Reviewer's report

Title: Low-dose endotoxin inhalation in healthy volunteers - a challenge model for early clinical drug development

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Reviewer: Neil Alexis

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Manuscript Review for BMC Pulmonary Medicine

Title: Low-dose endotoxin inhalation in healthy volunteers – a challenge model for early drug development

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Summary

Janssen et al performed 3 repeated low dose LPS (GMP grade 20,000 EU LPS/CCRE) inhalation challenges in healthy human volunteers to examine the reproducibility of the LPS-induced airways inflammatory response, specifically the neutrophil response. A baseline sputum with no LPS challenge was performed 2 months prior to the first LPS challenge to determine the ability of each subject to produce an adequate sputum sample, and a fourth follow-up sputum (without LPS challenge) was also conducted several months following the last LPS challenge. The purpose of the study was to determine the feasibility of using the inhaled LPS challenge as a model for early drug development for inflammatory based respiratory diseases like COPD and asthma. Prior to the third LPS challenge, an anti-inflammatory drug used to treat COPD (oral administration of Roflumilast) was introduced to determine its modulatory potential on the LPS-induced inflammatory response. Induced sputum (plug selection method) was performed 6h following LPS inhalation exposures and sputum markers of cellular and biochemical inflammation were evaluated as the primary endpoints. Systemic markers of inflammation were also evaluated in blood pre and post LPS inhalation as was exhaled breath temperature. Flow cytometry was also assessed on sputum derived cells to measure cell surface marker expression. LPS inhalation was administered using a controlled inhalation bolus delivery technique that controlled the flow rate and inhaled volume of the LPS delivery. The authors report a significant increase in percent neutrophils following each LPS challenge compared to baseline, with the largest response occurring after LPS1. PMN responses following LPS2 and LPS3 were very similar to one another and smaller than LPS1. A noted increase in monocytes and small macrophages was observed following LPS2. The authors report that the PMN and cumulative inflammatory responses were reproducible, while Roflumilast had no significant effect on inflammatory cell composition. Since LPS inhalation challenge in humans is being considered as a model to induce
transient neutrophil influx in the airways, most notably by Pharma for early phase
drug development, this study is important and timely in that it provides some
necessary information on within subject reproducibility and between subject
variability. The following points need to be addressed in order to enhance the
quality of this manuscript.

Comments:
1. Background: the authors need to clarify whether their rationale for performing/
recommending low dose LPS inhalation challenges is due to possible side effect
issues or limited availability of GMP grade LPS, or both – as written, it is not clear
which point the author is making.
2. Background: The authors used a controlled inhalation delivery technique (vs
uncontrolled inhalation) to administer the LPS to the lung, the purpose of which
was to augment LPS deposition and hence maximize the inflammatory response
generated. The delivery parameters however, (10 second inhalation cycle,
150ml/s flow rate; first 5 seconds inhale 750ml of air with LPS followed by 2
seconds of 300 ml air only, followed by a 3 second breath hold) support a
preferential deposition of the aerosol (particle size depending) to the
“peripheral/deep airways”, a location in the lung that sputum does not sample
(Alexis, AJRCCM, 2001). Indeed, the author’s demonstrated a robust
inflammatory response in sputum resulting from their use of this technique, but
this was likely due to the minimal loss of aerosol out of the mouth which this
procedure would have achieved, and not from preferentially depositing the
aerosol to the location of interest (central airways). The authors are referred to
the work by Alexis et al (AJRCCM, 2001) and more recently Zeman et al (J
Aerosol Med and Pul Drug Delivery, 2010) for delivery parameters that promote
deposition to the large central airways. As written, it is misleading for readers to
potentially interpret that the success of the inflammatory response was due to the
delivery technique’s inhalation parameters. Although the authors do not explicitly
state this, it can be inferred or interpreted this way. The authors should
acknowledge that their inhalation procedure was not designed to target the
central airways but rather the main advantage was to eliminate aerosol loss from
the mouth region. Furthermore, deposition location in the airways depends on
particle size. The authors should state the size of the aerosol particle that was
emitted from the nebulizer if they characterized it. If they didn’t, they should at
least state the nebulizer manufacturer’s specs of MMAD = 3.5 microns, which if
true, would definitely promote peripheral airways deposition and not central
airways.
3. Methods: Was cell viability included in the baseline sputum eligibility criteria?
4. Why was < 50% PMN used as an exclusion cut off level at baseline? Is there a
clinical rationale for this? If so, are there references to support this? Many
healthy individuals have >50% PMNs. Can the authors defend that this does not
introduce a potential subject selection bias?
5. Study Design: Include pre-challenge endpoints in the text as shown in Figure 2
6. Why was blood not sampled after visit 3? There is no reason given.
7. Pg 5, 6th line from bottom: “function” is not spelled with a “k”

8. Sputum Analysis: identification of monocytes and small macrophages in sputum can be difficult using just microscopy – since the authors also performed FACS analysis, they had the opportunity to confirm/compare their microscopy results with their FACS results when determining the % of monocytes and small macrophages – please include the FACS population percentages for monocytes and small macrophages.

