

## Bacteria-Mediated Acute Lung Inflammation

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### Abstract

Mouse models of acute lung inflammation are critical for understanding the role of the innate immune response to pathogen associated molecular patterns, bacteria, and sepsis in humans. Bacterial infections in the lung elicit a range of immune reactions, depending on the pathogen, the level of exposure and the effectiveness of the host response. In general, mice have proven to be an acceptable surrogate model organism for studying specific aspects of human lung pathogenesis, including localized and systemic inflammation, necrotizing pneumonia, bacteriemia, and survival. Here, we describe a highly versatile model utilizing the gram-negative bacterium *Klebsiella pneumoniae*. Following a single challenge with this bacterium, mice develop a robust Th1 mediated immune response and clinically relevant disease progression. While these protocols have been optimized for *K. pneumoniae*, they can be applied to any gram-positive or gram-negative organism of interest.

**Key words** *Klebsiella pneumoniae*, Necrosis, Lung inflammation, Pneumonia, Pulmonary infection, Macrophage, Neutrophil, Th1, Gram-negative bacteria

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### 1 Introduction

Acute respiratory diseases such as pathogen mediated infections and pneumonia represent a significant public health burden, which is expected to increase in the coming decades. Elucidating the underlying mechanisms associated with the host innate immune response to the agents associated with these diseases will facilitate the development of innovative prevention strategies and novel therapeutic agents targeting these infections. One pathogen of specific relevance to acute respiratory disease is *Klebsiella pneumoniae*. *K. pneumoniae* is a common gram-negative bacterium and is a normal component of the mouth, skin and intestinal microbiome. The bacteria are nonmotile, non-flagellated, and rod-shaped. *K. pneumoniae* is routinely encountered by clinicians and is a leading cause of respiratory infections [1]. It has been estimated that up to 23 % of all nosocomial infections are associated with *K. pneumoniae* and infection with this bacterium is of

particular concern as it carries a mortality rate of up to 50 % in the elderly or immunocompromised individuals [2]. Over the last decade, clinicians have reported a growing prevalence of antibiotic resistant strains of this species [3, 4]. In humans, pathogenic *K. pneumoniae* lung infections are capable of inducing severe bacterial pneumonia, which often progresses to bacteremia and sepsis [5]. The acute lung infection typically results in extensive tissue injury associated with excessive inflammation, hemorrhage and necrotic damage. These latter symptoms result in the characteristic thick, blood-laced mucus that is often described as “currant jelly” sputum. Significant progress has been made in characterizing the innate immune mechanisms associated with the diverse host responses elicited by *K. pneumoniae* [6–9].

Here, we describe a highly flexible model of acute lung inflammation that is based on *Klebsiella pneumoniae* infection. In our hands, this model successfully recapitulates many physiologically relevant aspects of gram-negative bacterial infection and acute necrotizing pneumonia in humans. The protocols presented here are designed to maximize the data generated from individual mice and minimize the number of animals required to complete studies. In addition, we also present alternative protocols to evaluate specific aspects of acute lung inflammation that are often overlooked by typical studies. This model is easily adaptable to evaluate other gram-negative and gram-positive bacterial infections in the lungs, as well as other viral, fungal, or damage associated airway inflammation models.

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## 2 Materials

### 2.1 Mice

1. Adult mice, 6–12 weeks old (*see Note 1*) that have been bred and housed under specific pathogen free conditions (*see Note 2*).
2. Mice should be acclimated to the housing facility for at least 5 days prior to the beginning of the experiment.

### 2.2 Reagents and Solutions

1. *Klebsiella pneumoniae*, serotype 2 (American Type Culture Collection (ATCC), VA, USA) (*see Note 3*).
2. Nutrient Agar (Becton, Dickinson and Company, NJ, USA).
3. Nutrient Broth (Becton, Dickinson and Company, NJ, USA).
4. Deionized water.
5. Gentamicin.
6. 1× Hank’s Buffered Saline Solution (HBSS).
7. 10× Phosphate Buffered Saline (PBS).
8. Sterile Water.
9. Isoflurane (*see Note 4*).

10. 10× Buffered Formalin.
11. Trypan Blue.
12. Formamide.
13. Diff-Quick Staining Kit.
14. Permount.
15. ELISA Kits for IL-1 $\beta$ , IL-6, and TNF $\alpha$ .
16. Liquid Nitrogen.
17. Standard reagents for RNA extraction, cDNA amplification, and real-time PCR analysis.
18. Standard reagents for protein extraction and Western blot analysis.

