

An outer membrane vesicle vaccine prevents lung pathology in a macaque model of pneumonic melioidosis

Corresponding Author: Professor Lisa Morici

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is an interesting and important paper which demonstrates that Rhesus macaques which were immunised with OMVs were protected from *Burkholderia pseudomallei* infection in an inhalation model. In the context of development of any vaccine against melioidosis this is a landmark study. The authors evaluated the protective efficacy of their OMV vaccine against inhalational melioidosis in rhesus macaques, a highly translational preclinical model. They assessed survivability, biotelemetry data, and pathology outcomes to determine vaccine effectiveness. They showed that the OMV vaccine is protective against inhalational melioidosis in macaques. Additionally, they employed serologic assays to measure systemic and mucosal IgG and IgA responses, linking protection to the most immunogenic surface antigens and polysaccharides reactive to IgG and IgA. However, while there is clear evidence of protection in 80% of animals up to 14 days, more information / discussion on the cellular immune responses and mucosal immune responses would strengthen this manuscript.

Specific points.

Major comment.

The study focusses on the humoral responses predominantly. While antibodies are recognised to contribute to protection, it is well recognised that Interferon gamma secretion correlates with survival in humans (Jenjaroen et al., 2015), but this is largely overlooked in this paper. This bias should be addressed in the introduction and discussion. In addition, the authors should present all available data and explain why more cellular responses were not evaluated. In addition, more non-LPS specific IgAs in BAL data should be presented.

Minor comments.

Line 119 insert "the" before study.

What is the rationale for immunising with two doses. Would they have observed better protection with a second boost?

Line 139 – due to the death of one animal in the immunised group, survivability data are present through to day 14 only. This is a very short timeframe. Although not explicitly stated, it is apparent that all animals were not killed on day 14 and if this is the case, the authors should show a more extended timeline beyond that date with the remaining mice (even in supplemental data). It would be very informative in the context of long term protection and would be maximise the data available from the surviving animals from an animal ethics perspective

Line 146 It is not clear why the Weight loss/gain data was not shown. Can it be included in supplemental data?

Line 166 and Figure 2 legend. The legend should be revised and clarified. It does not describe the C) or D) components presented in the figure.

Line 179. One animal exhibited anergy. How do the authors know the reason was T cell anergy and not exhaustion or senescence? Were markers for T cell anergy evaluated? If not, it should change it to "exhibited no or little response".

Discussion. The study focusses on the humoral responses predominantly. It is unfortunate that more cellular immune response parameters were not evaluated.

Line 252 – This discussion should include the seminal papers from the Dunachie group that compared cellular responses in survivors versus patients that died from melioidosis (Jenjaroen et al., 2015 and Ronkard et al., 2020).

Line 253. Comma missing – yet,

Line 261. Indicate that. (insert that).

Line 264-266. It seems quite apparent that the main distinguishing feature between the surviving immunised animals and the non-survivor was the presence of multiple antigen specific sIgA in the BAL (rather than serum IgGs). Can the authors comment on this? Mucosal IgA has not been adequately considered in the discussion "perhaps mucosal IgA".

Line 286 to 292: Perhaps the abundant LPS signalling via TLR4 is driving a more Th2-skewed response. Can the authors comment on whether incorporating a T cell-specific adjuvant might help address this issue by promoting a more balanced response or a Th1-biased immune response.

Figure 2 – The graphs are quite small and the numbers in the x axis are very difficult to read. Removal of the key from all but one figures could improve the available size and allow presentation of larger figures.

Figure 3. Can the authors show more data on the IgA antibodies against other antigens other than LPS-A and comment on their presence / absence in immunised versus sham animals? .

Line 282 – "Several other antigens of interest were detected by protein microarray that were not available for the study in the BurkPx assay" can the authors elaborate on what these are?

Methods:

The statistical analysis section is quite vague. While it is clear that GraphPad was used, can the authors detail what tests / post tests were used for the individual datasets presented within the methods?

Supplemental Figure S2 As with Figure 2, the legend should be revised and clarified. It does not describe the C) or D) components.

Supplemental Figure S4 – includes "lorem ipsum" on the figure. This should be removed.

The authors should comment on the variability of the secretory IgA responses in BAL, relative to the IgG in BAL, and discuss the potential correlates of both isotypes, given that the non- survivor showed reasonable IgG against at least 3 antigens. .

Table 2 – BPSL1743 is ArcA, not Arg

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

Previous studies on *Burkholderia pseudomallei* outer membrane vesicles (OMVs) have demonstrated that OMV-based vaccines can provide significant protection in murine models and cross-protection against inhalational glanders in mice and non-human primates. In this manuscript, Baker et al. present, for the first time, evidence that the OMV vaccine also confers protection against inhalational melioidosis in rhesus macaques. Rhesus monkeys vaccinated with the OMV vaccine demonstrated an increased survival rate, reduced lung inflammation, and a slower progression of clinical signs. The survival of these non-human primates was associated with significantly elevated levels of both systemic and mucosal IgG and IgA antibodies targeting OMV components, particularly LPS, OmpA, and FlgK proteins. A correlation between elevated levels of anti-LPS and anti-OmpA antibodies and survival has also been observed in the serum of melioidosis patients. I am fully convinced that the OMV vaccine represents the most promising candidate for combating melioidosis, and I commend the progress made in advancing vaccine development for this disease. However, there are several major concerns that need to be addressed to improve its potential further.

Major concerns

1. Elevated levels of anti-OMV antibodies are strongly associated with protection against death in both non-human primates and patients. However, the potential involvement of cellular immune responses, such as T cells, NK cells, and NKT cells, cannot be ruled out. Further studies are required to definitively confirm whether other cellular immune responses contribute to this protection or are entirely absent. I have some concerns regarding the cellular immune responses observed in this study. Previous research has shown that the OMV vaccine can induce both CD4+ and CD8+ T cells in mice. However, in this study, no OMV-specific CD4+ T cells were detected in the PBMCs of vaccinated monkeys. Additionally, the authors did not present data on CD8+ T cell responses. I suggest further investigation to confirm the absence of cellular immune responses, perhaps through a PBMC proliferation assay or by assessing the proportions of B and T cells before and after vaccination to determine whether only B cells, but not T cells, expanded. I also recommend evaluating T cell responses using either an ELISpot assay or flow cytometry targeting Bp Omp or FlgK, which are key targets of protective antibodies.
2. In line 247, the authors state that the macaques were also exposed to soil bacteria, and one vaccinated monkey suffered from an *S. aureus* infection. I recommend that the authors provide evidence to confirm that the observed effects in the macaques were due to *B. pseudomallei* rather than other bacterial infections. Would it be possible to measure the concentration of *B. pseudomallei* in the organs and/or blood to assess whether a bacterial burden was present exclusively in the control macaques, which could explain the severe conditions observed?
3. To complement the findings on antibody levels, I encourage the authors to include an assessment of functional antibody activity e.g. opsonophagocytic. This could provide a more comprehensive understanding of how these antibodies contribute to protection.
4. I suggest the authors provide additional explanation regarding observed discrepancy between KJ33 who exhibited elevated level of anti-OMV antibodies but was not protected, and KT39 who elicited low level of anti-OMV antibodies (only elevated level of FlgK was observed) yet demonstrated protection. Clarifying this distinction could offer valuable insights into

the role of antibodies specificity and other potential protective mechanisms beyond antibody level.

Minor comments

1. If I understand this correctly, it appears that KM49 is excluded from Figure 1a (that's why $n = 5$) and Figure 1b; however, the antibody levels of this macaque are still included in other figures. Could you kindly clarify this in the manuscript please?
2. I believe that Figures 3b and 3c represent data as medians. If this is the case, would you mind indicating this in the figure legend for clarity?
3. It would be helpful to differentiate the data points for succumbed and survived macaques in Figure 1f by using different pattern, similar to those in Figures 3b and 3c.

Reviewer #4

(Remarks to the Author)

Dear Authors and Editor,

Thank you for considering me to review this manuscript. Broadly this is a very well written manuscript and does indeed represent a first reported use of a *Burkholderia* vaccine in NHPs. I have very few minor concerns and one major concern. This manuscript predicated on vaccine that provides measurable protection. This is stated in the title "A *Burkholderia* OMV vaccine prevents inhalational melioidosis in non-human primates" and then later in the results section "OMV vaccination protects against inhalational melioidosis in macaques". The survival comparison yielded insufficient evidence ($p=0.15$) to dismiss the null hypothesis. However, it is clear that disease was reduced in these animals (fever, clinical score, etc). It would therefore be more accurate to say that vaccination reduced the impact of inhalational melioidosis and not protected against it. This doesn't invalidate all of the further interesting findings, but the authors need to rephrase their titles and target their article to a less prestigious journal. To be clear, I still think that these results are very worthy of publication and discussion. However, to qualify for publication in a high tier journal I would expect a survival benefit for the vaccine. If the authors are intent on publication in a high tier journal, I suspect that a repeat study and (providing a similar level of protection is observed) stratified analysis across both studies would demonstrate a statistical survival benefit. A statistician should be consulted to determine the require N for a follow up study.

- The authors need to state their tested hypothesis up front and how they determined that $n = 6$ would be suitable to prove their hypothesis. This is especially important when using primate species for experimental biology but should be a universal for the scientific method. If the researchers did not have a specific hypothesis (and this is an observational study) then this must be stated, and the authors need to concede that any beneficial effects could be due to confirmation bias and will need to be tested again in an independent trial. I don't believe that confirmation bias has occurred here but if shorter fever duration and lower clinical scores were not the intended primary outcome measure, then the possibility does exist, and this should be acknowledged.
- The authors haven't considered familywise error in their statistical analysis. The alpha rejection rate was set to $p<0.05$ throughout, giving a less than 1 in 20 chance of data occurring randomly. In several sections of the manuscript the authors perform multiple tests across similar data types (e.g. ELISA endpoint data, and MFI data). Either a familywise error adjustment should be performed, or some discussion should be given as why it is not need in this instance.
- In the reporting summary the authors state that the authors should record whether one- or two-sided tests were used and the journal requests test statistics. I could not find these. Largely the authors present raw data which I think negates the journal's requirement for measures of variation to be recorded.
- Figure 1A suggests that animals were kept for 3 weeks after challenge however figure 1B suggest 2 weeks. This is explained in the text being due to an animal being removed due to an unrelated health issue. In this instance the animal should be right censored, and the full curve given out to day 21.
- I think that Figure 3 needs the legend to go into further detail about methodology.
- The authors switch abbreviation for subcutaneous from sub-Q to SC in the methods section.

Best regards,

Reviewer

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is an interesting and important study.

It is a revised manuscript, however there was no document with tracked or otherwise highlighted changes incorporated, which was unfortunate.

The authors have addressed the reviewers' comments overall.

For the record the statement , " We agree with the reviewer that cellular immunity/IFN-g is very important in human melioidosis. We have now cited these seminal studies in the introduction and discussion" is incorrect. This statement was also repeated later in another response. The studies are cited in the discussion only and are not cited in the introduction. Cellular immunity is not mentioned in the introduction at all. The bias towards humoral responses is maintained.

In summary, the paper is now acceptable for publication.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

I appreciate the authors' efforts in revising the manuscript and addressing several of the initial concerns. The study presents important findings on vaccine-mediated protection against inhalational melioidosis in rhesus macaques, and the authors have made improvements to the clarity and presentation of their data.

