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A Method for Generating Pulmonary Neutrophilia Using Aerosolized Lipopolysaccharide

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Abstract:	Acute lung injury (ALI) is a severe disease characterized by alveolar neutrophilia, with limited treatment options and high mortality. Experimental models of ALI are key in enhancing our understanding of disease pathogenesis. Lipopolysaccharide (LPS) derived from gram positive bacteria induces neutrophilic inflammation in the airways and lung parenchyma of mice. Efficient pulmonary delivery of compounds such as LPS is, however, difficult to achieve. In the approach described here, pulmonary delivery in mice is achieved by challenge to aerosolized Pseudomonas aeruginosa LPS. Dissolved LPS was aerosolized by a nebulizer connected to compressed air. Mice were exposed to a continuous flow of LPS aerosol in a Plexiglas box for 10 minutes, followed by 2 minutes conditioning after the aerosol was discontinued. Tracheal intubation and subsequent bronchoalveolar lavage, followed by formalin perfusion was next performed, which allows for characterization of the sterile pulmonary inflammation. Aerosolized LPS generates a pulmonary inflammation characterized by alveolar neutrophilia, detected in bronchoalveolar lavage and by histological assessment. This technique can be set up at a small cost with few appliances, and requires minimal training and expertise. The exposure system can thus be routinely performed at any laboratory, with the potential to enhance our understanding of lung pathology.
Author Comments:	
Additional Information:	
Question	**Response**

Karolinska Institutet

Elizabeth Sheeley
Associate Editor
JoVE

Stockholm, July 29, 2013

Dear Ms. Sheeley,

Enclosed please find our manuscript entitled "A Method for Generating Pulmonary Neutrophila Using Aerosolized Lipopolysaccharide " for consideration for publication in your journal. The manuscript describes for the first time a detailed protocol for generating pulmonary inflammation by using aerosolized lipopolysaccharide. The methodology is easily accessible and generates a profound inflammatory response, with minimal variation. In addition, the described protocol can be modified to address various different scientific questions. In light of this, we believe that the method we describe is well suited for publication in JoVE, with the possibility of interesting researchers focusing on a wide array of topics.

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A Method for Generating Pulmonary Neutrophilia Using Aerosolized Lipopolysaccharide

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Keywords
Acute lung injury, Airway inflammation; Animal models, Bronchoalveolar lavage; Lipopolysaccharide; Neutrophils; Pulmonary delivery; Sterile inflammation.

Short abstract

We describe a method for inducing neutrophilic pulmonary inflammation by challenge to aerosolized lipopolysaccharide by nebulization, to model acute lung injury. In addition, basic

surgical techniques for lung isolation, tracheal intubation and bronchoalveolar lavage are also described.

Long abstract

Acute lung injury (ALI) is a severe disease characterized by alveolar neutrophilia, with limited treatment options and high mortality. Experimental models of ALI are key in enhancing our understanding of disease pathogenesis. Lipopolysaccharide (LPS) derived from gram positive bacteria induces neutrophilic inflammation in the airways and lung parenchyma of mice. Efficient pulmonary delivery of compounds such as LPS is, however, difficult to achieve. In the approach described here, pulmonary delivery in mice is achieved by challenge to aerosolized *Pseudomonas aeruginosa* LPS. Dissolved LPS was aerosolized by a nebulizer connected to compressed air. Mice were exposed to a continuous flow of LPS aerosol in a Plexiglas box for 10 minutes, followed by 2 minutes conditioning after the aerosol was discontinued. Tracheal intubation and subsequent bronchoalveolar lavage, followed by formalin perfusion was next performed, which allows for characterization of the sterile pulmonary inflammation. Aerosolized LPS generates a pulmonary inflammation characterized by alveolar neutrophilia, detected in bronchoalveolar lavage and by histological assessment. This technique can be set up at a small cost with few appliances, and requires minimal training and expertise. The exposure system can thus be routinely performed at any laboratory, with the potential to enhance our understanding of lung pathology.

Introduction

Lipopolysaccharide (LPS) is a cell wall component of gram negative bacteria[1]. Challenge to LPS is a well-documented model of acute lung injury, a syndrome characterized by acute neutrophilic inflammation and edema[2]. In addition, pulmonary neutrophilia is also a hallmark of chronic obstructive pulmonary disease (COPD)[3], and LPS challenge in humans has been used to model COPD exacerbations[4]. Thus, experimental models of LPS exposure are clinically relevant and valuable tools to understand human pathology.

The objective of the pulmonary delivery of aerosolized LPS described here is to generate a neutrophilic inflammatory response in the conducting and respiratory airways, without systemic involvement. Several techniques of LPS challenge have been described previously. Intra-venous injection of LPS is the most commonly used route of administration. Although this technique is easily accessible, the primary damage is to the endothelium, with secondary destruction of the pulmonary epithelium following neutrophil migration to the lung. Intra-venous administration also induces systemic inflammation[2], which may complicate the clinical picture in animal models. Systemic inflammation is in contrast not observed with intra-tracheal administration. This technique, however, is labor intensive and requires anesthetics as well as considerable training[5,6]. Furthermore, pulmonary deposition by this route of administration is dependent on breathing[7]. Thus, pulmonary deposition is affected by the depth of anesthesia needed for the intra tracheal administration and variable deposition in the airways may be observed. In contrast, pulmonary delivery with aerosolized LPS requires minimal training, and can easily be accomplished on a large number of animals with little or no variation between individuals[5,8]. A recent study confirms that aerosol delivery is superior to the intra-tracheal route with regard to deposition, and that more relevant doses of LPS induce neutrophilic inflammation with this model[8].

Previous studies have demonstrated that challenge to aerosolized *Psuedomonas aeruginosa* LPS generates a marked inflammatory response in the airway lumen and lung parenchyma, including the alveolar spaces[9,10]. The inflammation is characterized by a predominance of neutrophils and presence of pulmonary edema, and can thus be used to address pathogenesis of acute lung injury and gain further knowledge of the mechanisms contributing to disease pathology.

Protocol

The animal studies were approved by the Northern Stockholm animal welfare ethics committee. The experimental procedures were performed in compliance with Swedish law.

1. Generating a LPS Aerosol

1.1 Dissolve 0.5 g purified *P. aeruginosa* LPS in 50 ml sterile saline with gentle agitation and verify dissolution. Dilute 1 ml dissolved LPS in 9 ml saline, to a final concentration of 1 mg/ml. Protect from light with aluminum foil and store at -20 °C.

1.2 Thaw solubilized LPS in the dark at room temperature and mix well immediately before use.

1.3 In a ventilated level II biohazard hood, insert a red inlet into a nebulizer, and connect the nebulizer to pressurized room air via the tubing provided by the manufacturer (review scheme presenting the experimental devices in Figure 1). CAUTION: appropriate personal protective equipment, including a half face piece reusable respirator with particulate filters, goggles, gloves and protective garments should be used during the course of exposure.

[place figure 1 here]

1.4 Connect the outlet of the nebulizer to a mass flowmeter via an air filter. Connect the mass flowmeter to an electrical supply.

1.5 Adjust the airflow to 5 l/min, with pressure remaining at 1.0 - 2.0 bar.

1.6 Remove the mass flowmeter and disconnect the airflow.

1.7 Connect the outlet of the nebulizer to a 15.9 mm tube, which bifurcates and connects to two Plexiglas boxes with the dimensions: 150 x 163 x 205 mm, fitted with removable lids. Each box should have a 5 mm hole in the side opposing the inlet, to prevent pressure build-up.

1.8 Place up to 5 mice in each Plexiglas box and close the lids.

1.9 Open the nebulizer and fill the insert with at least 4 ml LPS dissolved in saline or vehicle alone (saline) (the volume should not exceed 8 ml). Re-connect the inlet to the air supply.

1.10 Allow the aerosol to flow into the closed Plexiglas boxes for 10 min. Monitor the animals continuously. Make sure the air supply remains tightly secured to the inlet of the nebulizer.

1.11 Disconnect the air supply. With the lids closed, let the animals remain in the Plexiglas boxes for 2 min.

1.12 Open the lids and allow for the aerosol to disperse, and return the animals to the cages. If the animals appear wet, place the cages on a heating pad set to low heat, to prevent hypothermia.

1.13 Monitor the animals continuously for the first 30 min, and thereafter every two hours for the first 6 hours.

2. Bronchoalveolar lavage (BAL)

2.1 At the experimental end point, sedate the animals with 3.5% isofluorane. Pinch the hind leg foot with forceps to ensure sufficient depth of anesthesia. Spray down the fur of the animals with 70% ethanol.

2.2 Open the abdomen using scissors, and euthanize animals by severing the aorta. Place a piece of tissue over the abdomen to soak up the blood.

2.3 Use a single anterior-posterior cut of the scissors to expose the thorax. Lift up the rib cage by the anterior tip of the sternum and use the scissors to puncture the diaphragm at the most ventral point, without cutting into any lung lobe. Open the ribcage by making two cuts in the anterior-posterior direction (meeting below the jaw).

2.4 Gently pull apart the rib cage using forceps, and cut the trachea below the larynx.

2.5 Lift up the trachea with the forceps and remove the lungs by cutting the ligaments connecting the lobes to the thoracic cavity, and gently pulling by the adipose and cardiac tissue.

2.6 Insert a polyethylene tube (inner diameter: 0.58 mm; outer diameter: 0.965 mm) into the trachea and secure the tube with a string of silk thread.

2.7 Tie off the multilobe (the four lobes of the right lung) with silk thread.

2.8 Insert a 23 gauge needle into the polyethylene tube and slowly inject 250 µl of ice-cold sterile PBS into the single lobe with a 1 ml syringe.

2.9 Carefully tap the lungs 30 times and collect the liquid through the syringe. Repeat the procedure with 200 µl PBS (approximately 300 µl PBS should be recovered from the single lobe from the total injected volume of 450 µl).

2.10 Keep the bronchoalveolar lavage fluid (BALF) on ice, or enumerate the BAL cells immediately. Count the cells with a heamocytometer, using Turk solution to stain the cells[11]. Calculate the total cell number by multiplying the number of cells with the dilution factor of the staining solution and the volume within the counted field of the heamocytometer.

2.11 Prepare differential cell counts from cytocentrifuged cells as described elsewhere[11].

3. Formalin fixation of lung tissue for histological assessment

3.1 Remove the multilobe and snap-freeze on dry ice. Store at -80 °C.

3.2 Mount a 60 ml syringe with the plunger removed on a metal support stand. Fill the syringe with 10% formalin to the height of 20 cm above the laboratory bench, representing 20 cm of constant pressure.

3.3 Connect the 23 gauge needle secured to the trachea to the 60 ml syringe via a plastic tube with a valve to control the flow of formalin.

3.4 Insufflate the lung lobe with formalin for 5 min. Disconnect the needle and remove it together with the polyethylene tube from the trachea while pulling on the silk thread to close the trachea and retain the pressure in the lung.

3.5 Submerge the lung lobe in formalin and fix for 24 hr at 4 °C.

3.6 Wash the fixed tissue three times for at least 20 min with 70% ethanol, and dehydrate to xylene through the following regimen (1 hr each):
3 x 70 % ethanol
3 x 95 % ethanol
3 x 100% ethanol
3 x xylene
1 x (liquid) paraffin

3.7 Embed dehydrated tissue in paraffin, cut in 4 - 5 µm sections, and stain with hematoxylin and eosin to allow for histological assessment.

Representative results

Challenge to aerosolized *P. aeruginosa* LPS usually yields a marked inflammatory response in the airway lumen and alveolar space, characterized by a predominance of neutrophils at both early and late time points.

