Author's response to reviews

Title: Comparison of the effect of LPS and PAM3 on ventilated lungs

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Version: 2 Date: 23 February 2010

Author's response to reviews: see over
The authors would like to thank the reviewers for critical and instructive discussion on our manuscript. We addressed the reviewers’ comments point by point. We would like to point out that additional experiments (for LPS) were performed and that more lung function parameters are now included (e.g. $R_n$, G, H). These parameters were already measured during the experiments but were not included in the first version of our manuscript. For better interpretation of the data we compared lung function parameters between time of challenge (30 min) and end of ventilation (120 min). We hope that the revised manuscript will now be acceptable for publication in the journal BMC Pulmonary Medicine.

**Reviewer’s report**

**Title:** Comparison of the effect of LPS and PAM3 on ventilated lungs  
**Version:** 1  
**Date:** 30 September 2009  
**Reviewer:** Graeme Žosky

**Reviewer’s report:**

The authors compared the progression of ventilator induced lung injury (VILI) in mice exposed to lipopolysaccharide (LPS), as a model for gram-negative bacterial pneumonia, or PAM3, as a model for gram-positive bacterial pneumonia. VILI was assessed by monitoring lung mechanics and inflammation (neutrophils in the lung and cytokine mRNA production). Most previous studies have used intratracheal or systemic instillation of LPS prior to the initiation mechanical ventilation. This study is novel in that it directly compared responses to gram-negative and gram–positive bacterial products after ventilation had begun. These aspects make the manuscript of interest, however there are a number of issues that need to be addressed by the authors.

**General comments**

**Introduction – the content of the introduction is appropriate and the aims of the study are clear**

**Methods – the methods lack detail about critical aspects of the study and need to be clarified**

This has been addressed.

**Results – I had trouble assessing the lung function figures due to the small symbols, font and legend. This needs to be addressed.**

This has been addressed.

**Discussion - It is unfortunate that you did not use some of the more sophisticated measurements available with the flexivent system that can separate the contribution of the conducting airways and smaller airways (where gas flow occurs by diffusion) to lung resistance. The interpretation of the results also needs to be toned down as you do not have any data comparing responses to LPS and PAM3 in unventilated mice. These insults alone (in the absence of mechanical ventilation) could explain the differences in inflammation you observed.**
More lung function parameters including Newtonian resistance, tissue damping and elastance have now been included (Results section and Fig. 2). This is now discussed (page 10, lines 19-23).

**Major Compulsory Revisions**

Methods, animal preparation. The level of anaesthesia cannot be assessed without giving the doses of ketamine and xylazine (e.g. mg/kg) and the timing/criteria for giving additional doses of anaesthetic throughout the ventilation period.
This has been clarified (page 4, lines 5-8).

Methods, animal preparation, last sentence. What were the animals covered with and how did you determine the body temperature was being maintained?
A homeothermic blanket system with flexible probe was used (Harvard Apparatus, Holliston, USA) (page 4, lines 8-9).

Methods, protocols. I am surprised at the low respiratory rate (120 bpm) that you were able to achieve. Was this done in the absence of paralysis which is usually reported in studies using similar respiratory rates?
Anaesthetics were given until full mechanical ventilation without breathing of the animals was achieved. Paralysis occurred (page 4, line 6).

Methods, protocols. How long was the nebulisation period required to deliver the 50uL solution of LPS/PAM3? Which version of the Scireq nebuliser was used for this study?
Nebulisation period was 10 sec. The nebuliser was Ana-B1/8 (page 4, lines 21-22).

Methods, protocols, last sentence. Was any form of instillation/inflation (intratracheal or vascular) of fixative used or were the lung samples simply placed in formalin?
Lungs were simply placed in formalin.

Methods, lung function measurements, last sentence. What was the volume displacement of the “forced manoeuvre” used to measure resistance and compliance?
The volume was approximately 200 µl (page 5, line 11).

Methods, histological measurements. As per my previous comments – was any form of standardised inflation of the lung used for fixation?
All lungs were inflated to total lung capacity prior to fixation (page 5, line 17).

Methods, QRTPCR, sentence 2. Some justification needs to be provided for the choice of these cytokines in the context of mechanical ventilation and known responses to LPS and PAM3.
The information has been introduced (page 6, lines 9-10).

Methods, statistics. The description of the statistical methods used needs to be clarified. For example, serial measurements of lung function should be compared using repeated measures ANOVA – was this the case? Also, which statistical package was used to conduct the analysis? Please justify the use of SEM – in
most cases you are simply reporting the variability of measurements within an individual group of mice so you should report the standard deviation not the SEM.