However, given the high standards of this journal, I would expect more conclusive data regarding the immune mechanisms responsible for protection. In particular, more definitive evidence is needed to support the authors' claims about the correlates of immunity. I outline my main concerns below:

Major Comments

Antibody-Mediated Protection

The authors suggest that vaccine-mediated protection is associated with elevated systemic and mucosal IgG and IgA levels. While high antibody titers can reflect vaccination and may be associated with protection, they are not sufficient on their own to establish a causal role in protection. In the absence of functional assays (e.g., neutralization, opsonophagocytosis, or bactericidal assays), it is difficult to assess the true protective capacity of these antibodies. Furthermore, it appears that 2 out of 6 vaccinated animals (KT39 and KP07) already had high baseline levels of anti-OMV IgG, which complicates the interpretation. I would strongly encourage the authors to include or discuss functional antibody data to support their conclusions.

T Cell Responses

The manuscript states that there was no significant difference in T cell responses measured in PBMCs between vaccinated and control animals, potentially due to late sampling. The authors note that vaccine-induced T cells might be retained in lymphoid tissues post-immunization. If lymphoid tissues were collected, it would be valuable to assess T cell responses directly in those tissues to strengthen the claim. Otherwise, the absence of a significant difference could reflect a limitation in study design or sampling strategy. This uncertainty weakens the conclusion that antibody responses alone mediate protection. If possible, I suggest examining T cell markers in formalin-fixed tissues to determine whether T cell responses were indeed comparable between groups.

Cytokine Responses and Pathogenesis

Since cytokine dysregulation, including cytokine storm, is a key driver of pathogenesis in melioidosis, I expected to see cytokine profiling data either in circulation or in relevant tissues. This would provide important insight into the host immune response and disease severity, and potentially correlate with protection. Including this data, or discussing its absence, would improve the manuscript's impact.

Minor Comments

Line 134: The term "unprotected" may be too strong or potentially misleading. I suggest revising it to "unvaccinated" for clarity and precision.

Overall, this study has the potential to make a meaningful contribution to the field. I encourage the authors to address the above points to strengthen the mechanistic understanding of the vaccine's protective effects.

Reviewer #4

(Remarks to the Author)
Dear Authors and Editor,

Thanks again for the chance to review this manuscript. It would seem that my fellow reviews directed some excellent changes, and I can see that this manuscript has improved. I especially appreciated the additions concerning T cell responses. I am pleased that the minor points that I made were easily resolved, thank you. I also am gratified by the debate regarding the implied meaning of the word "protects" and "prevents".

Unfortunately, also I feel uncomfortable about the word "prevents" in the title. Like the author states "prevents" means "stops something from happening in the first place". While day 21 pathology is statistically significantly reduced this does not preclude the possibility that some disease occurred and was healed by day 21. I can see no other way of reading "A Burkholderia OMV vaccine prevents inhalational melioidosis in non-human primates" than [to paraphrase] "we can provide statistical proof (that is based on our experimental data) that the disease has not occurred more frequently in vaccinated animals". I just do not accept this in the data presented. Titles are so import and this is especially true in an age where AI is being used to summarise and locate data. In order for me to support publication of this manuscript, I need to see a short qualifying statement in the title. For example: "...prevents lung pathology..." or "protects against the effects of...", etc.

I also appreciate that the authors have affirmed that "This study was designed around the central hypothesis was that there would be a positive immune response to vaccination, consistent with protection from disease. The measurements to be made, which were selected well in advance of the study, enable this hypothesis to be tested in multiple, direct and highly appropriate ways to answer the central hypothesis." However, the reader should be able to access to the rationale for the sample size and what the a priori determined success criteria were. My concern is now that there was a wide selection of measurements were taken and the authors have chosen to provide the end of experiment lung pathology score because they provide a low p value ($p < 0.05$). What if the authors had 20+ success criteria? Then it would then be a near certainty that at least one would present as statistically significant just through the process of random variation (i.e. be a false positive). I have no issue to the authors stating that this was a pilot study to consider the big picture of the infection with and without vaccine (i.e. no primary measure of success was assigned in the experimental design). Nor do I disagree with the interpretation; from the data I have seen it does look vaccination has had a positive effect. I just think that authors need to be upfront about the design of the experiment because this is so important in the interpretation of the data.

Ultimately the decision regarding whether this data has enough scientific kudos, is an editorial decision. In the interest of full disclosure, I have never published in a top tier journal. I had always assumed that I would need highly novel data with potent evidence across primary measures of effect (which for the reasons above, I am not wholly convinced is true here). However, as someone with no experience in this, perhaps I am not best placed to advise.

Best regards,

Reviewer 4

made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

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Reviewer #1 (Remarks to the Author):

This is an interesting and important paper which demonstrates that Rhesus macaques which were immunised with OMVs were protected from *Burkholderia pseudomallei* infection in an inhalation model. In the context of development of any vaccine against melioidosis this is a landmark study. The authors evaluated the protective efficacy of their OMV vaccine against inhalational melioidosis in rhesus macaques, a highly translational preclinical model. They assessed survivability, biotelemetry data, and pathology outcomes to determine vaccine effectiveness. They showed that the OMV vaccine is protective against inhalational melioidosis in macaques. Additionally, they employed serologic assays to measure systemic and mucosal IgG and IgA responses, linking protection to the most immunogenic surface antigens and polysaccharides reactive to IgG and IgA. However, while there is clear evidence of protection in 80% of animals up to 14 days, more information / discussion on the cellular immune responses and mucosal immune responses would strengthen this manuscript.

Response: We thank the reviewer for their comments and appreciate that they recognized the potential importance of this study to the field as one of the most robust preclinical models available for the assessment of protection against this infection.

Specific points.

Major comment.

The study focusses on the humoral responses predominantly. While antibodies are recognised to contribute to protection, it is well recognised that Interferon gamma secretion correlates with survival in humans (Jenjaroen et al., 2015), but this is largely overlooked in this paper. This bias should be addressed in the introduction and discussion.

Response: We agree with the reviewer that cellular immunity/IFN- γ is very important in human melioidosis. We have now cited these seminal studies in the introduction and discussion, and we have performed additional experiments to demonstrate the capacity of the OMV vaccine to drive human IFN- γ , TNF, and IL-2 producing CD4 and CD8 T cells.

