationship between aerosolization parameters (concentration, duration) and deposition.

The primary advantage of oropharyngeal aspiration is the ability to quickly get an infection model up and running without the need to optimize delivery parameters to achieve the desired inoculation. Additionally, higher loads of bacteria can be delivered by direct inoculation as opposed to aerosolization (Fig. 2.2). Finally, an argument can be made that oropharyngeal aspiration, which results in heterogeneous infection, better models clinical pneumonia. An example protocol for preparing *S. aureus* for infection is provided at the end of this chapter.

Hyperoxia-Induced Lung Injury

Prolonged exposure to high oxygen fractions causes lung injury in a strain- and sex-dependent manner that is thought to be secondary to generation of reactive oxygen species, leading to peroxidation of membrane lipids, proteins, and nucleic acids and promotion of both necrotic and apoptotic cellular death [38]. In contrast to bacterial or LPS-induced lung injury, hyperoxia is associated primarily with disruption of the alveolar capillary barrier, and neutrophilic inflammation develops late in moribund mice. Exposure of female C57BL/6 mice to an inspired oxygen

fraction of ≥ 0.95 results in measurable permeability changes by 36–48 h, neutrophilic emigration into the alveolar spaces by 72–84 h, and death by 96–120 h. In contrast, approximately 80 % of female C57BL/6 mice will survive exposure to an FiO_2 of ~ 0.8 [39]. Although hyperoxia exposure systems for mice are commercially available, it is also relatively straightforward to fashion one. A non-airtight Plexiglas box, sufficient to hold one or more micro-isolator mouse cages and with a hinged end for access, is connected to an oxygen concentrator, capable of delivering oxygen at 5 lpm. A vacuum line is also attached to the box with vacuum sufficient to result in ~ 3 lpm air flow. As long as the box is not airtight, oxygen delivery in excess of the flow through the vacuum system will vent to the room and also result in an elevated oxygen fraction in the chamber. However, in the event of failure of the oxygen concentrator, the vacuum line will pull sufficient fresh ambient air through the exposure chamber to prevent build-up of CO_2 . Fine tuning of the actual oxygen concentration in the chamber can be achieved by adjusting the inspiratory oxygen flow and/or the vacuum flow.

Bleomycin-Induced Lung Injury

Bleomycin is an anti-neoplastic drug isolated from *Streptomyces verticillus* [40]. Intratracheal administration of bleomycin is typically thought of as a model of pulmonary fibrosis; however, in fact it causes injury that follows a well-defined pattern of acute neutrophilic inflammation and disruption of the alveolar capillary barrier that peaks by day 3–7 followed by resolution of inflammation and development of a fibroproliferative phase that peaks around day 21–24, which then resolves over time [41–43]. Dosing of bleomycin can be challenging; there is a narrow dose range in which significant lung injury develops, but the majority of animals recover. This is further complicated by a significant sex-dependent variation in susceptibility with female C57BL/6 mice being significantly more resistant to bleomycin-induced lung injury than male mice. A prudent practice is to prepare bleomycin in quantities sufficient to complete a series of experiments, aliquot and store at -20°C , and then perform an initial dose response experiment to determine the optimal dose for that particular preparation. Additionally, because of the potential concern for injury to the pharynx and larynx, most investigators will intubate mice and administer bleomycin intratracheally as opposed to administration by oropharyngeal aspiration. Several different approaches to orally intubating mice have been described [44–46].

Ventilator-Induced Lung Injury

Mechanical ventilation is commonly employed, clinically, to support patients with ARDS. However, beginning in the 1990s, clinicians recognized that mechanical

