

Video Article

***Pseudomonas aeruginosa* Induced Lung Injury Model**

Varsha Suresh Kumar¹, Ruxana T. Sadikot², Jeanette E. Purcell³, Asrar B. Malik¹, Yuru Liu¹

¹Department of Pharmacology, University of Illinois College of Medicine, University of Illinois at Chicago

²Section of Pulmonary and Critical Care Medicine, Atlanta VAMC, Emory University

³Biologic Resources Lab, University of Illinois at Chicago

Correspondence to: Yuru Liu at yuruli@uic.edu

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Abstract

In order to study human acute lung injury and pneumonia, it is important to develop animal models to mimic various pathological features of this disease. Here we have developed a mouse lung injury model by intra-tracheal injection of bacteria *Pseudomonas aeruginosa* (*P. aeruginosa* or PA). Using this model, we were able to show lung inflammation at the early phase of injury. In addition, alveolar epithelial barrier leakiness was observed by analyzing bronchoalveolar lavage (BAL); and alveolar cell death was observed by Tunel assay using tissue prepared from injured lungs. At a later phase following injury, we observed cell proliferation required for the repair process. The injury was resolved 7 days from the initiation of *P. aeruginosa* injection. This model mimics the sequential course of lung inflammation, injury and repair during pneumonia. This clinically relevant animal model is suitable for studying pathology, mechanism of repair, following acute lung injury, and also can be used to test potential therapeutic agents for this disease.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52044/>

Introduction

Lungs are exposed to environmental pathogens and are susceptible to inflammation and injury¹⁻³. During pathological conditions such as pneumonia or Adult Respiratory Distress Syndrome (ARDS), pathogens as well as inflammatory factors released by leukocytes induce injury and death of alveolar cells¹⁻³. It is important to develop animal models of acute lung injury to facilitate the study of pathology of injury as well as mechanism of repair.

Currently, most people use hyperoxia and bleomycin induced mouse lung injury models⁴. However, the mechanisms of hyperoxia caused injury are not the same as most common lung injuries that occur during pneumonia or ARDS⁵. Bleomycin induced acute injury is rare in a clinical context⁴. Here we report a mouse lung injury model using intra-tracheal injection of *P. aeruginosa*^{6,7}. This model is clinically relevant, and mimics the processes that happen following pneumonia⁸.

As an opportunistic, nosocomial pathogen of immunocompromised individuals, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections⁶. The bacteria release virulence factor exotoxin A, multiply and trigger immune responses⁶. Intra-tracheal administration of *P. aeruginosa* reflects the situation in human exposure to the bacteria which cause pneumonia and the pathology is likely to be different from the recently reported influenza virus H1N1 induced lung injury model⁹. Since *P. aeruginosa* is an opportunistic pathogen, it is relatively safe to handle as compared to some of the more virulent pathogens. Here we used intra-tracheal injection to administer the bacteria because we observed that this method introduced more bacteria into the distal alveoli region of the lung compared with some other procedures such as using a catheter via mouth.

Compared with other acute lung injury models, the *P. aeruginosa* model described here is suitable for studying lung injury induced by bacteria and by excessive inflammation. Unlike other animal models that use *P. aeruginosa* to induce sepsis^{10,11}, here we use intra-tracheal injection of these bacteria to induce localized acute lung injury.

Protocol

The animal experiments were approved by the Animal Care Committee and Institutional Biosafety Committees of the University of Illinois at Chicago.

NOTE: All procedures involving pseudomonas should be performed with Biosafety Level 2 (BSL2) practices, which include but are not limited to: mask, eye protection, gown or jumpsuit, and double gloves. Work in certified Biosafety Cabinet. Treat instruments in contact with bacteria with bleach or chlorine dioxide based disinfectant. Use a sealed box for transport samples.

1. *P. aeruginosa* Culture and Growth

1. Store *P. aeruginosa* PA103 as a bacterial stock in a screw cap cryovial at -80 °C.
2. Place the vial in a rack in a box with 70% ethanol wetted paper towels and take to the Biosafety Level 2 (BSL2) laboratory.
3. Streak the bacteria onto sheep blood agar plates and grow at 37 °C for ~15 hr in an incubator.
4. In a BSL2 hood, scratch the bacteria from plate with bacteria loop and resuspend in 5 ml PBS.
5. Store the stock at 4 °C for up to 3 months. However, determine the titer every 2 weeks.
6. Serially dilute the bacteria in PBS (usually from 1:10⁴ to 1:10⁷) and plate out the known dilution on sheep blood agar plates.
7. Incubate the plates for ~15 hr. Count colonies and calculate Colony Forming Unit (CFU) to determine bacteria concentration.
8. Resuspend the appropriate concentrations (~5 x 10³ CFU/μl) in 0.5 ml PBS in 1.5 ml sterile screw cap cryovials. Seal the cryovials and place them in a cryovial rack on disinfectant laden paper towels in a snap lid box.
9. Transport the box to BSL2 animal facility. Once there, open the box in the BSL2 cabinet.

2. *P. aeruginosa* Instillation

1. Perform survival surgery aseptically (sterile gloves, sterile instruments, and aseptic techniques). Use a sterile drape to provide a working surface for sterile (autoclaved) surgical instruments. Sterilize instruments using a hot bead sterilizer between each tracheal instillation procedure.
2. Weigh mice prior to anesthetization. Anesthetize mice with ketamine (100 mg/kg), xylazine (5 mg/kg), in 0.1 - 0.2 ml PBS intraperitoneally (*i.p.*). Determine the effectiveness of the anesthetic by non-responsiveness to toe pinch. Use a vet ointment on eyes to prevent dryness under anesthesia.
3. Restrain mice on a surgical board in BSL2 cabinet.
4. Identify the area for the cut down. Shave this area and prepare the skin using alternating alcohol and povidone iodine swabs 3 times.
5. Treat the incision area with local anesthetic (lidocaine) as this anesthesia is sufficient for a minor surgery such as a skin incision to access the trachea.
6. Make a small incision (approximately 5 mm) at the midline of the neck, and use blunt forceps to gently move the muscle for access to the trachea. Expose the trachea by surgical dissection.
7. Draw bacteria solution into a 1 ml disposable syringe with 27 G needle. For each mouse, administer 20-30 μl of bacteria at the appropriate concentration (up to 10⁵ CFU each mouse).
8. Insert needle into the trachea. Inject solution slowly into the tracheal.
9. Ensure that the animal gasps, which usually indicates that solution has reached into the lung.
10. Close the wound with sterile sutures (6-0 monofilament) under aseptic conditions.
11. Use 0.1 mg/kg buprenorphine by subcutaneous injection as post-op analgesia to control post-surgical pain.
12. Ensure that the time required from initial anesthetic induction to incision closure is less than 15 min.
13. After injection, dispose of syringes and needles in an appropriate biohazard sharps container.
14. Treat the surgical instruments with chlorine dioxide based disinfectant for 15 min, rinse and return to the lab for sterilization and reuse as necessary.
15. House the mice singly in clean cages at warm environment.
16. Check on the animal every 30 min until it regains consciousness and starts to move; and later at 12 hr intervals for the first 3 days post-surgery.
17. Keep the mice in the animal BSL2 facility throughout the experiment. Ensure that only tissue contained in sealed box leaves the animal BSL2 facility. If the mice express any of the moribund behaviors (defined as respiratory distress, lethargy, failure to ambulate in response to gentle stimulation) at any point in the study, euthanize the mice by CO₂ inhalation from a bottled source followed by cervical dislocation.
18. Euthanize the experimental subject by exsanguination under anesthesia.
 1. Collect lung tissue from euthanized mice. Carry out these procedures in a BSL2 hood. If needed, transfer the sample using sealed tubes placed in a rack on disinfectant laden paper towels in a snap lid box for further process.

Representative Results

Starting from 24 - 72 hr post *P. aeruginosa* injection, increased cellularity was observed in lung sections (**Figure 1A-D**). The lung started to recover from 96 hr post injury (**Figure 1E**). At 7 days post *P. aeruginosa*, normal alveoli morphology was largely restored (**Figure 1F**). Tunnel staining using lung sections prepared at 24 hr post *P. aeruginosa* showed cell death in alveoli cells (**Figure 1G-I**). In order to study the repair process in this injury model, BrdU was injected into mice at 3 days post *P. aeruginosa*. Lungs were isolated at 5 hr post BrdU injection and processed for BrdU antibody staining. As shown in **Figure 1J and K**, significantly increased number of cells incorporated BrdU at 72 hr post *P. aeruginosa* administration indicating hyperproliferation.

To study the change in barrier permeability and inflammation post injury, bronchoalveolar lavage (BAL) fluid was collected at different time points post *P. aeruginosa* injection. The protein concentration in BAL was significantly increased at 48 hr post *P. aeruginosa* injection (**Figure 2A**), indicating epithelial barrier leakiness. The cell numbers in BAL also increased significantly at 48 hr post *P. aeruginosa* injection (**Figure 2B**), suggesting an inflammatory response. At 4 - 5 days post *P. aeruginosa* injection, both the BAL protein level and cell number started to decrease suggesting recovery (**Figure 2A,B**). Furthermore, lung lysate was collected at different points post *P. aeruginosa* administration and the level of macrophage inflammatory protein 2 (MIP2), a cytokine involved in neutrophil attraction¹², was measured by ELISA. Consistent with the BAL analysis results, MIP2 level significantly increased at 48 hr post *P. aeruginosa* injection and returned to basal level at 96 hr post *P. aeruginosa*

injection (**Figure 2C**). Furthermore, cells from BAL were fixed on glass slides and subjected to hematology staining. Without *P. aeruginosa*, there were only a small number of monocytes in BAL fluid (**Figure 2D**). In contrast, large amount of neutrophils were present in BAL isolated at 48 hr post *P. aeruginosa* injection (**Figure 2E**). By 96 hr post *P. aeruginosa* injection, the cell composition of BAL returned to the control level (**Figure 2F**). To summarize, results in **Figure 2** indicated an acute neutrophilic inflammatory response together with increased alveoli barrier permeability and increase in cytokine concentrations, which are all hallmarks of acute lung injury¹³. In all experiments, *i.t.* injection of saline was used as control.

Part of the data, including H and E staining, BrdU labeling, TUNEL assay, BAL analysis and MIP2 level measurement, were previously published⁷. In this article, we studied the role of FoxM1, a transcription factor, in the repair of alveolar injury using the *P. aeruginosa* lung injury model described here⁷.

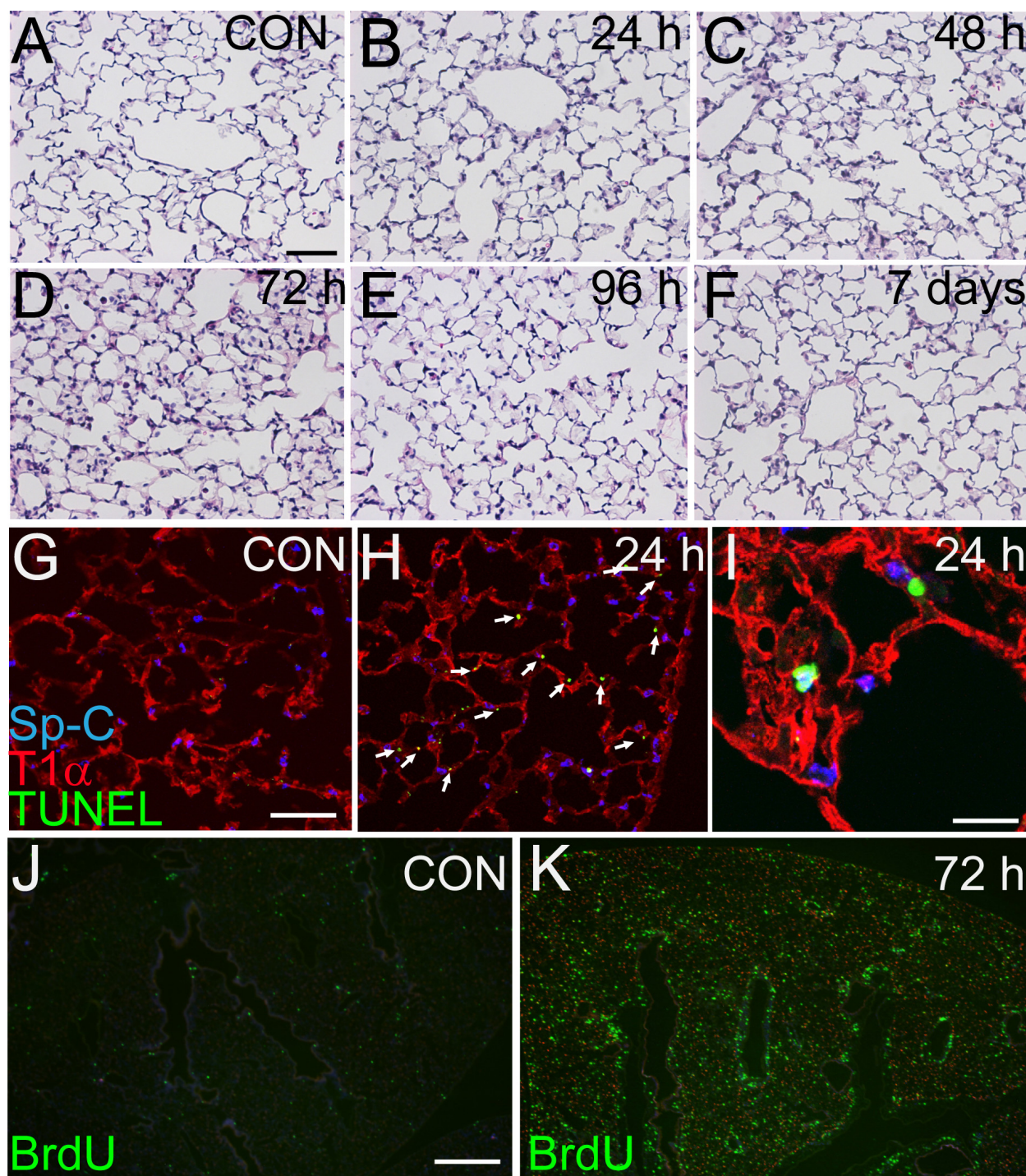


Figure 1. Histology, cell death and proliferation in *P. aeruginosa* mediated mouse lung injury model. (A-F) Lung isolation, sectioning and H/E staining were performed using non-*P. aeruginosa*-injected (non-PA) control lungs (A) as well as lungs at 24 hr (B), 48 hr (C), 72 hr (D), 96 hr (E), 7 days (F) post *P. aeruginosa* injection (post-PA). (G-I) Lung sections were prepared from control lungs (G) and 24 hr post-*P. aeruginosa* injected lungs (H, I) and proceed for TUNEL assay to detect cell death. Lung sections were stained for lung epithelial cell marker Sp-C (blue), T1α (red) to show morphology and also stained for TUNEL (green). Arrows in (H) indicate TUNEL positive cells. (J, K) BrdU was injected in non-*P. aeruginosa* injected mice (J) and 72 hr post *P. aeruginosa* injected mice (K) by *i.p.*, lungs were prepared at 5 hr post BrdU injection and processed for antibody staining against BrdU (green staining). Scale bar = 60 μm for A-F, 50 μm for G and H, 20 μm for I, and 100 μm for J and K. This figure has been modified from Liu *et al.*¹. [Please click here to view a larger version of this figure.](#)