Repeated measurements of lung function parameters were omitted. For better interpretation of data lung function parameters at time of challenge (30 min) and at the end of ventilation were compared (120 min). SEM was changed to SD. Statistical package is mentioned (page 7, lines 1-2).

Results, lung function parameters. What is your explanation for the significant increase in lung mechanics for the saline exposed group compared to the controls? Looking at the associated figure it seems to me that the increase is transient and returns to control levels. It was extremely difficult to understand which groups were represented on this graph due to the minute symbols and low quality legend.

Resistance increased over time of ventilation in all groups. To better compare and interpret the data figure 1 was edited and a new figure (fig. 2) was added. In figure 2 values are now compared between time of challenge (30 min) and the end of ventilation (120 min). This was done because resistance reached a plateau after 30 min of ventilation (page 4, line 22).

Results, cytokine expression. The fold increases in cytokine expression compared to unventilated animals are unclear in the figure as the mRNA expression in the controls appeared to be zero – I suggest changing these graphs to a log scale. Due to the substantial differences in variability between groups (and the apparent zeroes in the w/o group) the statistical comparisons made between groups here needed to be done on transformed data or an ANOVA on ranks – was this the case?
The graphs have been changed to log scale (fig. 3). An ANOVA on ranks was performed.

Results, neutrophil inflammation. See previous comments re: inflation during fixation. In order to compare these groups based on “fields” the level of inflation needs to be standardised. Even when inflation during fixation is standardised at the very least the cell counts should be standardised to the area of the lung tissue (mm2), not the field of view as you have done here.
The fields for counting were 0.25 mm2 (page 5, lines 19-20).

Discussion, second paragraph, last sentence. You cannot comment on the “synergistic” effect without having measured the response to LPS alone in unventilated mice.
This has been omitted.

Discussion, third paragraph. See previous comment – these interpretations of the results need to be discussed in light of the fact that you do not have data comparing inflammatory responses to PAM3 and LPS in unventilated mice.
This has been clarified in the text (page 9, lines 23-25).

Discussion, third paragraph, last sentence. Please expand on your discussion of the limitations of your results i.e. you did not measure cytokine production just mRNA expression.
Minor Essential Revisions

Introduction, paragraph 2, second sentence. Please insert the reference indicating the incidence rates of VAP in the ICU “…from 8% to 28%.”. The reference has been inserted (page 3, line 9).

Introduction, paragraph 3, first sentence. Insert comma after LPS and insert reference showing that gram-positive bacteria are important in VAP. This has been added (page 3, line 21).

Introduction, paragraph 3, last sentence. Delete “which has not been investigated very well until yet”. This has been deleted (page 3, line 24).

Results, oxygen saturation. Spelling of “oxygen”. This has been corrected (page 7, lines 22-23).

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests: I declare that I have no competing interests

Reviewer’s report

Title: Comparison of the effect of LPS and PAM3 on ventilated lungs
Version: 1 Date: 23 December 2009
Reviewer: Rosalinda Sorrentino

Reviewer’s report:
Major Compulsory Revisions:
1. Fig 1 is very hard to read and thus it is very difficult to follow the whole stating. Figure 1 has been edited and a new figure (figure 2) has been included to facilitate interpretation of results.

2. Mechanistic insights should be provided. This is now discussed (page 10, lines 18-23).

3. Number of animals for LPS treatment is very low. Additional experiments with LPS challenge have been performed and data have been included (page 4, line 24).

4. Protein levels for MiP-2a and TNFa (Figure 2) are needed because it is not true that the mRNA levels always correspond to the protein levels as the authors state into the discussion section.
The authors agree with the reviewer that mRNA and protein levels may differ. However, as stated in the text, previous studies already reported increased protein levels of cytokines following LPS challenge. Therefore we did not include protein levels. It is now clearly mentioned that this is a limitation of our study (page 9, lines 20-21).

5. Do the authors observe the same effect when they use FSL-1, another TLR2 ligand? This type of experiments should also be considered in order to distinguish between TLR2/1 and TLR2/6 implication in pulmonary resistance during lung ventilation.
This is a new set of experiments we would like to address in the future. In previous experiments we found increased expression of TLR-2 mRNA after stimulation with either NaCl, LPS or PAM3 compared to ventilation alone. Since it is known that ventilation increases TLR-2 expression we would like to further evaluate the TLR-2 pathway and its possible implication in lung function.

6. Do the authors have longer time point experiments? Do they observe the same effect?
Some additional experiments were performed with longer time points (3h and 4h). The effects of LPS and PAM3 remained the same.

Level of interest: An article of limited interest
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests