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.  
  
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."   
  
  
**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*](#_ENREF_12)*,*[*13*](#_ENREF_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