In addition, the authors should present all available data and explain why more cellular responses were not evaluated.

Response: The goal of the study was to demonstrate the protective capacity of the OMV vaccine in NHPs and to identify potential vaccine correlates of protection using non-invasive techniques such as blood draws which could then be incorporated in planned human clinical trials. We did attempt to measure vaccine-specific T cells in the blood

(Supplemental Fig. S3) of NHPs. Our failure to detect memory CD4 T cells in vaccinated macaques is likely a result of 1) tissue source (blood for NHPs) compared to mice using lymphoid organ (spleen) and 2) time of sampling as it is likely that circulating vaccine-specific T cells in the blood were too rare 2 weeks post-immunization. Measurement of recall responses during active infection of NHPs, particularly in sick animals, was not feasible for ethical and safety reasons. However, we recognize the importance of cellular immunity. To address the reviewer's concern (as well as that of Reviewer #3), we performed additional experimentation using individual human donors (n=20) and Sanofi's MIMIC system (PMID: 19807200) to demonstrate that the OMV vaccine can drive vaccine-specific IFN- γ , TNF- α , and IL-2 secreting CD4 and CD8 T cells. This data is now presented as supplemental figure S4a,b.

In addition, more non-LPS specific IgAs in BAL data should be presented.

Response: We have added the data for the other antigens to Figure 3.

Minor comments.

Line 119 insert "the" before study.

Response: This has been added.

What is the rationale for immunising with two doses. Would they have observed better protection with a second boost?

Response: The Burkholderia pseudomallei vaccine target product profile developed by the Defense Threat Reduction Agency (funding agency) indicated a maximum of two doses so we sought to achieve this.

Line 139 – due to the death of one animal in the immunised group, survivability data are present through to day 14 only. This is a very short timeframe. Although not explicitly stated, it is apparent that all animals were not killed on day 14 and if this is the case, the authors should show a more extended timeline beyond that date with the remaining mice (even in supplemental data). It would be very informative in the context of long term protection and would be maximise the data available from the surviving animals from an animal ethics perspective.

Response: The reviewer is correct and one NHP (KM49) that survived acute melioidosis was humanely euthanized on day 14 due to a culture-proven S. aureus eye infection unrelated to the inhaled bacterial infection. All other animals were kept until day 21. Longer timeframes of observation were simply not feasible due to ethical and financial constraints

related to NHP select agent studies such as these. We have extended the survival curve graph to day 21 and right censored animal KM49 as suggested by Reviewer #4.

Line 146 It is not clear why the Weight loss/gain data was not shown. Can it be included in supplemental data?

Response: We have added the weight data for each animal that survived to day 21 as supplemental table 1.

Line 166 and Figure 2 legend. The legend should be revised and clarified. It does not describe the C) or D) components presented in the figure.

Response: We apologize for the confusion. There are only 2 panels (a and b). The box surrounds the OMV vaccinated animals to distinguish them from sham vaccinated. We have added more clarification to the figure legend.

Line 179. One animal exhibited anergy. How do the authors know the reason was T cell anergy and not exhaustion or senescence? Were markers for T cell anergy evaluated? If not, it should change it to “exhibited no or little response”.

Response: The reviewer makes an excellent point here, and we have changed the language based on this recommendation.

Discussion. The study focusses on the humoral responses predominantly. It is unfortunate that more cellular immune response parameters were not evaluated.

Response: As detailed above, we now include OMV-elicited CD4 and CD8 T cell responses using human donors in the MIMIC system and include as supplemental Fig. S4a,b. We also have expanded on the role of cellular immunity in the discussion.

Line 252 – This discussion should include the seminal papers from the Dunachie group that compared cellular responses in survivors versus patients that died from melioidosis (Jenjaroen et al., 2015 and Ronkard et al., 2020).

Response: We appreciate the reviewer highlighting these seminal studies and now reference these important papers in the introduction and discussion.

Line 253. Comma missing – yet,

Response – corrected.

Line 261. Indicate that. (insert that).

Response – corrected.

Line 264-266. It seems quite apparent that the main distinguishing feature between the surviving immunised animals and the non-survivor was the presence of multiple antigen specific sIgA in the BAL (rather than serum IgGs). Can the authors comment on this?

Response – We agree that IgA and IgG responses in the BAL may be more indicative of protection than serum IgG but further study is warranted to ultimately determine this. Relative roles for each may also be related to the route of infection used in this study. We have elaborated more on the potential role of mucosal immunity in the discussion.

Mucosal IgA has not been adequately considered in the discussion “perhaps mucosal IgA”.

Response: We agree and have elaborated more on the potential role of mucosal immunity in melioidosis in the discussion.

Line 286 to 292: Perhaps the abundant LPS signalling via TLR4 is driving a more Th2-skewed response. Can the authors comment on whether incorporating a T cell-specific adjuvant might help address this issue by promoting a more balanced response or a Th1-biased immune response.

Response: Previous studies have shown that LPS/lipid A signaling via TLR4 skews toward a Th1 response (PMID: 21198672). We have tested both Th1 and Th2-skewing adjuvants in combination with the OMV vaccine and do not see much of an effect, likely due to the potent and inherent adjuvanticity of the Burkholderia OMVs (PMID: 33498352). As shown in our new data, the OMV vaccine drives potent Th1 cellular immunity (IFN- γ , TNF- α , and IL-2 producing CD4 and CD8 T cells) in the human MIMIC system.

Figure 2 – The graphs are quite small and the numbers in the x axis are very difficult to read. Removal of the key from all but one figures could improve the available size and allow presentation of larger figures.

Response: We thank the reviewer for this suggestion and have revised the figures accordingly.

Figure 3. Can the authors show more data on the IgA antibodies against other antigens other than LPS-A and comment on their presence / absence in immunised versus sham animals?

Response: We have added this data to Fig. 3 and the results.

Line 282 – “Several other antigens of interest were detected by protein microarray that were not available for the study in the BurkPx assay” can the authors elaborate on what these are?

Response: We have provided a list of these proteins in Table 1. We did not elaborate on many of them as their functions remain unknown.

Methods:

The statistical analysis section is quite vague. While it is clear that GraphPad was used, can the authors detail what tests / post tests were used for the individual datasets presented within the methods?

Response: The statistical tests and post-test information have been provided in the figure legends.

Supplemental Figure S2 As with Figure 2, the legend should be revised and clarified. It does not describe the C) or D) components.

Response: We apologize for the confusion. There are only 2 panels (a and b). The box surrounds the OMV vaccinated animals to distinguish them from sham vaccinated. We have added more clarification to the figure legend.

Supplemental Figure S4 – includes “lorem ipsum” on the figure. This should be removed.

Response: Corrected.

The authors should comment on the variability of the secretory IgA responses in BAL, relative to the IgG in BAL, and discuss the potential correlates of both isotypes, given that the non- survivor showed reasonable IgG against at least 3 antigens. .

Response. We thank the reviewer for this suggestion. We have elaborated more on the potential roles of IgG and IgA in our study and human melioidosis in the discussion.

Table 2 – BPSL1743 is ArcA, not Arg

Response: Thank you to the reviewer for catching this which has now been corrected.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you!

Reviewer #3 (Remarks to the Author):

Previous studies on *Burkholderia pseudomallei* outer membrane vesicles (OMVs) have demonstrated that OMV-based vaccines can provide significant protection in murine models and cross-protection against inhalational glanders in mice and non-human primates. In this manuscript, Baker et al. present, for the first time, evidence that the OMV vaccine also confers protection against inhalational melioidosis in rhesus macaques. Rhesus monkeys vaccinated with the OMV vaccine demonstrated an increased survival rate, reduced lung inflammation, and a slower progression of clinical signs. The survival of these non-human primates was associated with significantly elevated levels of both systemic and mucosal IgG and IgA antibodies targeting OMV components, particularly LPS, OmpA, and FlgK proteins. A correlation between elevated levels of anti-LPS and anti-OmpA antibodies and survival has also been observed in the serum of melioidosis patients. I am fully convinced that the OMV vaccine represents the most promising candidate for combating melioidosis, and I commend the progress made in advancing vaccine development for this disease. However, there are several major concerns that need to be addressed to improve its potential further.

Response: Thank you for your supportive and constructive review!

Major concerns

1. Elevated levels of anti-OMV antibodies are strongly associated with protection against

death in both non-human primates and patients. However, the potential involvement of cellular immune responses, such as T cells, NK cells, and NKT cells, cannot be ruled out. Further studies are required to definitively confirm whether other cellular immune responses contribute to this protection or are entirely absent. I have some concerns regarding the cellular immune responses observed in this study. Previous research has shown that the OMV vaccine can induce both CD4+ and CD8+ T cells in mice. However, in this study, no OMV-specific CD4+ T cells were detected in the PBMCs of vaccinated monkeys. Additionally, the authors did not present data on CD8+ T cell responses. I suggest further investigation to confirm the absence of cellular immune responses, perhaps through a PBMC proliferation assay or by assessing the proportions of B and T cells before and after vaccination to determine whether only B cells, but not T cells, expanded. I also recommend evaluating T cell responses using either an ELISpot assay or flow cytometry targeting Bp Omp or FlgK, which are key targets of protective antibodies.

Response: We agree with the reviewer that cellular immunity is a critical feature of protection in melioidosis. We did attempt to measure vaccine-specific T cells in the blood of NHPs (Supplemental Fig S3). Our failure to detect memory T cells in vaccinated macaques is likely a result of 1) tissue source (blood for NHPs) compared to mice using lymphoid organ (spleen) and 2) time of sampling as it is likely that circulating vaccine-specific T cells in the blood were too rare 2 weeks post-immunization. Measurement of recall responses during active infection of NHPs, particularly in sick animals, was not feasible for ethical and safety reasons. However, we recognize the importance of cellular immunity. To address the reviewer's concern (as well as Reviewer #1), we performed additional experimentation using individual human donors (n=20) and Sanofi's MIMIC system (PMID: 19807200) to demonstrate that the OMV vaccine does drive vaccine-specific IFN- γ , TNF- α , and IL-2 secreting CD4 T and CD8 T cells. This data is now presented as supplemental figure S4a,b.

2. In line 247, the authors state that the macaques were also exposed to soil bacteria, and one vaccinated monkey suffered from an *S. aureus* infection. I recommend that the authors provide evidence to confirm that the observed effects in the macaques were due to *B. pseudomallei* rather than other bacterial infections. Would it be possible to measure the concentration of *B. pseudomallei* in the organs and/or blood to assess whether a bacterial burden was present exclusively in the control macaques, which could explain the severe conditions observed?

Response: Animals were experimentally infected with aerosolized B. pseudomallei, with disease signs consistent with pneumonic melioidosis. Animals that succumbed by day 4

had bacteremia and were culture positive for B. pseudomallei in the lung and blood. All study animals were born and raised in outdoor caging units with earthen floors at the Tulane Center until young adulthood and thereafter assigned to this study. They passed all health checks and showed no signs of disease based on biotelemetry and blood chemistry measurements taken at baseline. It is therefore highly unlikely that there is any correlative effect of a S. aureus infection as all study animals were continuously exposed to soilborne bacteria during rearing for several years. S. aureus infections of unknown etiology in nonhuman primates are relatively common, and although worthy enough to mention in this manuscript, should not be considered a confounder of overall study objectives.

3. To complement the findings on antibody levels, I encourage the authors to include an assessment of functional antibody activity e.g. opsonophagocytic. This could provide a more comprehensive understanding of how these antibodies contribute to protection.

Response: We agree that functional antibody assays would be informative and plan to study these responses in depth in future work.

4. I suggest the authors provide additional explanation regarding observed discrepancy between KJ33 who exhibited elevated level of anti-OMV antibodies but was not protected, and KT39 who elicited low level of anti-OMV antibodies (only elevated level of FlgK was observed) yet demonstrated protection. Clarifying this distinction could offer valuable insights into the role of antibodies specificity and other potential protective mechanisms beyond antibody level.

Response: We have commented on KJ33 and KT39 in the results and discussion because, as the reviewer points out, these animals displayed little to no response in different ways. We have provided some comments on how the responses could have affected outcomes, but we hesitate to draw further conclusions without over-reaching.

Minor comments

1. If I understand this correctly, it appears that KM49 is excluded from Figure 1a (that's why n = 5) and Figure 1b; however, the antibody levels of this macaque are still included in other figures. Could you kindly clarify this in the manuscript please?

Response: We apologize for the confusion. We have clarified the group numbers in the methods and results and modified Figures 1a and 1b.

2. I believe that Figures 3b and 3c represent data as medians. If this is the case, would you mind indicating this in the figure legend for clarity?

Response: Done, thank you!

3. It would be helpful to differentiate the data points for succumbed and survived macaques in Figure 1f by using different pattern, similar to those in Figures 3b and 3c.

Response: Thank you for the excellent suggestion. We made this change.

Reviewer #4:

Dear Authors and Editor,

Thank you for considering me to review this manuscript. Broadly this is a very well written manuscript and does indeed represent a first reported use of a *Burkholderia* vaccine in NHPs. I have very few minor concerns and one major concern.

This manuscript predicated on vaccine that provides measurable protection. This is stated in the title “A *Burkholderia* OMV vaccine prevents inhalational melioidosis in non-human primates” and then later in the results section “OMV vaccination protects against inhalational melioidosis in macaques”. The survival comparison yielded insufficient evidence ($p=0.15$) to dismiss the null hypothesis. However, it is clear that disease was reduced in these animals (fever, clinical score, etc). It would therefore be more accurate to say that vaccination reduced the impact of inhalational melioidosis and not protected against it. This doesn’t invalidate all of the further interesting findings, but the authors need to rephrase their titles and target their article to a less prestigious journal. To be clear, I still think that these results are very worthy of publication and discussion. However, to qualify for publication in a high tier journal I would expect a survival benefit for the vaccine. If the authors are intent on publication in a high tier journal, I suspect that a repeat study and (providing a similar level of protection is observed) stratified analysis across both studies

would demonstrate a statistical survival benefit. A statistician should be consulted to determine the required N for a follow up study.

Response: The authors thank the reviewer for the extensive comment concerning appropriate hypothesis testing involving nonhuman primates and a highly infectious disease such as pneumonic melioidosis. Our response addresses each part of the comment in an attempt to stratify each reviewer criticism within the context of the scope and thrust of our manuscript.

For the comment “This manuscript predicated on vaccine that provides measurable protection. This is stated in the title “A Burkholderia OMV vaccine prevents inhalational melioidosis in non-human primates” and then later in the results section “OMV vaccination protects against inhalational melioidosis in macaques”. The survival comparison yielded insufficient evidence ($p=0.15$) to dismiss the null hypothesis. However, it is clear that disease was reduced in these animals (fever, clinical score, etc). It would therefore be more accurate to say that vaccination reduced the impact of inhalational melioidosis and not protected against it. “

*Response: The authors agree that the use of the word ‘protect’ may not be appropriate and have modified the text by replacing with the word ‘prevent’. The use of the word ‘prevent’ in the title, and the use of ‘protect’ in the results section are related, however can construe differing meanings; that is ‘prevent’ stops something from happening in the first place whereas ‘protect’ reduces or block harm if the threat is already present. The authors feel that the use of ‘prevent’ in the title is appropriate as the results of the NHP study clearly demonstrate benefit to the vaccinee by stimulating an immune response prior to experimental exposure. The authors agree that the primary endpoint – survival – was not statistically met, however secondary endpoints in the animals were robustly and clearly a demonstration of an immune response that is consistent with prevention of disease. There are two important factors in this study in particular 1) the use of the nonhuman primate and the individualized immune response in this highly complex species (*M. mulatta*) that rarely is congruent with the homogeneity that one may experience when experimenting with rodent species, and 2) testing a vaccine product against a disease that is not only severe in pathogenesis, but also insidious; that is spontaneous recrudescence and bacterial seeding of multiple organ systems makes complete protection from disease by vaccination a mighty hill to climb, especially when using the most severe of all the disease forms*

(pneumonic) that challenges even the most competent immune system response. When considering the natural variability in immune response in NHP, which emulates distributions that one may expect in humans, in the context of pneumonic melioidosis, the homogeneity observed in the vaccine cohort of animals is remarkable. Further, the intention of this study was to identify and explore immune response from vaccination in this species, which is clearly stated in the introduction, therefore relying upon an accumulation of all of the data, not just the primary endpoint, to draw conclusions about the study, especially in the context of the sophisticated disease model species used in this study, and the severity of the challenge modality (aerosol exposure). The authors recognize that the primary endpoint was not statistically met and state as much in the manuscript and note in the figure, however, when considering all supporting data (e.g., physiological response, bacteriology, and pathology), it is hard to agree with the reviewer suggestion that this manuscript should be relegated to a lower-tier journal simply by not achieving statistical significance of the primary endpoint. Rather, in exploratory investigations such as this (e.g., this is not a regulated study), the accumulation of positive secondary endpoints promotes the posit that the immune response resulting from this vaccine ‘prevents’ melioidosis, but not necessarily death, at least statistically.

Addressing the second portion of reviewer comment “...However, to qualify for publication in a high tier journal I would expect a survival benefit for the vaccine. If the authors are intent on publication in a high tier journal, I suspect that a repeat study and (providing a similar level of protection is observed) stratified analysis across both studies would demonstrate a statistical survival benefit...”. The authors disagree that qualification for publication in a high tier journal is a survival benefit, especially in the context of an array of such promising positive secondary endpoint data in the vaccine cohort. It is rare in exploratory vaccine studies involving NHP and severe infectious disease models to observe a clear survival benefit, especially considering how rarely primates are used to evaluate an experimental Burkholderia vaccine. Further, in modern NHP studies involving deadly infectious disease (e.g., pneumonic melioidosis), USDA animal use guidelines, expert veterinary care, and adherence to the ARRIVE tenets of animal use dictate that once an animal experiences a combination of signs consistent with a rapid decline due to (induced) illness, the PI and attending veterinarian authorize euthanasia. In this study, all non-survivors were euthanized based upon nearly constant visualization and active monitoring, and survival curves are reflective of a timed euthanasia after appropriate anesthesia. The artificiality associated with this approach is an absolute requirement in the use of NHP in biomedical research, especially within the context of such a deadly disease as melioidosis. The authors recognize that not all reading this work may be familiar with the granular

mechanics of primate research, and the context in which one should appreciate survival curves considering the requirement for euthanasia. Accordingly, the authors have added specific descriptive language to reinforce these aspects of the study in the results section.

The authors need to state their tested hypothesis up front and how they determined that $n = 6$ would be suitable to prove their hypothesis. This is especially important when using primate species for experimental biology but should be a universal for the scientific method. If the researchers did not have a specific hypothesis (and this is an observational study) then this must be stated, and the authors need to concede that any beneficial effects could be due to confirmation bias and will need to be tested again in an independent trial. I don't believe that confirmation bias has occurred here but if shorter fever duration and lower clinical scores were not the intended primary outcome measure, then the possibility does exist, and this should be acknowledged.

Response: This study was designed around the central hypothesis was that there would be a positive immune response to vaccination, consistent with protection from disease. The measurements to be made, which were selected well in advance of the study, enable this hypothesis to be tested in multiple, direct and highly appropriate ways to answer the central hypothesis.

Confirmation bias would require a scenario in which a larger set of measurements had been made, from which we selected only the subset that supported our central hypothesis. This is, of course, not the case.

The authors haven't considered familywise error in their statistical analysis. The alpha rejection rate was set to $p < 0.05$ throughout, giving a less than 1 in 20 chance of data occurring randomly. In several sections of the manuscript the authors perform multiple tests across similar data types (e.g. ELISA endpoint data, and MFI data). Either a familywise error adjustment should be performed, or some discussion should be given as why it is not needed in this instance.

*Response: For experiments with multiple parallel comparisons, such as ELISA endpoint studies in Fig 2 and supplemental Fig. 2, we corrected the α -rate cutoff for significance using the approach of Bonferroni, such that $\alpha(\text{corrected}) = \alpha(\text{standard})/N$, where N is the number of pair-wise comparisons. Ultimately, this made no difference in our conclusions because all samples with significant differences in ELISA measurements (designated **** in Fig. 2) had p -values between 1×10^{-12} and 1×10^{-5} compared to a Bonferroni-corrected α cutoff for 8 pairwise comparisons of $0.05/8 = 0.00625$.*

The mean fluorescence intensity (MFI) data from the microarray experiments were used only to rank order the observed response to antigen molecules from a set that was selected from a list of likely/suspected antigens.

In the reporting summary the authors state that the authors should record whether one- or two-sided tests were used and the journal requests test statistics. I could not find these. Largely the authors present raw data which I think negates the journal's requirement for measures of variation to be recorded.

Response: All statistical tests in this paper are 2-sided. We thank the reviewer for pointing out that this was not mentioned, an omission that is corrected in the revised version.

Figure 1A suggests that animals were kept for 3 weeks after challenge however figure 1B suggest 2 weeks. This is explained in the text being due to an animal being removed due to an unrelated health issue. In this instance the animal should be right censored, and the full curve given out to day 21.

Response: We have revised the figure as suggested by the reviewer.

I think that Figure 3 needs the legend to go into further detail about methodology.

Response: We have added additional detail to the legend, but more detailed methodology can also be found in methods section.

The authors switch abbreviation for subcutaneous from sub-Q to SC in the methods section.

Response: This inconsistency has been fixed.

Authors Response to Reviewers:

Reviewer #1 (Remarks to the Author):

This is an interesting and important study.

It is a revised manuscript, however there was no document with tracked or otherwise highlighted changes incorporated, which was unfortunate.

The authors have addressed the reviewers' comments overall.

For the record the statement, " We agree with the reviewer that cellular immunity/IFN-g is very important in human melioidosis. We have now cited these seminal studies in the introduction and discussion" is incorrect. This statement was also repeated later in another response. The studies are cited in the discussion only and are not cited in the introduction. Cellular immunity is not mentioned in the introduction at all. The bias towards humoral responses is maintained.

In summary, the paper is now acceptable for publication.

Response: Thank you again for your constructive feedback. We apologize that our tracked changes to the word document were not captured in the pdf document. In the newest version, we have highlighted all changes in the word document so that the pdf will reflect all tracked changes.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you again for your support.

Reviewer #3 (Remarks to the Author):

I appreciate the authors' efforts in revising the manuscript and addressing several of the initial concerns. The study presents important findings on vaccine-mediated protection against inhalational melioidosis in rhesus macaques, and the authors have made improvements to the clarity and presentation of their data.

However, given the high standards of this journal, I would expect more conclusive data regarding the immune mechanisms responsible for protection. In particular, more definitive evidence is needed to support the authors' claims about the correlates of immunity. I outline my main concerns below:

Major Comments

Antibody-Mediated Protection

The authors suggest that vaccine-mediated protection is associated with elevated systemic and mucosal IgG and IgA levels. While high antibody titers can reflect vaccination and may be associated with protection, they are not sufficient on their own to establish a causal role in protection. In the absence of functional assays (e.g., neutralization, opsonophagocytosis, or bactericidal assays), it is difficult to assess the true protective capacity of these antibodies. Furthermore, it appears that 2 out of 6 vaccinated animals (KT39 and KP07) already had high baseline levels of anti-OMV IgG, which complicates the interpretation. I would strongly encourage the authors to include or discuss functional antibody data to support their conclusions.

Response: We now present data demonstrating OMV vaccine-induced sera antibody effector responses in vitro. As melioidosis is not considered a toxin-mediated disease (eg. anthrax, tetanus), neutralization assays are not standard in the field. In addition, others have shown that B. pseudomallei is inherently resistant to the effects of complement even in the presence of immune sera (PMID: 33986290). Therefore, we elected to perform opsonophagocytosis assays using commercially sourced macaque macrophages. The data is now presented in Fig. 3f and shows that the serum antibodies from vaccinated macaques are indeed opsonophagocytic and likely to be functionally protective.

T Cell Responses

The manuscript states that there was no significant difference in T cell responses measured in PBMCs between vaccinated and control animals, potentially due to late sampling. The authors note that vaccine-induced T cells might be retained in lymphoid tissues post-immunization. If lymphoid tissues were collected, it would be valuable to assess T cell responses directly in those tissues to strengthen the claim. Otherwise, the absence of a significant difference could reflect a limitation in study design or sampling strategy. This uncertainty weakens the conclusion that antibody responses alone mediate protection. If possible, I suggest examining T cell markers in formalin-fixed

tissues to determine whether T cell responses were indeed comparable between groups.

Response: Lymph nodes were only collected at necropsy after infection. As some animals succumbed to overwhelming infection within 3-4 days and others survived until 21 days, we do not feel that comparing responses in lymph nodes at these different timepoints would be pertinent as this would introduce significant survivor bias. Additionally, it would be impossible to separate vaccine induced responses from infection induced responses. In response to the original critique about absence of cellular immune responses, we partnered with Sanofi to examine human cellular responses to the OMV vaccine in the MIMIC system using appropriate controls. We believe the T cell data is quite robust and provide that data as supplementary figures. We also dedicate several sentences in the discussion about the limitations of measuring cellular responses in vaccinated macaques.

Cytokine Responses and Pathogenesis

Since cytokine dysregulation, including cytokine storm, is a key driver of pathogenesis in melioidosis, I expected to see cytokine profiling data either in circulation or in relevant tissues. This would provide important insight into the host immune response and disease severity, and potentially correlate with protection. Including this data, or discussing its absence, would improve the manuscript's impact.

Response: Our study was not focused on pathogenesis, however we have added the absence of NHP T cell and cytokine data as a limitation of the study and point out the need for future studies to consider these in our revised discussion.

Minor Comments

Line 134: The term “unprotected” may be too strong or potentially misleading. I suggest revising it to “unvaccinated” for clarity and precision.

Response: We used the word “unprotected” to encompass both vaccinated and sham animals that did not survive. However, we have changed the wording as suggested by the reviewer.

Overall, this study has the potential to make a meaningful contribution to the field. I encourage the authors to address the above points to strengthen the mechanistic understanding of the vaccine's protective effects.

Response: Thank you for your constructive feedback to help improve our manuscript.

Reviewer #4 (Remarks to the Author):

Dear Authors and Editor,

Thanks again for the chance to review this manuscript. It would seem that my fellow reviews directed some excellent changes, and I can see that this manuscript has improved. I especially appreciated the additions concerning T cell responses. I am pleased that the minor points that I made were easily resolved, thank you. I also am gratified by the debate regarding the implied meaning of the word “protects” and “prevents”.

Unfortunately, also I feel uncomfortable about the word “prevents” in the title. Like the author states “prevents” means “stops something from happening in the first place”. While day 21 pathology is statistically significantly reduced this does not preclude the possibility that some disease occurred and was healed by day 21. I can see no other way of reading “A Burkholderia OMV vaccine prevents inhalational melioidosis in non-human primates” than [to paraphrase] “we can provide statistical proof (that is based on our experimental data) that the disease has not occurred more frequently in vaccinated animals”. I just do not accept this in the data presented. Titles are so import and this is especially true in an age where AI is being used to summarise and locate data. In order for me to support publication of this manuscript, I need to see a short qualifying statement in the title. For example: “...prevents lung pathology...” or “protects against the effects of...”, etc.

Response: We have revised the title according to the reviewer's suggestion. We have also added in all of the lung gross pathology images to Figure 1 to highlight the findings further.

I also appreciate that the authors have affirmed that “This study was designed around the central hypothesis was that there would be a positive immune response to vaccination, consistent with protection from disease. The measurements to be made, which were selected well in advance of the study, enable this hypothesis to be tested in multiple, direct and highly appropriate ways to answer the central hypothesis.” However, the reader should be able to access to the rationale for the sample size and what the a priori determined success criteria were. My concern is now that there was a wide selection of measurements were taken and the authors have chosen to provide the end of experiment lung pathology score because they provide a low p value ($p < 0.05$). What if the authors had 20+ success criteria? Then it would then be a near certainty that at

least one would present as statistically significant just through the process of random variation (i.e. be a false positive). I have no issue to the authors stating that this was a pilot study to consider the big picture of the infection with and without vaccine (i.e. no primary measure of success was assigned in the experimental design). Nor do I disagree with the interpretation; from the data I have seen it does look vaccination has had a positive effect. I just think that authors need to be upfront about the design of the experiment because this is so important in the interpretation of the data.

Response: We present the experimental design in Fig. 1a. The study utilized a small cohort (n=5-6 per group) of macaques and experimental readouts which are standard for studies involving BSL-3 select agents and high containment research with NHPs. We have added to the final paragraph in the discussion that the small sample size is a limitation of the study and only serves as proof of concept. We have also revised wording throughout the manuscript, being mindful not to overstate the results.