Aerosolized LPS induces pulmonary neutrophilia

C57BL/6by and BALB/c mice were exposed to aerosolized *P. aeruginosa* LPS or vehicle alone and neutrophils were enumerated in BALF. The total cell number in BALF of C57BL/6by mice exposed to an aerosol generated with vehicle only is typically around or below 200,000 cells and the cells consist of 95-100% mononuclear cells, with only few lymphocytes (0.5-5%), and no neutrophils in the BALF (Figure 2 A-C). Mice challenged with aerosolized LPS exhibit an increased total cell number in BALF, typically >500,000 cells after 6 hr. The cell infiltrates remains high after 24 hr. The cellular profile in BALF is shifted towards a predominance of neutrophils (80-95%) following LPS exposure (Figure 2 B and C).

[place figure 2 here]

A comparable increase in inflammatory cells in BALF is observed in LPS-challenged BALB/c mice (Figure 3 A). In addition, the percentage of neutrophils and mononuclear cells

in BALF after LPS challenge is comparable in C57BL/6 and BALB/c mice (Figure 3 B and C).

[place figure 3 here]

Similar inflammatory cell profile and pulmonary neutrophilia is observed with nebulization of 5 mg/ml LPS (Figure 4 A-C) and by intranasal delivery of LPS, as previously reported [12,13].

[place figure 4 here]

Pulmonary localization of neutrophils in LPS-challenged

Neutrophils are observed in the epithelial submucosa, as well as spaces surrounding the conducting airways and blood vessels of LPS-challenged mice (Figure 5). Dispersed neutrophils are also detected in the parenchyma and alveolar region.

[place figure 5 here]

Concentration of neutrophil chemoattractants in BALF

The total protein content in the BALF of LPS-challenged mice is increased compared to mice exposed to saline (Figure 6). Also, the expression of the neutrophil chemoattractants chemokine (C-X-C motif) ligands (CXCL) 1 and CXCL2 are increased in LPS-challenged mice [10] (Figure 7 A and B).

[place figure 6 here]

[place figure 7 here]

Figure legends

Figure 1. Schematic presentation of the experimental devices used for generating an aerosol. The inlet of the nebulizer is connected to an air supply. The outlet of the nebulizer is first connected to a flow meter via a 15.9 mm tube and an air filter, and airflow is adjusted to 5.0 l/m at 2 kbar pressure. The outlet is next connected to a Plexiglas box fitted with removable lids and 5 mm holes to prevent pressure build-up.

Figure 2. Pulmonary neutrophilia in C57BL/6by mice challenged with 1 mg/ml aerosolized LPS. C57BL/6by mice were exposed to 1 mg/ml aerosolized *P. aeruginosa* LPS or vehicle (saline, white bar) alone for 10 minutes. Bronchoalveolar lavage (BAL) was performed after 6 hr or 24 hr and the leukocytes were enumerated in BAL fluid (BALF). (A) Total cell number (TCN), (B) neutrophils, and (C) mononuclear cells (MNC) in BALF. Significant differences were analysed using un-paired t-tests. n=3-4, * indicates $p<0.05$, ** indicates $p<0.01$.

Figure 3. Pulmonary neutrophilia in BALB/c mice challenged with 1 mg/ml aerosolized LPS. BALB/c mice were exposed to 1 mg/ml aerosolized *P. aeruginosa* LPS or vehicle (saline, white bar) alone for 10 minutes. Bronchoalveolar lavage (BAL) was performed after 6 hr or 24 hr and the leukocytes were enumerated in BAL fluid (BALF). (A) Total cell

number (TCN), (B) neutrophils, and (C) mononuclear cells (MNC) in BALF. Significant differences were analysed using un-paired t-tests. n=3, ** indicates $p<0.01$, *** indicates $p<0.001$.

Figure 4. Pulmonary neutrophilia in BALB/c mice challenged with 5 mg/ml aerosolized LPS. BALB/c mice were exposed to 5 mg/ml aerosolized *P. aeruginosa* LPS or vehicle (saline, white bar) alone for 10 minutes. Bronchoalveolar lavage (BAL) was performed after 24 hr and the leukocytes were enumerated in BAL fluid (BALF). (A) Total cell number (TCN), (B) neutrophils, and (C) mononuclear cells (MNC) in BALF. Significant differences were analysed using un-paired t-tests. n=3, *** indicates $p<0.001$.

Figure 5. Pulmonary localization of neutrophils in LPS-challenged mice. Hematoxylin and eosin staining of formalin-fixed lung tissue from (A) C57BL/6by mice exposed to vehicle alone or (B) 1 mg/ml aerosolized LPS sacrificed after 6 hr or (C) 24 hr. Arrow indicates a neutrophil. Bar indicate 200 μm.

Figure 6. Increased total protein concentration in bronchoalveolar lavage fluid (BALF) of LPS-challenged C57BL/6by mice. Total protein content in BALF of mice challenged to 1 mg/ml aerosolized LPS or exposed to vehicle (saline, white bar) alone was measured by spectrophotometric analysis. Significant differences were analysed using un-paired t-tests. n=3-4, ** indicates $p<0.01$.

Figure 7. Increased expression of CXCL1 and CXCL2 in bronchoalveolar lavage fluid (BALF) of LPS-challenged mice. Expression of (A) CXCL1 and (B) CXCL2 in BALF of mice challenged to 1 mg/ml aerosolized LPS or exposed to vehicle (saline, white bar) alone quantified by ELISA. Significant differences were analysed using un-paired t-tests. n=3.

Discussion

Aerosolized LPS generates an inflammatory response in the airways, characterized by neutrophils in the epithelial submucosa, spaces surrounding the conducting airways, as well as the alveolar spaces. This is, together with the increased total protein content in BALF, indicative of plasma leakage, representative of the pathology of acute lung injury. As LPS induces a sterile inflammation, the reaction is independent of the adaptive immune response, and there are limitations to the relevance to bacterial infections. The technique may, however, be used to dissect inflammatory mechanisms by excluding adaptive immune responses.

Although the methodology is simple and easily adapted to answer different scientific questions, the choice of nebulizer and tubing is critical. The deposition of LPS and resulting neutrophilia must be validated with inlets, nebulizers and tubing other than what is described here. Furthermore, as less common mouse strains may display different responses to LPS, optimal doses of LPS should be determined for each strain. Moreover, the neutrophilic inflammation generated with aerosolized LPS is comparable with the inflammation induced by intranasal delivery of LPS, as observed by others [12,13]. Although intranasal administration easily is performed, the methodology requires anesthetics and could potentially introduce the microbial flora of the nasal cavity to the lungs, as the nasal cavity is not sterile and the technique requires a large volume of vehicle.