ventilation can also worsen injury in patients with ARDS, culminating in a large, multi-center trial that demonstrated a large mortality benefit associated with reducing tidal volumes in patients with ARDS [47]. Additionally, retrospective studies have suggested that mechanical ventilation can increase the likelihood of ARDS developing in patients with ARDS risk factors [3, 4]. Similarly, mechanical ventilation in mice will induce lung injury as measured by histological changes, neutrophilic inflammation, and disruption of the alveolar capillary barrier. Mouse models of mechanical ventilation have used large tidal volumes and low or absent positive end-expiratory pressure (PEEP) in isolation to induce lung injury; however, this approach is limited by several factors, including relative resistance of mice to lung injury from ventilation alone, potential impact on hemodynamics, which are challenging to quantify and adjust in mice, and questions regarding the clinical relevance of such models. In contrast, mechanical ventilation with moderate tidal volumes in the range of 10–15 mL/kg synergistically increases lung injury when combined with bacterial products [26, 28, 48], infections [29], hyperoxia [32], and acid instillation [30]. The mechanisms by which mechanical ventilation interacts with other forms of lung injury to amplify the injury response are unclear and remains an active area of investigation. Mechanical ventilation, using parameters that do not result in overt lung injury, results in transcriptional activation of pathways associated with inflammation and may synergistically upregulate pro-inflammatory mediators associated with lung injury and development of extra-pulmonary organ dysfunction [29, 49–52]. Additionally, mechanical strain likely causes physical disruption of cellular membranes, particularly in lung epithelial cells [53, 54], resulting in release of damage-associated molecular patterns and inflammatory activation [28]. Mechanical ventilation in mice is technically challenging, particularly for periods longer than 4 h. A number of pitfalls can significantly increase mortality. For example, body temperature is difficult to regulate in a mouse that is anesthetized and supine, and typical warming pads placed under the mice are insufficient. Temperature regulation is better if the mouse is prone on a warming pad but limits access to the mouse. We have found that a heat lamp with a rheostat is the most effective method for maintaining body temperature of mechanically ventilated mice in the supine posture. However, body temperature must be monitored via a rectal thermistor or similar method to insure that it remains within an appropriate range. A second important issue is control of mouse respiratory effort. In our experience, total suppression of mouse respiratory effort is not feasible with anesthesia alone. Given that mouse ventilators generally are simple volume adjustable syringe pistons with a user set frequency, discordance between spontaneous respiratory effort and the ventilator's respiratory cycle are inevitable. This is easily observed as variation in airway pressure over short intervals. To prevent spontaneous respiratory effort, neuromuscular paralysis with a non-depolarizing paralytic agent, such as pancuronium, vecuronium, or rocuronium is required. Such agents require a plan for monitoring adequacy of anesthesia, which is challenging in a paralyzed mouse. Our experience is that non-invasive blood pressure measurements are unreliable in anesthetized, mechanically ventilated mice. Invasive monitoring of blood pressure would be sufficient but is technically challenging and significantly increases

preparation time and risk of unintended death. One option is to monitor heart rate via EKG and develop an algorithm with the Institutional Animal Care and Use Committee to identify parameters for anesthesia adjustment. For example, increases in heart rate of 20 % or more could trigger an increase in inhaled isoflurane concentration by 0.5 % or an additional dose of systemic anesthetic at half the original induction dose. Finally, monitoring for continued mouse viability is important. In our experience, anesthetized, paralyzed, ventilated mice can die on the ventilator without recognition by performing personnel. If airway pressure is monitored, then a rapid, large increase in airway pressure typically signals death. However, measurements that are more robust include monitoring exhaled CO₂ or monitoring the EKG. A general description of a mouse mechanical ventilation setup is included at the end of this chapter.

Assessment of Lung Injury

A comprehensive workshop report by the American Thoracic Society has been published outlining assessment of lung injury in animal models and should be referred to for an in-depth discussion [7]. However, to summarize, the primary features of experimental acute lung injury in animals were identified as histological evidence of tissue injury, alteration of the alveolar capillary barrier, evidence of an inflammatory response, and evidence of physiological dysfunction. Of these parameters, quantitative assessment of alveolar capillary barrier and quantitative assessment of inflammation are the most commonly employed and easiest to measure. In our opinion, histological evidence of lung injury is most useful for a qualitative demonstration of injury and to identify potential anatomical localizations of specific processes, e.g., by IHC. This position paper proposed a semi-quantitative histology system that has been commonly employed; however, without a systematic sampling system such as designed-based stereology, the quantitative value of this approach is questionable [55].

Multiple approaches can be used to assess alveolar capillary barrier integrity, including assessment of extravascular fluid accumulation and permeability of the lung to macromolecules. The most common measure of extravascular fluid accumulation is wet lung weight at the time of necropsy, normalized either to the dry lung weight, obtained by drying the lung in an oven until weight no longer changes, or to pre-experiment total body weight. Disruption of the alveolar capillary barrier is commonly measured by presence of serum proteins in the alveolar compartment. The simplest, yet reliable, measure is to assay total protein in cell-free bronchoalveolar lavage fluid by the bicinchoninic acid or BCA assay. A brief protocol for mouse bronchoalveolar lavage is included at the end of this chapter. Another approach is to quantify IgM in bronchoalveolar lavage fluid, using a commercially available ELISA (e.g., Bethyl Laboratories). IgM exists in a pentameric form with a molecular weight

of ~900 kD. It is not normally present in the alveolar compartment, and elevated levels imply frank disruption of the alveolar capillary barrier. Other techniques include injecting mice with Evan's Blue dye at a dose of 20 mg/kg. Evan's Blue dye labels albumin, *in vivo*, and translocates to the lung with albumin during lung injury. Permeability is then measured by flushing the pulmonary vasculature via the right ventricle, post-mortem, extracting the dye with formamide, quantifying by spectrophotometry [56]. An additional method is to intravenously inject mice with 100 μ L of a 14.4 mM solution of a fluorescence-labeled 70 kD dextran (ThermoFisher Scientific) 1–3 h prior to euthanasia. Injections are most consistently accomplished via the retro orbital sinus in anesthetized mice. Degree of alveolar capillary permeability can be assessed by measuring the specific fluorescent label in bronchoalveolar fluid with a fluorimeter [28].

Assessment of cellular lung inflammation is easily assessed in bronchoalveolar lavage fluid. Because the number of cells present in the alveolar compartment of normal lungs are relatively low, counting can be facilitated by spinning out the cells from the lavage fluid and then suspending them in a smaller volume (0.2–0.5 mL). Total cell count is made using a hemocytometer, and percentage of neutrophils and mononuclear cells can be assessed on a cytospin preparation of 30,000 cells, using a modified Wright stain. Percentages of other cell types, such as lymphocytes and eosinophils are also often made; however, reliably distinguishing these specific cell types can be challenging and flow cytometry analysis should be considered. Release of pro-inflammatory cytokines can also be assessed via standard ELISA or multiplex assays on cell-free bronchoalveolar lavage fluid as well as tissue homogenate.

Assessment of physiological dysfunction is the most challenging lung injury parameter to obtain in mice. Measurement of gas exchange can be attempted with trans-cutaneous oximeters; however, these devices are frequently unreliable in anesthetized mice. We have found that the best reading is obtained on a mouse thigh after removing hair by a depilatory. Arterial blood gas measurements can also be attempted via left ventricular cardiac puncture. More recently, computer-controlled mechanical ventilators have been developed (e.g., flexiVent, SciReq), which allow application of defined forced oscillatory waveforms to mice. Measurement of pressure fluctuations with changing volume allows estimation of lung mechanics, including elastance and resistance [57]. These newer ventilators also allow measurement of more traditional pressure–volume curves for estimating lung compliance.

Many of these measures can be done in the same animal and a general guideline is that measuring lung injury by multiple different methods will increase confidence in the overall conclusions of the study. We routinely combine measurements of elastance over time, lung extravascular water as estimated by lung weight normalized to body weight, alveolar capillary disruption by measurement of total protein, IgM concentration, and extravasation of 70 kD fluorescence-labeled dextran, leukocyte total and subset counts in the bronchoalveolar lavage fluid, and assessment of pro-inflammatory cytokine release in bronchoalveolar lavage fluid.

Example Protocols

Oropharyngeal Aspiration (Fig. 2.3)

Materials needed

- Stand for suspending a mouse via front incisors (available commercially but a metal bookend, bent to a 75° angle with a rubber band stretched between two bolts works well)
- Two small forceps
- 200 μL pipet
- Anesthesia.

Oropharyngeal aspiration is a straightforward and relatively quick method to deliver material to the lungs. The primary limitation to this method is that distribution of material to the lungs is heterogeneous with a tendency toward greater delivery to the left lung. In a study comparing oropharyngeal aspiration to intratracheal instillation and nose-only aerosol delivery, the fractional distribution of a $^{99\text{m}}$ technetium-labeled sulfur colloid to the lungs by oropharyngeal aspiration was equivalent to that of intratracheal instillation and significantly higher than that observed with aerosolization [58]. Importantly, the optimal volume for aspiration in this study was identified as $\sim 50 \mu\text{L}$.

