>First and foremost we would like to extend our gratitude to you for the constructive criticisms given by the reviewers. We have addressed all issues that were brought up by the reviewers and we feel that our manuscript has greatly profited from these modifications.<

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

The manuscript by Jung et al. summarizes a protocol of their recently published study in PLoS Pathogens 2016, in which the effect of outer membrane vesicles (OMVs) isolated from Legionella pneumophila (L. pneumophila) on monocyte-derived cells as a model of interaction of this pathogen with alveolar macrophages in the lung was studied. The topic is very important, the concept of OMVs as bacterial messengers and modulators of host-microbe interactions is fascinating, and the protocol is indeed timely as it further alerts scientific community about this important problem and provides a straight-forward way to approach it. However, there are a number of questions, which should be addressed in order to make this protocol a meaningful resource and high quality reference material for other investigators.

*Major Concerns:*

1. More attention should be paid to definition of the macrophage (considering recent advancements in macrophage nomenclature). THP-1 cells and THP-1-derived cells are unlikely macrophages in this classical sense, unless the authors provide evidence that they have features of alveolar macrophages, the target cell for this pathogen. It is unclear why mouse bone marrow-derived macrophages were proposed to be used? Do they represent a model for L. pheumophila host-pathogen interactions? Are they similar in any way to alveolar macrophages? Given than alveolar macrophages are the primary target cell for L. pneumophila relevant to disease pathogenesis, and these cells can be easily isolated from the lung by bronchoalveolar lavage, it is unclear why the authors did not use these primary cells? Description of this methodology will significantly increase the impact and clinical relevance of this protocol.

>Thank you very much for putting forth your concerns. We took great care to address these issues adequately by including a paragraph in the discussion section to address these questions. Isolation of alveolar macrophages is only easily done in mice and even there the obtained amount of cells is limited. We wanted to keep the amount of mice per experiment to a minimum, so we refused to do it in this experimental setup.<

2. Since interaction of L. pneumophila-derived OMVs with macrophages is the focus of this protocol, could the Authors provide more insights into how these vesicles are detected by macrophages prior to engulfment. Does this process involve innate immune recognition? Can this be studied and, most importantly, modulated therapeutically.

>We appreciate the reviewer’s indication and included information in the introduction and comments in the discussion section. Therapeutically, OMVs might be used for vaccine development in the future, as it is already possible for *Neisseria meningitides* OMVs (Boxsero, Novartis, <https://www.novartis.com/news/media-releases/novartis-bexsero%C2%AE-vaccine-approved-fda-prevention-meningitis-b-leading-cause>). <

3. The protocol will benefit from adding notes related to the specific purpose of each step (i.e., what is the goal of each specific procedure in terms of generation, isolation, purification, quality control, quantification of OMVs). If some steps are original or represent modification of previously published protocols, please more emphasis on those steps and explain the innovation. If the procedures have been previously described, please specify and provide references. Since some major OMV methodological papers have been published (Jäger J and Steinert M., Methods Mol Biol 2013; Chutkan H et al, Methods Mol Biol 2013; Klimentova et al, Microbiol Res, 2015), it would be important to mention these protocol, as well as discuss what similarities and differences (particularly with Jäger J and Steinert M., Methods Mol Biol 2013).

>We appreciate the reviewer’s indication and included information in discussion section.<

4. Discussion of naturally secreted (native) and induced (bioengineered) OMVs would be important, and different procedures are needed to obtain these different types of OMVs. Moreover, they may have different composition and biological properties. Have the Authors compared these two types of OMVs? Even if they haven't, this should be discussed in the protocol. How closely stress-induced OMVs mimic in vivo lung environment?

>Following the reviewer’s suggestion, we added a comment on that in the discussion section. As it is very challenging to obtain a bronchoalveolar lavage from a patient with Legionnaire’s disease, we unfortunately could not investigate the effects of in vivo generated OMVs so far despite the indisputably great scientific interest. An alternative might be to obtain OMVs from patients in other disease circumstances.<

5. Given that expected size of OMVs is up to 300 nm, a 0.22 μm sterilization filter (step 3.2) might capture larger OMVs and lower the yields. Have the Authors consider this possibility and, if so, is there any way to overcome this potential problem?