In addition to the relevance to acute lung injury, the technique may be further developed to include multiple challenges with aerosolized LPS. The methodology may thus be used to

study pathogenic mechanisms in the chronic inflammation of COPD, which is associated with persisting neutrophilia[14], together with reoccurring bacterial infections or permanent microbial colonization[15]. Thus, there is a particular relevance for the neutrophilic inflammation of LPS-challenged mice to the bacterial infections associated with COPD exacerbations, which are central for disease progression[16,17].

Challenge with aerosolized LPS can be set up at a small cost with few appliances and requires minimal training. Furthermore, the technique can thus be routinely performed on a large scale at any laboratory, with little or no variation between individuals and is thus superior to other routes of pulmonary delivery.

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Disclosure

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Figure 1

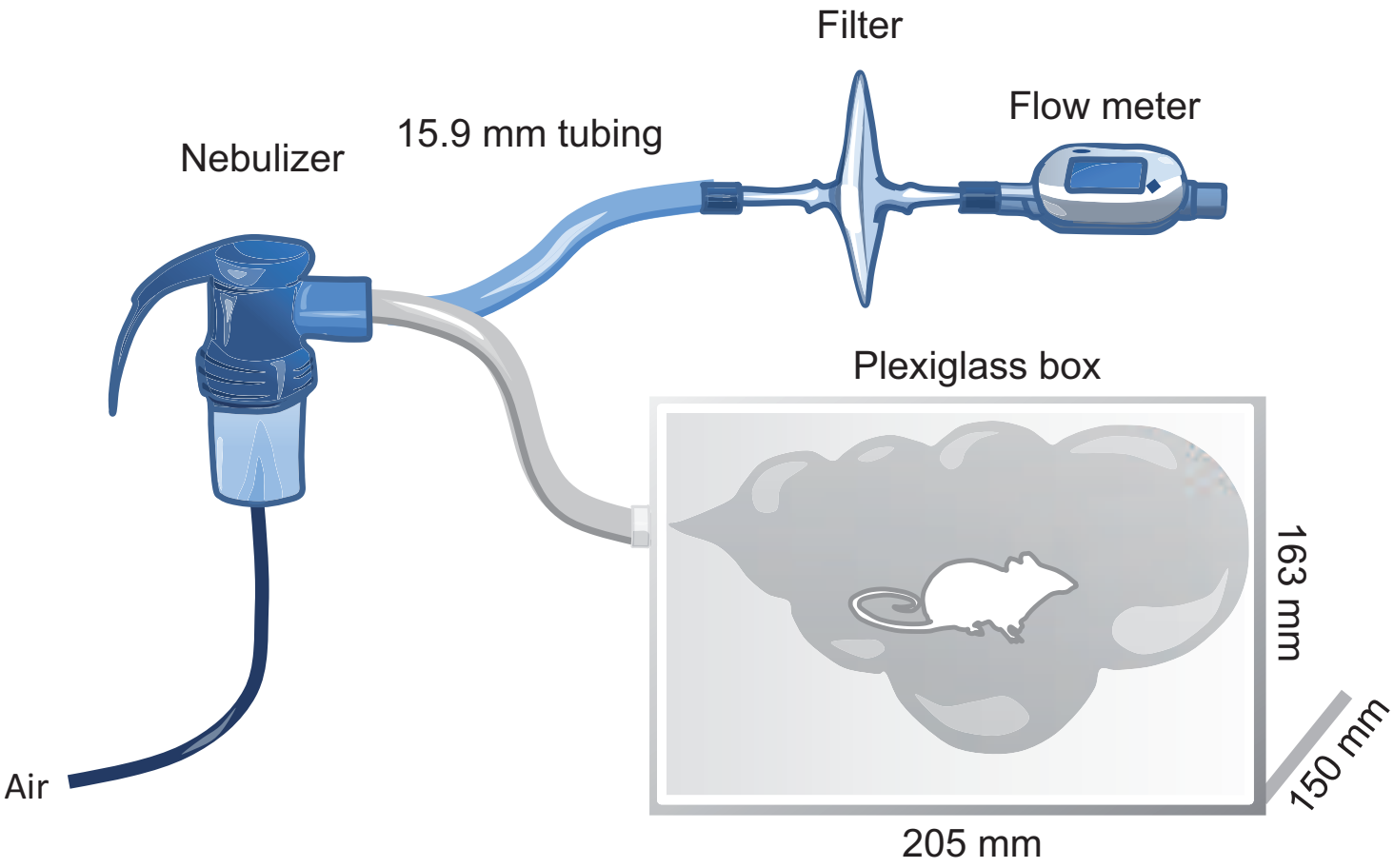

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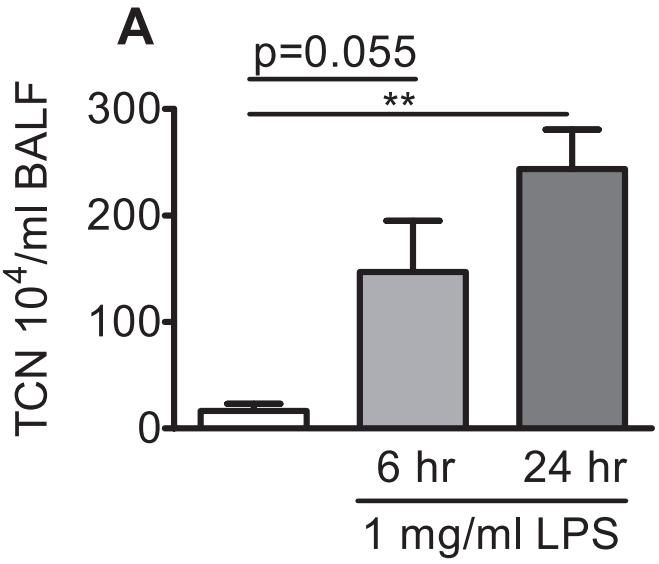
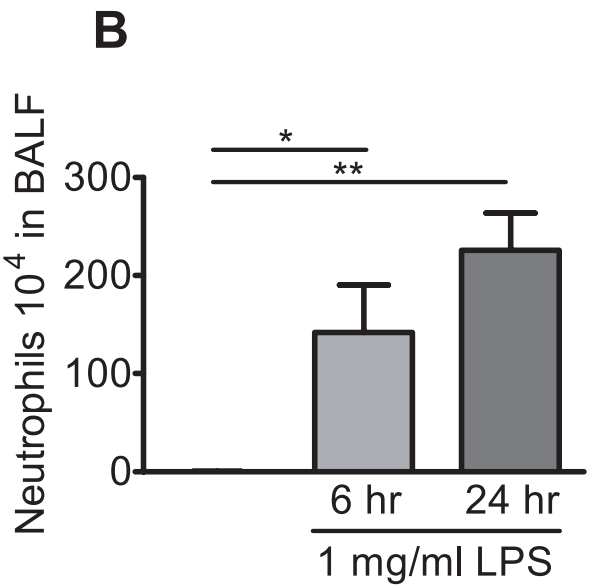
Figure 2