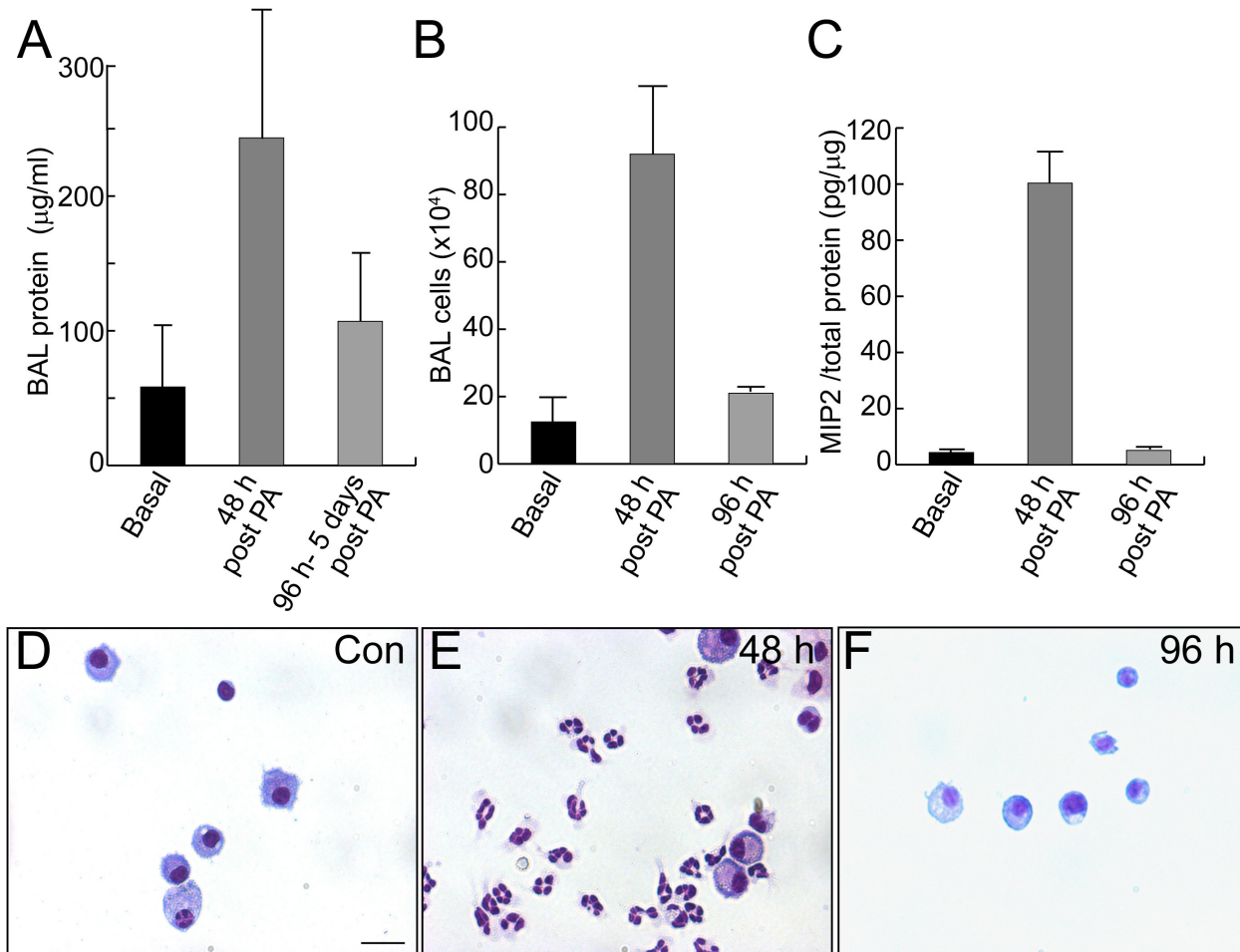


Figure 2. Alveolar barrier permeability and inflammation following in *P. aeruginosa* induced lung injury. (A,B) BAL was collected from control and *P. aeruginosa* treated lungs. (A) Protein concentration in BAL increased at 48 hr post *P. aeruginosa* injection and decreased at 96 hr, 5 days post *P. aeruginosa* injection. (B) Total cell numbers in BAL increased at 48 hr post *P. aeruginosa* injection (B) and returned control level at 96 hr post *P. aeruginosa* injection. (C) MIP-2 levels were measured using lung lysates isolated from control mice as well as mice at 48 hr and 96 hr post *P. aeruginosa* injection. Data were presented at mean \pm SE, $n \geq 3$. (D-F) Cells from BAL were centrifuged and fixed on glass slides and subjected to HEMA3 staining. A small number of monocytes were present in BAL of control mice (D). Large amount of neutrophils were present in BAL isolated at 48 hr post *P. aeruginosa* injection (E). By 96 hr post *P. aeruginosa* injection, there were a small number of monocytes in the BAL (F). Scale bar=10 μm , these results are representative of at least 5 independent experiments. This figure has been modified from Liu *et al.*⁷. Please click here to view a larger version of this figure.

Discussion

The *Pseudomonas* mouse lung injury model that we describe here mimics the whole process of inflammation, lung injury, repair, and resolution that occur following acute lung injury or pneumonia. It has unique advantages comparing with several other injury models in that it is clinically relevant and relatively safe and easy to handle.

The critical step in the procedure is that the injection of bacteria solution needs to be very slow. If injection is too fast, the mice are likely to die by choke. After a successful injection, the mice usually show several deep breathes and gasp, which will help the bacteria particles get into the lung.

The strain we used was PA103⁶. One of the critical points of this model is that the titer of *P. aeruginosa* needs to be tightly controlled. Batch of *P. aeruginosa* from different source could show different degrees of inflammation and damage even when used at the same CFU. Therefore, it is recommended that every new batch of bacteria be tested for their causative effects (such as inflammation, cell proliferation, etc.), and accordingly adjust the number of CFU to be used. If inconsistency of results occurs by using refrigerated bacteria stock, one can use fresh stock obtained by growing bacteria from a fresh plate for each experiment. In addition, the growth phase of the bacteria and choice of media can also have an impact on the effect on the host response. Therefore, care should be taken to ensure that bacterial inoculations occur during the same phase of the bacterial growth cycle.

There might be a difference in the response to *P. aeruginosa* by different strains of mice. The strain we used was a mixture of C57BL/6 and FVB/N. The appropriate *P. aeruginosa* titer should be determined for different mice strains.

One factor to consider for this model is that the damage of the bacteria is not limited to one cell type. For example, epithelial, endothelial, fibroblasts could all have been damaged. Therefore, this model is not suited for to study the effects of cell-type specific lung injuries. The bacteria

cause lung cell death by stimulating inflammatory responses as well as by secreting toxins into tissue⁶. Live bacteria are usually cleared from the lung within 48 hr post injury^{7,14}. A high concentration of dead bacteria can also cause damage by inducing inflammatory responses¹⁵.

The mechanisms of lung injury and repair are likely to be different in response to different types of lung injury. Therefore, it is important to compare several injury models. The *P. aeruginosa* model described here is a nice addition to the other models.

Disclosures

The authors have nothing to disclose.

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