### **2.3 Materials and Equipment for Inoculation**

1. 37 °C incubator for culturing bacteria, which should be separate and distinct from any utilized for routine tissue culture.
2. Platform shaker.
3. 50 ml flask.
4. UV/Vis spectrophotometer with cuvettes.
5. Glass beads or plate spreader for plating bacteria.
6. Chamber for drop method anesthesia (i.e., 500 ml beaker with lid).
7. p200 pipette.
8. Forceps.
9. Rodent intubation stand.

### **2.4 Materials and Equipment for Post-innoculation Monitoring**

1. Small animal rectal thermometer.
2. Digital scale.

### **2.5 Materials and Equipment for Animal Harvest**

1. 1 ml Syringe (with 27 gage needle).
2. 1.5 ml microcentrifuge tubes.
3. Microcentrifuge.
4. p1000 pipette.
5. 10 ml Syringe (with 27 gage needle).
6. Three 1 ml Syringes (without needles).
7. 15 ml conical tubes.
8. Tracheal Cannula (*see Note 5*) (Harvard Apparatus, MA, USA).
9. 4-0, Silk Surgical Suture.
10. Refrigerated benchtop centrifuge (with rotor to accommodate 15 ml conical tubes).

11. Hemacytometer.
12. Microscope (10× and 20× objectives).
13. Cytospin.
14. Microscope Slides.
15. Coverslips.
16. Coplin Jars.
17. 10 ml syringe with a 6 in. piece of medical tubing fitted at the hub.
18. 20 ml disposable glass scintillation vials with lids.
19. Tissue homogenizer.
20. 5 ml syringe (without needle).
21. Portable Liquid Nitrogen container or bucket.
22. Fine tipped indelible marker.
23. 2 ml screw-cap cryo tubes.
24. Mouse necropsy tools: 1 pair of large blunt scissors, 1 pair of straight forceps, 1 pair of blunt 90° angled forceps, 1 pair of sharp 90° angled scissors, 1 pair of slightly curved blunt scissors.

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### 3 Methods

#### **3.1 Prepare *K. pneumoniae* and Culture Bacteria in Nutrient Broth**

1. With a sterilized inoculating loop, streak a nutrient agar plate to isolate single colonies from the bacteria stock. Incubate the plate at 37 °C overnight.
2. Select a single colony from the plate using a sterilized pipette tip or inoculating loop and inoculate 20 ml of nutrient broth in a small flask. Seal the remaining plate with paraffin and store at 4 °C for up to 1 week.
3. *K. pneumoniae* can be cultured in a variety of broths and agars. However, we recommend using the suggested reagents from the bacteria stock supplier, in this case ATCC. Bacteria growth is rapid at 37 °C and oxygenation of the cultures is recommended though shaking during incubation. Under these conditions, we have found that *K. pneumoniae* divide in broth culture approximately every 20–30 min.
4. Monitor and quantify bacteria growth during early log-phase cultures using light absorbance at  $A_{600}$ . In our experience, 1 OD<sub>600</sub> =  $3 \times 10^8$  bacteria/ml. However, this was determined through empirical testing and should be evaluated prior to the start of these experiments.
5. Dilute the bacteria to deliver  $7.4 \times 10^4$  CFUs of *K. pneumoniae* in 50 µl of 1× PBS/mouse, as previously described [9]. Store on ice until ready for use.

**3.2 *K. pneumoniae*  
Lung Inoculation  
Using i.t.  
Administration  
(See Note 6)**

1. Baseline body weight and body temperature should be recorded immediately prior to *K. pneumoniae* instillation.
2. Withdraw the 50  $\mu$ l bacteria dose from the inoculum prepared above.
3. Anesthetize a mouse using drop method isoflurane in the 500 ml beaker with glass cover (*see Note 7*).
4. Mice should be placed on the intubation stand and gently secured by their incisors as directed by the stand's manufacturer.
5. Gently pull the mouse's tongue straight out using forceps. This effectively repositions the epiglottis and allows access to the trachea.
6. While still holding the tongue in position, dispense the 50  $\mu$ l bacteria dose in the back of the mouth and throat. Place fingers over the mouse's nose. The mouse will eventually aspirate the bacteria inoculum, which typically results in a characteristic crackle sound that is audible shortly after inspiration.
7. Once the mouse has aspirated the bacteria dose, gently remove the animal from the intubation rack and place it back in a clean cage for recovery.
8. Immediately following i.t. administration, a subset of animals should be euthanized and the lungs harvested to evaluate bacteria CFUs, as described below, to determine the actual dose given to each animal.
9. Survival, body weight and body temperature are effective surrogate markers of disease progression [9]. Within the first 4–6 h, animals will demonstrate a significant decrease in body temperature that will peak within the first 24 h after challenge. Body temperature will gradually recover within 48 h as the animals begin to clear the bacteria. Concurrent with temperature decreases, body weight will also begin to decline within the first 24 h and peak at approximately 48 h following bacteria challenge. Thus, within the first 72 h, mice should be monitored up to three times per day and moribund animals should be sacrificed following appropriate institutional specific guidelines.

**3.3 Tissue Collection  
to Evaluate the Host  
Innate Immune  
Response**

1. 48–72 h following the bacteria exposure, euthanize the mice following appropriate institutional guidelines (*see Note 8*). This time range represents the typical peak in the host innate immune response to the bacteria. However, this timing should be modified based on the experimental goals of each individual study.
2. For systemic assessments of circulating cytokines, whole blood should be collected utilizing cardiac puncture immediately following euthanasia (*see Note 9*). The whole blood should be

allowed to coagulate at room temperature for at least 30 min prior to serum isolation.

3. Perfuse the animals using 1× HBSS. Carefully open the peritoneal cavity and cut the portal vein leading to the kidney (either side). This will allow the remaining blood to drain from the animal during the perfusion. Without opening the chest, carefully move the liver to expose the diaphragm. The lungs and heart should be visible behind the translucent diaphragm. Carefully clip the diaphragm at the point of contact with the sternum, making a small nick to access the chest. Once the nick is generated, the lungs and tissues should resend into the chest cavity. The bottom of the heart should now be visible. Using a 10 ml syringe with 27 gage needle attached, slowly and carefully inject the heart. Gently inject 1–3 ml of HBSS. The lungs should begin to change color from red to pinkish/white and the liquid flowing from the excised kidney should change from red to clear. Use caution when injecting HBSS into the heart. If too much pressure is applied to the syringe, saline can be forced into the airways and compromise additional data collection.
4. Once the animal has been perfused, the chest cavity can be exposed. Using a pair of blunt scissors, carefully open the chest cavity and remove each side of the rib cage as completely as possible and without damaging the lungs. Next, carefully remove the collar bones, taking care to not damage the underlying trachea. Using blunt tipped forceps, separate the salivary glands and remove the thin layer of muscle that lies overtop of the trachea in the mouse's neck. The trachea should now be exposed from the lungs to the larynx.
5. Using the 90° angled sharp scissors, make a small incision in the trachea 1–3 tracheal rings below the larynx. The incision should be just large enough to insert and secure the tracheal cannula. Do not sever the trachea as this will cause the trachea to retract into the chest cavity. Insert the tracheal cannula into the incision. Brace the trachea with the straight blunt forceps. Using the 90° angled blunt forceps, thread the suture directly under the trachea and securely tie the cannula into place.
6. Collect the BALF. Fill a 50 ml conical tube with 1× HBSS. Fill three 1 ml syringes (without needles) with 1 ml of HBSS. Ensure that no bubbles are present in the syringe, and that the HBSS is flush with the end of the syringe. Gently attach the hub of the syringe to the tracheal cannula and slowly inject 900 µl of HBSS into the mouse lungs in one continuous motion. The lungs should visibly inflate with no obvious leaks. Immediately withdraw the fluid, also in one slow and continuous motion. Deposit the BALF into a 15 ml conical tube on ice. Repeat this process with the other two syringes. However, subsequent lavages should utilize the full 1 ml of HBSS per

lavage. Record the final volume of BALF collected for each animal. The final volume should be approximately 3 ml total. Keep the BALF on ice until ready for further evaluation.

7. Inflate the lungs and fix for histopathology. Fill the 10 ml syringe with attached tubing with 10 % buffered formalin. Brace the trachea with the straight blunt forceps. Using the 90° angled blunt forceps, thread a second suture directly under the trachea and below the end of the cannula. Loop the suture in a half tightened knot. Do not completely tighten the knot. Insert the tube from the 10 ml syringe into the cannula hub. Gently inflate the lungs with approximately 1 ml of 10 % buffered formalin. Do not overinflate the lungs as this will result in distortions in the lung histopathology. Once the lungs are inflated, secure the knot on the half tied suture. Remove the syringe, tubing and cannula from the trachea. Grasp the excess suture thread with the forceps and gently lift the trachea. Using the curved blunt scissors, slowly sever the trachea while lifting the inflated lungs out of the chest cavity. Carefully excise the lungs (with the heart still attached) without cutting them. Gently remove the inflated lungs from the mouse. Place the inflated lungs in a 20 ml disposable glass scintillation vial containing approximately 10 ml of 10 % buffered formalin. Place a lid on the vial and label with an indelible pen.
8. Properly dispose of the remaining mouse carcass.

### **3.4 Tissue Collection to Evaluate Bacteria Clearance and Gene Expression Profiling**

1. In addition to evaluating the host innate immune response, it is also desirable to evaluate bacteria growth and clearance in the lung, as well as, bacterial translocation to distal tissues including the liver and spleen [10]. To evaluate these parameters, subsets of animals from each experimental and control group should be harvested for tissue extraction and homogenization.
2. Euthanize the mice, collect the whole blood via heart stick and perfuse the mice as described in Protocol 3.3, steps 1–3. Once the animal has been perfused, the chest cavity can be exposed. Using a pair of blunt scissors, open the chest cavity and remove each side of the rib cage as completely as possible. Remove the heart and carefully remove each lung lobe individually, taking care to ensure that no lymph nodes or portions of the thymus are included. Place the lung lobes in labeled tubes on ice until ready to homogenize. In our experience, 5 ml round bottom snap cap tubes work well for this procedure.
3. Inside of a BSL2 rated biological safety cabinet, add 1 ml of 1× sterile PBS to each lung sample and completely homogenize the lungs using a tissue homogenizer. Determine lung CFUs by generating serial dilutions from the lung homogenates and plating on nutrient agar [9, 10] (*see Note 10*).

4. In addition to lung CFUs, the liver and spleen should also be harvested, homogenized and plated as described above to determine bacteria dissemination.
5. Following homogenization and plating, the remaining homogenates can be separated into two additional aliquots and centrifuged at  $200\times g$  in a microcentrifuge. Remove the supernatants and store the pellets at  $-80\text{ }^{\circ}\text{C}$  until ready for RNA or protein extraction.

### 3.5 Sample Analysis

1. Analyze cytokine profiles in the serum. After allowing the whole blood to coagulate at room temperature for at least 30 min, spin the samples in a microcentrifuge at maximum speed ( $\sim 16,200\times g$ ) for 5 min. Label a 1.5 ml microcentrifuge tube for serum collection with the indelible pen, one tube for each serum sample. Carefully remove the tubes containing the now separated whole blood from the centrifuge. Note the separation of the blood into two distinct phases. The serum is isolated in the top layer. Carefully remove the serum from the tube using a p1000 pipette and transfer the serum to the newly labeled microcentrifuge tube and keep on ice until ready for storage. The recovered volume of serum should be approximately equal to 20 % of the total volume of whole blood. Store the serum at  $-80\text{ }^{\circ}\text{C}$  until ready for use.
2. For cytokine analysis by ELISA, the serum should be diluted 1:5–1:20 (depending on the assay). These dilutions should be empirically determined prior to running the bulk of the samples. Due to the low volume of serum collected, most sample volumes can be reduced by half for loading on the ELISA plate (i.e., most commercial ELISAs utilize 100  $\mu\text{l}$  volumes of standards and samples; for serum, load 50  $\mu\text{l}$  of standards and diluted samples). Common ELISAs for serum include IL-1 $\beta$ , IL-6, and TNF $\alpha$  [9, 10].
3. Analyze cytokine levels in cell free BALF. Remove 500  $\mu\text{l}$  of BALF. Conduct a serial dilution of the samples for plating on nutrient agar to evaluate CFUs (*see Note 10*). Spin the remaining BALF in their respective 15 ml conical tubes in a refrigerated tabletop centrifuge at  $200\times g$  for 5 min to pellet the cells. Label two 1.5 ml microcentrifuge tubes with the indelible pen. Two tubes for each sample. Carefully remove the 15 ml tubes from the centrifuge without disturbing the cell pellet. Carefully transfer the BALF supernatant to the 1.5 ml microcentrifuge tubes and keep on ice. Store the BALF at  $-80\text{ }^{\circ}\text{C}$  until ready for use.
4. For cytokine analysis by ELISA, the BALF should be used neat or diluted 1:5 (depending on the assay). These dilutions should be empirically determined prior to running the bulk of the samples. Unlike the serum, the BALF should yield ample volume for ELISA and western blot analysis. However, most

sample volumes can also be reduced by half for loading on the ELISA plate, as discussed for the serum. Common ELISAs for the BALF include IL-1 $\beta$ , IL-6, and TNF $\alpha$  [9, 10].

5. Lyse the red blood cells in the BALF using hypotonic saline (*see Note 11*). Following centrifugation and the complete removal of the HBSS, resuspend the cells in 900  $\mu$ l of distilled water. Immediately add 100  $\mu$ l of 10 $\times$  PBS. Samples should be lysed one at a time. If samples contain excessive amounts of red blood cells, the cells can be pelleted in the table top centrifuge at 400  $\times g$  for 5 min and the red blood cell lysis protocol can be repeated.
6. Determine the total BALF cellularity in the 1 ml suspension using a hemacytometer under 10–20 $\times$  magnification with Trypan Blue staining. These data can be evaluated as cells/ml [9, 10].
7. Collect cells for differential staining (*see Note 12*). Label standard microscope slides using a pencil or solvent resistant pen. Secure the slides into the cytospin holder and funnel. Remove 150  $\mu$ l of BALF and cytospin at 100  $\times g$  for 5 min. The volume spun down onto the slides can be reduced if cell density is too great to evaluate cell morphology. Allow the slides to air dry overnight.
8. Differential stain the slides following the manufactures protocols. Allow the slides to air dry overnight. Coverslip the slides using permount. Evaluate the slides using a microscope equipped with a 20 $\times$  and 40 $\times$  objective.
9. Harvest the remaining cells for subsequent analysis, such as FACS, electron microscopy, confocal microscopy, RNA extraction for gene expression evaluation, and/or protein extraction for Western Blot. In general, these subsequent assays such as flow cytometry will be limited by the number of cells collected in the lavage. For most protocols, the cells can be concentrated by centrifugation at 200  $\times g$  for 5 min, the supernatant removed and samples stored at –80  $^{\circ}$ C until ready for use.
10. Prepare the lung for histopathology evaluation. After 24–48 h of formalin fixation, the whole inflated lungs should be ventrally orientated and embedded in paraffin. The resultant blocks should be cut to expose the main conducting airway. Increased scoring accuracy can be achieved by orientating the lungs in the same position and cut to the same depth. Five micron serial sections of the lungs should be cut and stained with H&E. Additional sections can be cut and prepared for in situ hybridization using standard protocols.
11. Evaluate lung histopathology using H&E scoring and evaluation. H&E staining is extremely useful in the evaluation of overall lung inflammation. The most efficient technique to evaluate

H&E staining in these types of assays is through semi-quantitative inflammation scoring of the left lung lobe. Sections of the left lobe should be cut to yield the maximum longitudinal visualization of the intrapulmonary main axial airway and evaluated by an experienced pathologist. Histopathology can then be evaluated by the following inflammatory parameters, which are scored between 0 (absent) and 3 (severe): mononuclear and polymorphonuclear cell infiltration; airway epithelial cell hyperplasia and injury; extravasation; perivascular and peribroncheolar cuffing; and the percent of the lung involved with inflammation. These parameter scores can then be averaged for a total histology score or used individually to quantify specific aspects of disease progression. Scoring should always be conducted in a double blind fashion, with reviewers blinded to both genotype and treatment. This scoring system has been previously described [9, 10].

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## 4 Notes

1. We have successfully utilized 6–12-week-old C57Bl/6, 129SvEv, and BALB/c mice in these assays. If strain is not a limiting factor, C57Bl/6 mice are preferred due to their Th1 skewing and robust response. It is possible that some aspects of this protocol may need to be adjusted and further optimized when using mice from different genetic backgrounds.
2. All studies should be conducted in accordance with the local and institutional animal care and use guidelines and in accordance with the prevailing national regulations.
3. *K. pneumoniae* is infectious and is classified as a BSL-2 pathogen. Materials in this category present a moderate risk to laboratory personnel and should be handled under standard BSL-2 guidelines. Additional pathogen specific institutional, local, and national regulatory guidelines apply. All infectious materials should be handled under the direct supervision of competent and knowledgeable laboratory personnel. A materials transfer agreement (MTA) with ATCC is required for the use of this pathogen.
4. 2,2,2 Tribromoethanol (Avertin) is a common substitute for drop method isoflurane anesthesia in acute airway inflammation protocols. However, in our experience, the deep plain of anesthesia induced by avertin can actually reduce the effectiveness of the intratracheal administrations.
5. We recommend the use of specialized, commercially available tracheal cannulas. However, 16 gage needles can be used as substitutes. In our experience, this alternative works best when the needles are ground down to a blunt end.

6. Intratracheal administration requires extensive practice to achieve proficiency. Improper technique can result in injury to the mouse and inefficient lung delivery. However, once proficient, this technique is ideal for delivering infectious agents to the lungs and has been found to be more accurate than intranasal inoculation. In our hands, we have found that Evans Blue Dye (EBD) is an effective training tool. A 1 % solution of EBD in 1× PBS can be generated, filter sterilized and administered i.t. To quantify the efficiency of the i.t. administration, the lungs can be removed and incubated in formamide for 48 h at room temperature to extract the EBD. The absorption of Evans blue can be measured using a standard plate reader at 620 nm and deposited Evans blue can be calculated against a standard curve to quantify technique efficiency.
7. Drop method isoflurane induces a low level of anesthesia that is recommended for this procedure. We have found that light anesthesia allows for more effective i.t. instillation compared to other techniques, which often suppress breathing volumes and rates. Drop method isoflurane induces anesthesia within 30 s and will lightly anesthetize the mouse for approximately 30 s. Each individual institution will have specific guidelines regarding the use of drop method anesthesia.
8. Note that inhalation anesthetics, such as isoflurane, may result in confounding issues when studying lung physiology. Therefore, ensure that control animals are properly utilized and limit the animal's exposure to the anesthetic as much as possible.
9. The blood should be harvested by heartstick using the 1 ml syringe with a 27 gage needle attached. There are multiple approved methods of conducting the heartstick. We have found that it is most effective when performed prior to making any incisions on the animal. Immediately after removal from the CO<sub>2</sub> chamber, ensure proper euthanasia by toe pinch reflex and pin the mouse to a surgical board. Spray the animal with 70 % ethanol and locate the base of the sternum. Insert the needle between the last 2 ribs and slightly to the right of the center. Using a controlled and singular motion, begin withdrawing the blood from the heart. With practice, this procedure can typically recover 500–800 µl of whole blood. Transfer the blood from the syringe to a labeled 1.5 ml microcentrifuge tube. Remove the needle from the syringe prior to transferring the blood. Forcing the blood through the needle will induce cell lysis and inhibit serum collection.
10. In addition to lung CFUs generated from the lung homogenates, the BALF will also contain live bacteria and can effectively be utilized to determine bacteria load. However, BALF CFUs are often more variable than lung CFUs.

11. There are many different protocols for red blood cell lysis. The protocol described here is optimized for the subsequent basic morphology assessments by differential staining and total cell counts. However, this procedure is often considered suboptimal for higher resolution analyses, such as FACs. Red blood cell lysis via ACK lysing buffer is a viable alternative for procedures requiring less background and higher resolution.
12. Differential staining allows for morphology based identification of BALF cellularity. To ensure the optimal results, the samples should be cytospun on the same day they are collected and the staining reagents should be prepared fresh prior to each use. DiffQuick based protocols allow the differentiation of eosinophils (granules stain red) and neutrophils (granules do not stain). Monocytic cells can be easily identified, but are difficult to distinguish. Therefore, these cells should be identified as monocytes, rather than macrophages. Likewise, lymphocytes are also commonly observed in the BALF. However, it is also unlikely that typical researchers can distinguish T-cells from B-cells based on morphology alone. Thus, many investigators have modified these procedures for use with flow cytometry. The only limiting factor is the low number of total cells typically harvested from control animals. Even with flow cytometry, differential staining should be used to confirm the results.

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