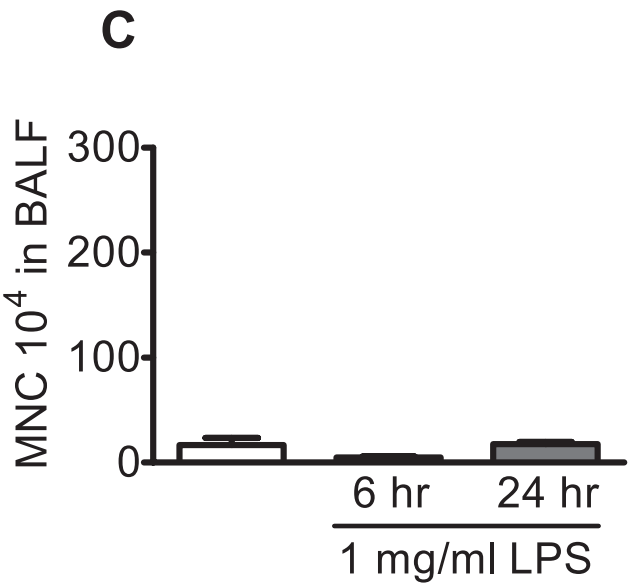


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Figure 3

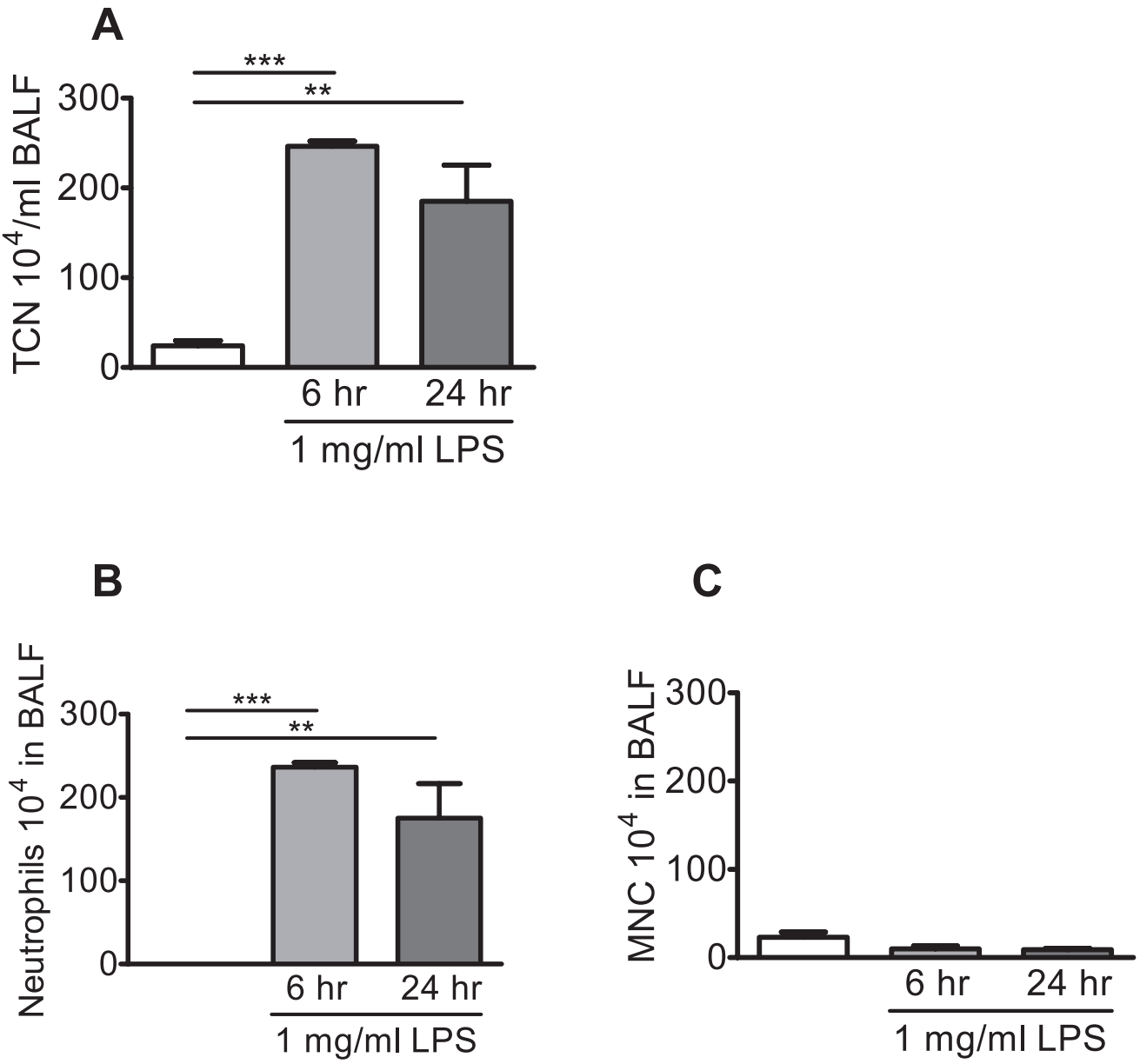

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Figure 4

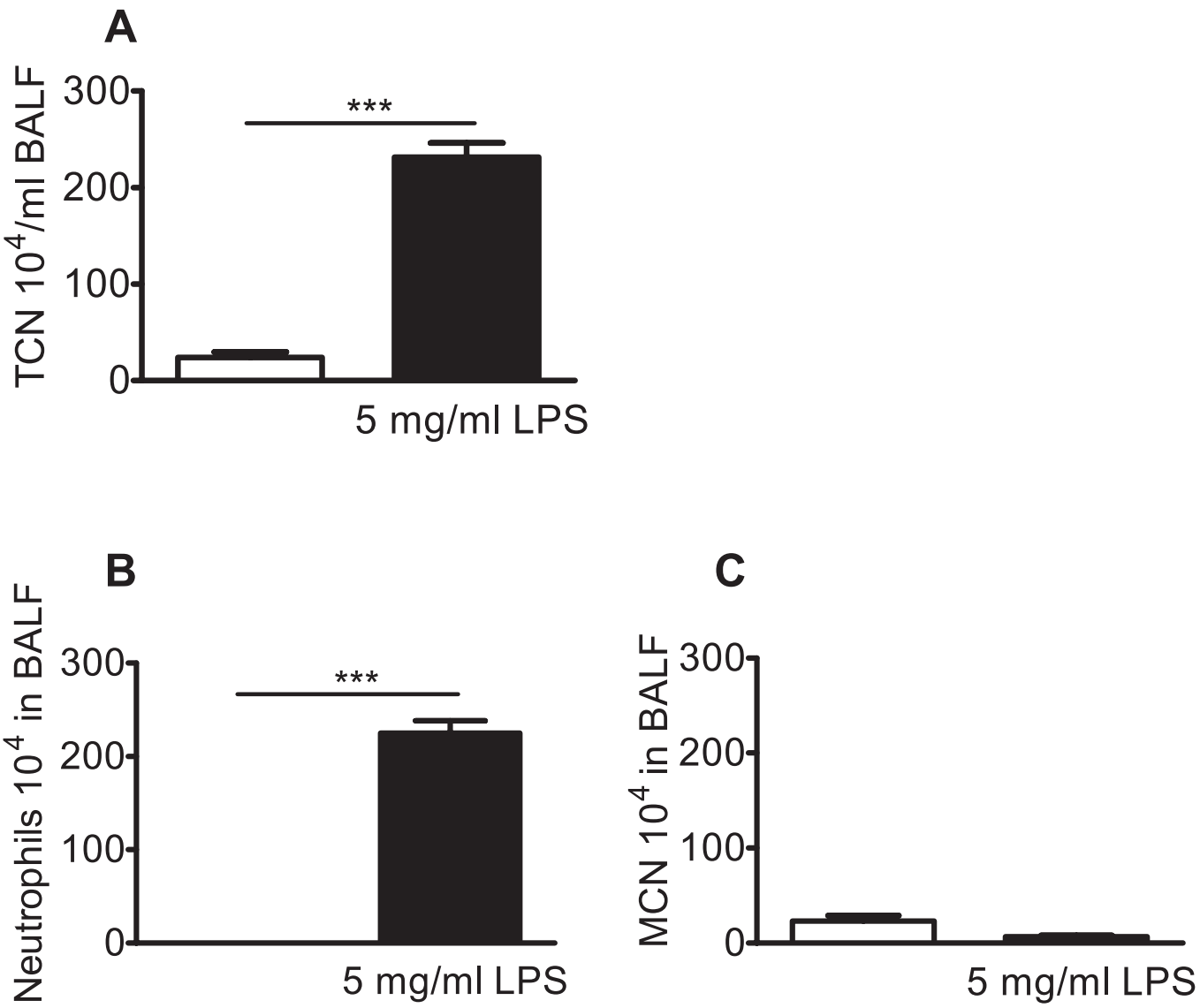