>Thank you very much for putting forth your concerns. We addressed this in the discussion section. We know that this filtration step leads to the loss of the largest OMVs (>0.22µm), but we wanted to ensure that the obtained OMV preparation is bacteria free. A bacterial contamination of the OMV pellet would reduce the comparability and accuracy of the performed experiments.<

6. It is possible that a total amount of OMV in the media is low. Would a subsequent pre-concentration step be required?

>Thank you very much for putting forth your concerns. We addressed this in the discussion section. We know that an additional concentration step is possible, even though we never performed it. When the OMV yield was lower as expected, we did not use it for further downstream experiments, as we considered this preparation to have failed. Changes in *Legionella pneumophila* metabolism due to e.g. media composition, temperature changes, etc might have caused the low OMV yield, but for the moment we chose not to analyze this in our experiments.<

7. It is unclear to a non-experienced reader how unwanted extracellular materials that may potentially contaminate OMV preparations (pili, flagella, fimbria, pili, large protein complexes or aggregates) will be removed to ensure that they do not contribute to unexpected immunomodulatory effect of OMVs, such as pro-inflammatory response in macrophages that the Authors propose to evaluate. Would any additional purification steps be necessary (such as density gradient centrifugation or gel filtration)?

>Thank you for your suggestion. We changed the manuscript accordingly, by adding a paragraph in the discussion section.<

8. OMV quantification requires more details. What methods the Authors would suggest? What yields that would consider satisfactory in order to proceed with the protocol? Would any troubleshooting be needed depending on quantification results?

>Thank you very much. We added the method of choice (bicinchronic acid assay) in the manuscript, but it is also possible to perform a Bradford assay to quantify the protein amount. The expected yield is added in the “NOTE” at the end of paragraph 3. As mentioned above, a lower yield was expected as a failed experiment and OMVs were discarded.<

9. Step 4. Pre-treat macrophages. Appropriate terminology should be used to accurately describe these cells. Note that some readers (particularly students and junior investigators) would consider this article as a "gold standard" textbook protocol and their future understanding of science might be altered if the terminology is used improperly. The authors should explain that this a leukemia monocytic cell line, used as a model; explain the reason why PMA is used; how to validate they are macrophages after PMA stimulation; can PMA affect the ability of cells to respond to OMVs; why these cells can be utilized as a model. References to previously published literature containing evidence-based answers to these questions may be used, but explanations are needed.

>Thank you very much for putting up your concerns. We changed the manuscript accordingly and provided literature in the discussion section stating that THP-1 cells are an appropriate model cell line for *L. pneumophila* studies and also for the use of mBMDM.<

10. Step 4.1.2. Why the media should be high-glucose and contain 10% FBS? Does this mimic the environment of alveolar macrophages? Have the Authors observed similar effects using serum-free medium?

>Thank you for this indication. We used RPMI 1640 supplemented with FCS as this is the suggested media by the provider. THP-1 cells do not proliferate without FCS. We did not perform stimulation experiments without FCS, as it might be that the change in media composition is altering the ability of THP-1 cells to adequately respond to the applied stimuli. The restriction of growth factors by serum-free media was not in the scope of this study.

The composition of the alveolar environment is of course different from commercially available media. It is not trivial to find the exact same composition as the alveolar lining fluid. Currently we are trying to mimic the composition of the alveolar lining fluid to better recapitulate the *in vivo* setting.<

11. If LPS is deactivated in the media containing OMVs, would THP1-derived cells show comparable pro-inflammatory response to OMVs?

>Thank you for this comment. We do not expect that we co-purified free LPS shed by the Legionella, as the purification of this LPS includes several concentration steps of the bacteria-free media. Additionally, the OMVs present there are discarded. The flow-through of 100kDa cut-off filters is used for further concentration in 10kDa cut-off filters. The concentrated retentate from the 100kDa filters contains the OMVs (described in Lück, C and Helbig, JH; Methods Mol Biol 2013). Furthermore, accidentally co-purified LPS might again be lost in the second round of ultracentrifugation (step 3.3). This PBS washing steps is improving the purity of the obtained OMV pellet, as protein aggregates and LPS are once more depleted.<

12. What is the source of flagellin-lacking L. pneumophila mutant?

>We apologize for this inconvenience and added this information in the included table.<

13. Apart from the parameters of host-pathogen interactions described by the Authors in the original paper in PLoS Pathogens, what other important aspects of innate immunity and microbial pathogenesis can be studied?

>Thank you for your suggestion. We included this in the discussion section.<

14. Discussion of limitations of the protocol and alternative approaches is missing.

>Thank you for this indication. We have added this in the discussion section.<

15. Discussion of therapeutic relevance of studies using this protocol would significantly increase the impact of this paper.