9. Flow Cytometry of Sputum Cells: please include in the text the antibody combinations that were used to identify specific leukocyte subpopulations.

10. Was CD45 (pan leukocyte) used to identify and differentiate inflammatory cells from debris, dead cells? The authors are referred to Lay et al. Flow cytometry of sputum: assessing inflammation and immune response elements in the bronchial airways. Inhal Toxicol. 2011 for a detailed explanation of flow gating strategies for sputum leukocyte identification

11. How were the isotype control values used to determine the MFI? were they subtracted from their respective MFI values?

12. Statistical Analysis: include “arithmetic and geometric” before the word “mean” in the first sentence since both were used.

13. Results: Systemic Effects of LPS: pg 9. Reference your figure or text (pg 5) that “pre-challenge” values were performed.

14. Airway Inflammation Induced by Low Dose LPS Challenge: second line – “….easier for subjects” – than what?

15. The “cumulative inflammatory response” requires more explanation if it is going to be used as a metric of inflammation – are there references for its use? If so, they need to be included. What is the rationale for only including neutrophils, monocytes and small macrophages? Why aren’t other cell types included?

16. I would like to see a table that included the side by side comparison of microscopic data and flow data for differential cell counts – this is valuable information for those using flow cytometry in sputum.

17. Although monocytes and “small macrophages” were grouped and counted together as one CD14+ve population, the authors should be clear about the fact that these are not one in the same cells, as they have different functional capabilities and express different surface phenotypes from one another. Earlier work by Alexis and colleagues (JACI, 2005) demonstrated the presence of cells in sputum after LPS inhalation that were monocyte-like but were in fact mature DCs (mDCs). Like reported here, these cells expressed elevated levels of HLA-DR and CD86, both markers of DC maturation. Furthermore, that study also showed that monocytes and macrophages had the ability to express DC-like surface phenotypes following LPS, so the cells identified in this study as “small macrophages” may be mDCs described previously by Alexis and colleagues.

18. Pg 14, 3rd paragraph, 5th sentence: “However, if a change in the proportion of different cell types…….potentially decreased” – this statement does not make sense. One can determine which cell type changes from examining the
differential cell count and one can determine changes in cell number from knowing the total cell count – so I’m unclear what the authors are saying here.

19. The 10.8 percentage point decrease in PMNs after LPS2 was only partly due to the increase in monocytes (4%) as stated by the authors. The authors should add that the majority of the change was due to the 6.8% increase in macrophages.

20. Re: Tolerance: the fact that there is a 28 day interval between exposures makes tolerance somewhat unlikely, so I would suggest toning down the discussion that tolerance is occurring.

21. Figure 2 shows that the follow-up sputum occurred at least 56 days after the last LPS inhalation/sputum. However, the text in the Discussion (pg 15) states a median of 111 days (3.7 months) elapsed between late summer and early spring. Late summer to early spring is 6-7 months apart. Can the authors clarify when the follow up sputum occurred for each of the 11 subjects? These results should be entered in Table 2.

22. Re: Roflumilast treatment: since the authors state that this drug showed little or no effect (ability to modulate PMNs) in humans with a 2 or 4 week treatment period, I’m unclear why they expected a 5 day treatment to work.

23. Can the authors speculate what the drug may have done to pre-exposure levels of PMNs? Is it not possible that the drug lowered baseline PMN levels prior to the third LPS exposure and therefore a robust PMN response did in fact occur but was masked by a transiently lower baseline due to the drug?

24. I’m unclear of the significance of the exhaled breath temperature finding. The authors do not devote much if any discussion to this endpoint. I suggest more explanation or deleting it from the paper.

25. The authors should offer a biological explanation for their CD86 and HLA-DR findings. As written, it appears their value was to prove subject compliance of the drug. This is an unsatisfactory use of this endpoint especially since there is published data for these endpoints in humans following LPS inhalation.

26. Table 2: the authors used arithmetic means for the % cells and geometric means for the number of cells. What was the rationale for this? Was there an issue with high variability in cell number ranges?

27. Table 2: LPSTx vs LPS1 for %PMN was statistically significant – LPS2 is essentially identical to LPSTx and even has a lower SEM than LPSTx, but was not statistically significantly different than LPS1 -how is this possible?

28. Figure 3: scatter plots showing individual data would be more meaningful since mean data are already shown in Table 2

**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: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

no competing interests to declare