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Figure 5

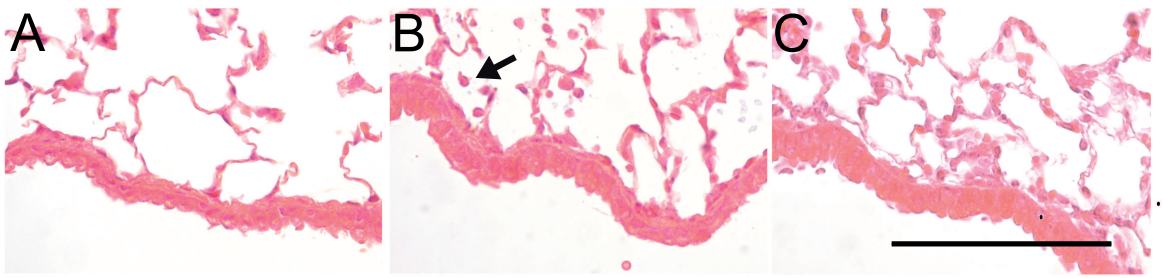

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Figure 6

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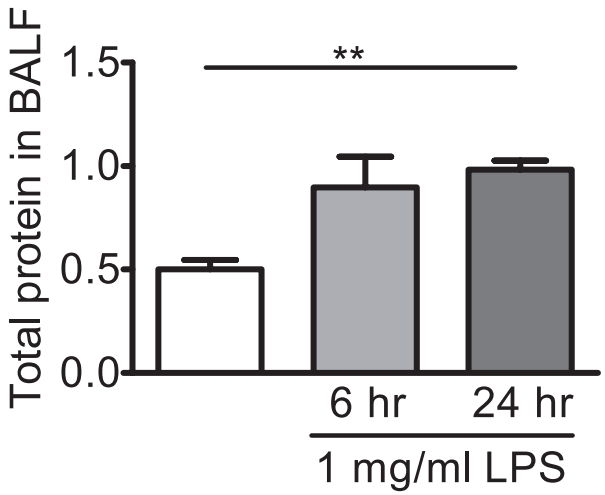