>Thank you for this indication. We have included information on this.<

*Minor Concerns:*

1. Title: Influence on what? (perhaps, should be specified; or re-phrased)

>Thank you. We have changed this accordingly.<

2. Abstract (2nd sentence) and Introduction (line 92): using the phrases "cannot only" and "also" makes it difficult to understand the meaning. "Their role" should be changed to "the role of OMVs"

>Thank you. We have changed this accordingly.<

3. Abstract: "Furthermore, the reaction of macrophages to a subsequent infection with L. pneumophila was studied by colony forming unit assays" ("the reaction of macrophages" - too broad term for such a specific assay; please specify)

>Thank you. We have changed this accordingly.<

*Additional Comments to Authors:*

N/A  
  
  
**Reviewer #2:**

*Manuscript Summary:*

Manuscript describes the method of isolation of OMV of Legionella pneumophila, and analysis of the pro-inflammatory effect on certain macrophages

*Major Concerns:*

N/A  
  
*Minor Concerns:*

Title: Analyzing the influence of Legionella pneumophila outer membrane vesicles  
Suggestion: Legionella pneumophila outer membrane vesicles: isolation and analysis of their proinflammatory potential on macrophages.

>Following the reviewer´s suggestions, we have changed the title.<

SHORT ABSTRACT:

We describe here the purification of Legionella pneumophila (L. pneumophila) outer membrane vesicles (OMVs) from liquid cultures. These purified vesicles were then used for the treatment of macrophages to analyse their proinflammatory potential.

>Thank you. We have changed this accordingly.<

L68 L. pneumophila > Legionella pneumophila

>Thank you. We have changed this accordingly.<

L96 macrophages, even though its natural host are freshwater amoebae. L. pneumophila > Legionella pneumophila

>Thank you. We have changed this accordingly.<

L123 Fill up to 1 L with distilled water and sterile-filter it; followed by filter sterilization (

>Thank you. We have changed this accordingly.<

L130 Add 10 mL L-cystein (0.4 g in 10 mL distilled water) and 10 mL Fe(NO3)3x9H2O (0.25 g in  
10 mL distilled water) to cooled BCYE. How were these solutions sterilized? Explain. L-cystein >L- cysteine

>Thank you. We have changed this accordingly.<

L133 Spread L. pneumophila strain Corby (wildtype) on BCYE agar plates > (wild type) also at other places

>Thank you for this indication. We have changed the manuscript accordingly.<

L138 agar plate. Incubate over-night at 37 °C. > overnight

>Thank you. We have changed this accordingly.<

L148 supernatant in fresh centrifuge tubes, discard the bacterial pellet and centrifuge again (4,000 x g for 20 min). Transfer supernatant, discard bacterial pellet and repeat centrifugation (4,000 x g 150 for 20 min).

Better supernatant in fresh centrifuge tubes, discard the bacterial pellet and centrifuge again (4,000 x g for 20 min). Repeat this step once.

>Thank you. We reworded this accordingly.<

L204 Count formed colonies by eye > Count formed colonies at sight

>Thank you for this indication. We have changed this.<

L209 to analyze their influence on macrophages > to analyze their influence on pro-inflammatory response of macrophages

>Thank you for this comment. We have changed the sentence.<

L215 L. pneumophila (figure 4 A). The impact of TLR signaling explain abbreviation TLR > Toll like receptor (TLR) signaling

>Thank you, we have changed this.<

L226 Scale bar represents 2.5 cm. There is no scale bar in this figure

>Following a suggestion by the editorial office, we included a scale bar at figure 1B to highlight the size of the ultracentrifugation tube and the obtained OMV pellet.<

280 DISCUSSION:

281 OMVs of Gram-negative pathogens and the impact of these membrane vesicles on their target cells are currently studied intensively. For example, Clostridium perfringens-derived OMVs: remark Clostridium perfringens is not a Gram-negative bacterium

>Thank you for this comment. We rephrased this sentence.<

L284 remark: Borrelia burgdorferi is not classified as a gram positive or gram negative bacterium

>Thank you for this comment.<

Fig. 2, 3 and 4 have been used in another paper of the same authors (PLoS Pathog 12(4): e1005592). Reference has been given in the text but not at the figure itself. No direct reference to this figure in the current manuscript. Copyright??

>The figures 2-4 are already published in PLoS Pathog 12(4):e1005592 as you correctly mentioned. The figure legends have this reference included. (Re-print with permission from 19.)<