For oropharyngeal instillation, prepare the instillate at a concentration for which 50 μL or 2 $\mu\text{L/g}$ body weight of the mouse will result in the desired dose. Load 50 μL or 2 $\mu\text{L/g}$ body weight into a P200 pipet. Anesthetize the mouse. Although systemic anesthesia such as ketamine/xylazine will work, use of an inhaled anesthetic such as isoflurane in an exposure chamber provides adequate duration of anesthesia for the procedure and results in more rapid recovery of the mouse. The level of anesthesia should be sufficient that the mouse is unresponsive to handling,

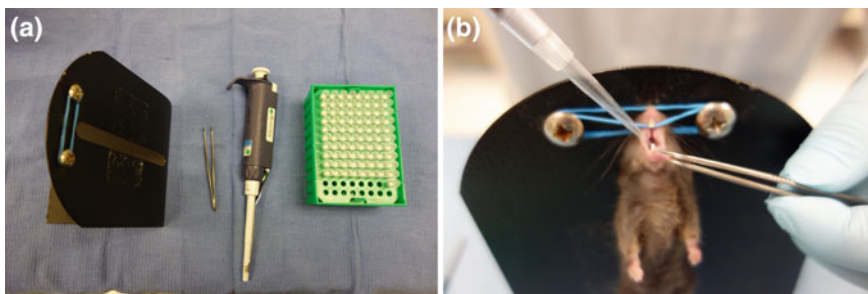


Fig. 2.3 Setup for oropharyngeal aspiration. **a** Equipment needed from *left to right* include an angled stand from which to suspend a mouse by its front incisors, a curved micro-forceps, a 200 μL pipet with aerosol-resistant tips. **b** Anesthetized mice are suspended by their front incisors. The tongue is gently extended with the forceps, and 50 μL of instillate is introduced to the oropharynx. The tongue must be kept extended until the liquid is completely aspirated (~ 20 respiratory cycles)

and respiratory rate has decreased and is notably deeper. At this level of anesthesia, the mouse is suspended by its front incisors from the stand. Using the two forceps, the oral cavity is exposed and the tongue is fully extended. This is a critical step as lack of tongue extension will result in swallowing of the instilled material as opposed to aspiration. The pressure exerted with the forceps must be sufficient to keep the tongue fully extended without causing significant tissue trauma, resulting in edema and impairment of subsequent food and water intake. Once the tongue is extended, the pipet tip is inserted into the posterior oropharynx and the liquid is instilled. At this point, the tongue must be kept extended until the liquid is fully aspirated as determined visually by clearance from the oropharyngeal cavity. Our practice is to typically keep the tongue extended for 5 breaths following clearance of the liquid from the oropharynx. Failure to keep the tongue extended during this period will result in variable amounts of the instillate being ingested as opposed to aspirated. Following instillation, the mouse is removed and recovered from anesthesia.

Protocol for Preparing S. aureus

Materials needed

- *S. aureus* stock (commercial or clinical)
- Tryptic soy (TS) broth
- Sheep blood agar plates
- Sterile 50 % glycerol
- 100 mm Petri dishes
- 50 mL conical tubes
- sterile 250 mL Erlenmeyer flasks
- sterile 0.9 % saline
- sterile, distilled water
- spectrophotometer (optional).

To infect mice with *S. aureus*, first obtain bacteria from either a commercial or clinical source. Bacteria are then streaked out on a sheep blood agar plate and allow to grow until discrete colonies are present. Then, pick one colony with a loop or a sterile toothpick and inoculate into 50 mL of TS broth. Culture overnight in a 37 °C shaking incubator. The following morning, prepare *S. aureus* aliquots by adding 0.5 mL of bacterial suspension to 0.5 mL of sterile 40 % glycerol in a sterile screw-top tube and vortex. Glycerol stocks can then be stored at –80 °C for years. To prepare bacteria for experiments, thaw one aliquot and add 100 µl of *S. aureus* stock into 10 mL of TS broth. Incubate for 6 h in a 37 °C shaking incubator. Take 500 µL of this culture and add to 50 mL of TS broth and incubate for 16 h in a 37 °C shaking incubator.

To prepare the expanded culture for infection in mice, divide into two 50 mL conical tubes and add 25 mL of sterile 0.9 % saline to each tube, mixing thoroughly. Centrifuge at 3000 rpm for 15 min and remove supernatant. Re-suspend pellet in each tube with 1 mL of sterile saline and mix thoroughly. Add sterile saline up to 50 mL in each tube, and centrifuge at 3000 rpm for 15 min. Re-suspend each pellet again in 1 mL of saline and combine. Bring up to 50 mL with saline. Centrifuge at 3000 rpm for 15 min. Remove supernatant and re-suspend in 1 mL of sterile water. This should result in a stock of $\sim 10^{11}$ cfu/mL of *S. aureus* that is depleted of bacterial debris. However, to improve accuracy and consistency of bacterial dosing across experiments, a reasonable initial step is to prepare serial log dilutions of prepared stock in water. The turbidity is quickly measured by the OD₅₄₀ for each dilution (be careful not to let the bacteria settle, which will affect the reading), and 100 μ L of each dilution is added to bacterial culture plates with LB agar. Allow cultures to grow and count colonies. A standard curve can be prepared so that, with future experiments, the OD₅₄₀ can be used to give consistent bacterial doses to mice.

Protocol for Mouse Mechanical Ventilation

Materials needed

- Mouse mechanical ventilator capable of delivering desired tidal volumes (e.g., 300 μ L provides 12 ml/kg for a 25-g mouse). Typically, the syringe piston has a volume of 1 mL. A number of models are commercially available, ranging from simple volume cycled pistons with manually adjusted volumes and frequencies (e.g., mouse ventilator, Ugo Basile) to computer-controlled models that can apply various ventilator perturbations, allowing calculation of lung mechanics (e.g., flexiVent, SciReq).
- Positive end-expiratory pressure (PEEP) water column—can be as simple as a 50 mL conical tube with cap and an inlet through which expiratory tubing is passed into the water to the desired depth and an outlet (connected to a scavenging device if inhaled anesthetic used)
- Rectal thermistor probe for monitoring body temperature
- Electrodes and amplifier for monitoring EKG (may be included with computer-controlled ventilator systems or purchased separately (e.g., PowerLab system with Bio Amp, ADInstruments))
- Pressure transducer and amplifier for measuring pressure (may be included with computer-controlled ventilators or purchased separately (e.g., Bridge Amplifier, ADInstruments))
- Small animal isoflurane vaporizer (e.g., tabletop non-rebreathing anesthesia machine, Harvard Apparatus) with appropriate scavenging system (F/AIR canister or exhausted to fume hood)
- Anesthesia induction chamber

- Anesthesia nose cone delivery system
- Isoflurane
- Vecuronium
- Heating lamp with rheostat
- Endotracheal tube—(e.g., 0.5" 18–20-gauge blunt needle with Luer stub adapter, VWR)
- Y connector and tubing to connect ventilator to endotracheal tube and endotracheal tube to PEEP chamber
- Microtip forceps $\times 2$
- Microtip surgical scissors
- 4-0 silk suture.

Prior to intubation, mice are weighed to allow calculation of tidal volumes and drug doses. Mechanical ventilators should be set up prior to mouse anesthesia induction with appropriate ventilation parameters. In our system, we have found that a tidal volume of 12 mL/kg and a respiratory rate of 150 yields a left ventricular PCO₂ of approximately 35–45 mmHg. However, this is dependent on the dead space of the ventilator circuit and maintenance of a core body temperature between 36 and 38 °C. Additionally, changing the tidal volume will obviously require adjusting the rate. For any planned mechanical ventilation experiments, preliminary studies with arterial blood gas determination from left ventricular puncture is recommended to insure that appropriate ventilation is achieved. Determination of desired PEEP and adjustment of depth of exhaled gas tubing in the PEEP water column should also be done at this time. A typical value for mice that limits progressive atelectasis is a PEEP of 3 cm H₂O, requiring the tip of the exhaled gas tubing to be inserted 3 cm into the water column.

For anesthesia induction and intubation, our lab typically exposes a mouse to 5 % isoflurane for ~3–4 min or until it is immobile with visually apparent reduced respiratory rate. The mouse is then removed to a nose cone exposure system, and anesthesia is maintained at 3 % isoflurane. Mouse limbs are secured with tape to a board, allowing full exposure of the ventral mouse. A midline skin incision is made over the neck and the trachea is bluntly dissected free with forceps. 4-0 silk suture is passed underneath the trachea. A T-shaped incision is made in the trachea, and the endotracheal tube is inserted and secured by tying the suture around the trachea, containing the endotracheal tube. The mouse is then quickly transferred to the mechanical ventilator, and ventilation is initiated with inhaled isoflurane at 1.5 %. Once in place, neuromuscular blockade is induced with vecuronium, 0.6 mg/kg, via intraperitoneal injection. This process requires either multiple isoflurane vaporizers or some planning and practice to switch the isoflurane flow from exposure chamber to nose cone delivery system, to mechanical ventilator inlet port. An alternative to this is to use a systemic anesthesia such as ketamine and xylazine for induction and then transition to isoflurane to maintain anesthesia during mechanical ventilation.

Once the mouse is secured on the ventilator, monitoring probes should be placed, including rectal thermistor probe and EKG leads. The initial body temperature will likely be less than 36 °C because of anesthesia induction and supine positioning

during intubation. Re-warming with a heat lamp should be initiated as soon as possible and monitored closely to prevent over-warming. Initial heart rate with isoflurane anesthesia will be in the 350–500 range. If ketamine and xylazine are used, it will be in the 200–300 range due to negative chronotropic effects of xylazine. Airway pressures are typically in the 12–16 cm H₂O range. If they are significantly higher, this likely represents placement of the endotracheal tube tip against the airway wall, and careful repositioning of the mice may alleviate this issue.

For prolonged mechanical ventilation, airway pressures, heart rate, and body temperature should be monitored and recorded at a minimum of every 15 min. Continuous monitoring with audible alarms at set thresholds is safer. Body temperature should be maintained between 36 and 38 °C. Increases in heart rate of 20 % or higher prompt an increase in isoflurane by 0.5 % or re-dosing of systemic anesthesia at half the induction dose. Vecuronium or other neuromuscular blocking agent should be redosed at 50 % of the original dose if ventilator dysynchrony is observed—either visually by watching the mouse's chest movement or by observing breath to breath variation in peak and/or end-expiratory airway pressures. For prolonged ventilation beyond 4 h, consideration of i.p. fluids, e.g., 0.2 mL of sterile saline, can be considered to prevent hypovolemia and impaired perfusion. For mechanical ventilation beyond 4 h, particularly with volume resuscitation, we have noted the development of significantly reduced heart rate which can progress to cardiac arrest. This appears to be associated with urinary retention and bladder distension. If this is observed, the bladder can sometimes be palpated and expressed, resulting in normalization of the heart rate. At the conclusion of the planned ventilation period, mice are euthanized by anesthesia overdose followed by confirmatory sternotomy.

Post-mortem Bronchoalveolar Lavage Protocol

Materials needed

- 0.5" 20-gauge blunt needle with Luer stub adapter (VWR)
- Microtip forceps ×2
- Microtip surgical scissors
- 4-0 silk suture
- 1 mL syringe
- Lavage fluid (e.g., PBS with 0.6 mM EDTA) warmed to 37 °C
- Eppendorf tubes
- Centrifuge.

After euthanasia, if the mouse is not already intubated, perform a midline incision in the skin over the neck. Using the forceps, bluntly dissect free the trachea and pass a piece of suture around it. Make a T incision, insert the 20-gauge blunt needle, and secure in place with the suture. To lavage both right and left lungs,

gently instill 1 mL of lavage fluid, leave in place for 10 s, and then slowly withdraw until any resistance is met. Quantify the amount returned lavage fluid in the syringe and place in a collecting tube on ice. Bronchoalveolar lavage may then be repeated with pooling of returned fluid. Additional lavages increase the total number of cells collected but decreases the concentration of any proteins which may be of interest such as cytokines or IgM. A common practice is to perform lavage two or three times. However, if the goal is to obtain alveolar cells for subsequent analysis, then repeating lavage up to 5 times will increase yield.

Once all lavage samples are collected, place samples in a centrifuge at 4 °C and spin at $400 \times g$ for 5 min to pellet cells. The cell-free supernatant can then be removed, aliquoted, and stored at -80°C for future assays. The cells are then suspended in 0.5 mL of PBS for counting and differential.

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