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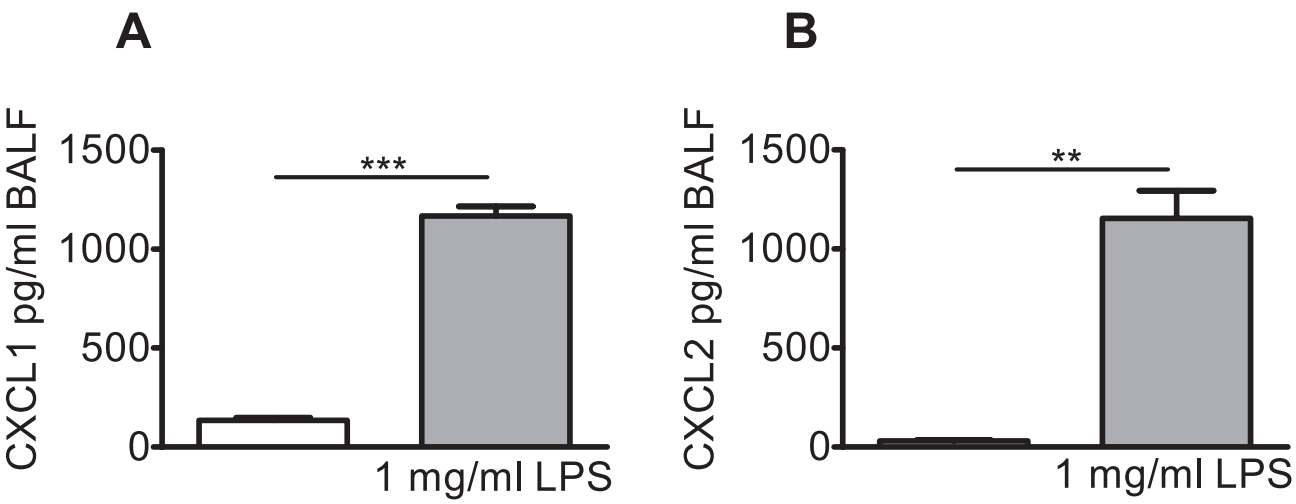

*Excel Spreadsheet- Table of Materials/Equipment
Click here to download Excel Spreadsheet- Table of Materials/Equipment: Materials JOVE_v1.xlsx

Name of the material/equipment
Purified *Pseudomonas aeruginosa* LPS
Pari LC sprint star nebulizer
TSI mass flowmeter 4040
Saint-Gobain 15.9 mm Tygon tube
Plexiglas boxes with removable lids
3M Half Facepiece Reusable Respirator
3M Advanced Particulate Filters (P100)
Sissors
Forceps
Intramedic PE50 polyethylene tube
Ethicon 2-0 Perma-hand silk tread
26 ½ gage needle
1 mL BD slip-tip syringe, non-sterile
60 mL BD Luer-Lok syringe, non-sterile, polypropolene
Fluka Hematoxylin-Eosin
Türk's solution
Table top centrifuge
Cytospin 4 cytocentrifuge
HEMA-3 stat pack
Formalin solution, neutral buffered, 10%

Company	Catalog number
Sigma-Aldrich	
PARI Respiratory Equipment Inc.	023G1250
TSI	4040
Sigma-Aldrich	Z685704
Custom built	N/A
3M	7503
3M	2291
VWR	233-1104
VWR	232-1313
BD	427411
VWR	95056-992
BD	301025
BD	301035
Sigma-Aldrich	3972
Merck Millipore	109277
Thermo Scientific	A78300003
Fisher Scientific	23-123-869
Sigma-Aldrich	HT501128

Comments/Description
Harmful. Recomended purification. LPS purified from other bactria may be used.
Alternative product from supplier may be used.
Recomended brand.
150 x 163 x 205 mm (a 2 mm hole on the side).
Recomended brand.
Recomended brand.
Preferred scissors may be used.
Preferred forceps may be used.
Recomended brand.
Recomended brand.
Alternative suppliers exist.
Alternative suppliers exist.
Alternative suppliers exist.
Alternative suppliers exist.
Alternative manufacturers exist.
Alternative centrifuge can be used.
Alternative staining kits exists.
Alternative suppliers exist.

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Author(s): A Roos, T Berg, K Ahlgren, J Grunewald, M Nord

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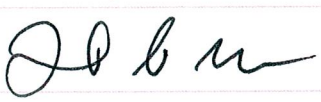
AUTHOR:

Name: Abraham Roos

Department: Karolinska Institutet

Institution: Dept of Medicine

Article Title: A Method for Generating Pulmonary Neutrophilia Using Aerosolized LPS

Signature: 

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Dear Dr. Zaman

We are grateful for the time and effort invested by the reviewers and editoral team at JOVE, and very pleased with the interest in our manuscript as well as the favorable outcome of the review. We agree with the reviewers that adding key pieces of data and making changes to how some data is presented, will improve the significance of the manuscript. Please see responses to specific comments below.

On behalf of the authors,

Abraham Roos

Editorial comments:

1) The formatting of your manuscript has been modified by your editor and minor copy edits were made. The following changes were made to your manuscript:

a) The word neutrophilia was incorrectly spelled as "neutrophila" in the title of your manuscript. This was corrected.

b) The JoVE format does not include a list of abbreviations. Therefore, this section was removed. The word counts from each section and the header at the top of the page were also removed.

c) Single-spaced text and 12 pt font was used throughout the manuscript and the margins were adjusted to 1 inch on each side.

d) The "Methods" section was re-named "Protocol"

e) Minor copy-edits were made to correct spelling errors e.g. steps 2.3.1 and 2.3.2: "silk thread"; steps 2.3.3 and 3.2.2: "23 gauge needle"

f) Figure Legends were moved to directly below the Representative Results section. Keywords were moved to above the Short Abstract.

R: We apologize for these mistakes and are grateful for the corrections.

2) Please maintain the current formatting throughout the manuscript. You can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions.

3) Please revise Figures 1 and 2 so that the panels are correctly labeled.

R: The correct labels have been inserted

4) Please disregard the comment below if all of your figures are original.

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Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Manuscript by Abraham Roos and al. proposes a model of aerosolized Lipopolysaccharide administration to study lung inflammation. Their readout comprises bronchoalveolar lavage fluid cell count and protein concentration and tissue histology.
The authors compare the advantages of the aerosol nebulization (local, non invasive) over LPS treatment by intravenous route (systemic) and intra tracheal (lot of practice required). However, authors may also need to mention and compare with intra nasal route, which is commonly used too (non invasive, no special equipment and easy technique).
Nevertheless, as stated by the authors, aerosol nebulization could be used on a large number of animals at the same time and with minimal variation between individuals.
Hence, the manuscript could deserve its publication in the Journal of Visualized Experiments once the following points have been addressed.

Major Concerns:
- The manuscript relevancy would highly benefit from the addition of data comparing the presented technique with intranasal or intratracheal administration of LPS at least in terms of neutrophil recruitment.

R: We appreciate that the reviewer brought this to our attention and agree that a comparison could be of value to the reader. As our current ethical permit and animal utilization protocol

does not allow for intranasal administration of LPS or the higher doses of LPS that are used with this route of delivery, and the limited time at our hands, we are however at this time unable to provide experimental data supporting similar neutrophilic inflammation with aerosolized and intranasal LPS. The process of amending an ethical permit is a very long process in Sweden. On behalf of this, we have included references to papers describing airway neutrophila with intranasal LPS. Please see line in results section (line 253) and in the discussion: "Moreover, the neutrophilic inflammation generated with aerosolized LPS is comparable with the inflammation induced by intranasal delivery of LPS, as observed by others [12,13]. Although intranasal administration easily is performed, the methodology requires anesthetics and could potentially introduce the microbial flora of the nasal cavity to the lungs, as the nasal cavity is not sterile and the technique requires a large volume of vehicle" on line 325.

- Figure 5 legend stated: "Also, the expression of the neutrophil chemoattrantants chemokine (C-X-C motif) ligands (CXCL) 1 and CXCL2 is increased in LPS-challenged mice." Please include the figures. In addition, well-established LPS mediated cytokine productions such as IL-6 and IL-1b measurement could be included.

R: We have included quantification of CXCL1 and CXCL2 by ELISA (please see Figure 7).

- A scheme presenting the experimental devices used for the nebulization would greatly help reader and even more scientist interested to reproduce proposed method (Protocol points 1.2.1 to 1.4.2).

R: This is an excellent suggestion. We have included an illustration of the equipment (Please see Figure 1).

- Due to expected massive neutrophil influx in the bronchoalvelar space following LPS administration, the % of mononuclear cells in this compartment dropped but the overall cell number could remain the same or even be increased. Presentation of data with total neutrophil and mononuclear cell numbers instead of percentage might clarify it.

R: We agree that making these changes will clarify that the number of mononuclear cells remains stable following LPS challenge (and that any changes would be very small in comparison to the massive number of infiltrating neutrophils). We do believe, however, that it is important to point out that there is a shift in the percentage of neutrophils and mononuclear cells. As this is already stated in the main text, we have changed the Figure 2, 3 and 4 to depict absolute numbers of cells.

- Number of mice per experiment, number of repeats and statistical significance should be included.

R: All experiments were performed at least twice. To implement the three Rs in our experiment, we included as few mice as possible in the analysis. Therefore, the number of

animals in each group is low, n=3-4 for BALB/c and n=3 for C57BL/6. Although we recognize that these groups are smaller than what is commonly used in experimental studies with animals, the limited number of mice and significant induction of neutrophils in both mice strains highlight the robust pulmonary neutrophilia generated by aerosolized LPS. The number of mice and statistical significance has been included in the figures and legends.

Minor Concerns:
- Abstract: please correct "neubulizer" by "nebulizer"

R: Corrected, we appreciate the comment.

- Why to protect LPS form dark?

R: Although the supplier does not recommend storing LPS in the dark, we always took precaution based on recommendations by the researchers at AstraZeneca R&D.

- Were truly the mice monitored every two hours after LPS administration when the end point was 24 hours later? Do you expect a lot of distress and or pain? Please explain

R: Mice did not display any clinical presentation and we agree that monitoring mice every two hours over night is excessive. We monitored mice during office hours, and then left them un-monitored over-night. We have changed the text to reflect this.

- The explanation for "multilobe" ("four lobes of the right lung") needs to be placed in protocol point 2.3.2 instead of 3.1.1

R: This has been changed accordingly.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The authors present a method to induce pulmonary inflammation by challenge to aerosolize LPS via nebulization. This technique is presented as a model for acute lung injury and an alternative to intra-venous injection and intra-trachael administration. It allows the user to generate neutrophilic inflammatory responses in respiratory airways without systemic involved. This method is widely applicable and this experimental model of LPS exposure will facilitate further investigation of fundamental aspect of human pathology.

Major Concerns:
I have no major concerns

Minor Concerns:

The article is well written. The introduction presents the context for the method within the field. The protocol is written clearly and concisely to be replicated properly, and the results demonstrate the expected outcome. The authors also mention steps that are critical to the success of the protocol in the discussion to further guide the user.

A few minor suggestions:
(1) The note of caution in 1.4.5 may be better placed earlier in the protocol to ensure that all users are appropriately protected.

R: We appreciate this suggestion. The note has been inserted at point 1.2.1.

(2) Add commas to numbers 1,000 and higher - for instance 200 000 and 500 000 in the results.

R: The text has been changed accordingly.

(3) Be consistent with italicizing P. aeruginosa in the manuscript.

R: We appreciate that this reviewer brought this to our attention and we have adjusted the text.

(4) Carefully proofread the text and correct any errors such as
- Line 83 "the primarily damage is to the endothelium,"

- Line 294 "arrow indicate a neutrophil"

R: The errors have been corrected.

Additional Comments to Authors:
N/A