

Outcome of SARS-CoV-2 reinfection depends on genetic background in female mice

Corresponding Author: Dr Michael Schotsaert

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)

In the manuscript titled "Host immune responses associated with SARS-CoV-2 Omicron infection result in protection or pathology during reinfection depending on mouse genetic background." Singh et.al. describe the different pathologies in K18 and 129S1 mice caused by reinfection by different SARS-CoV-2 variants. The authors find an interesting phenotype that Omicron (BA.1) preinfection, while providing similar antiviral protection against B.1.351 secondary infection, leads to enhanced lung pathologies in mice of 129S1 background. The authors hypothesize that the differential responses are due to CD11c+ cells which they show have persistent inflammatory signatures after 30 days after infection in 129S1 mice that is not present in K18. The authors thus show that ex vivo CD11c+ cells from either K18 or 129S1 mice respond differently from either mock or pre-infected animals when treated with LPS. Overall, the manuscript is well written, and experiments well done. The manuscript is deficient in a few key areas outlined below.

Major issues

1) Broad applicability of model/phenotype. While the authors acknowledge that "pre" infection with BA.1 and then reinfection with B.1.351 a contrived experimental model they don't address how this phenotype may be broadly applicable or just an observation of the model? The study seems to suggest some sort of difference in trained immunity between K18 and 129S1 mice. Is this trained immunity pathogen specific, ie does pre infection with another respiratory virus such as Flu or just intranasal PAMP (polyIC or other) also lead to a similar exacerbation on pathology with B.1.351 challenge. Do pretreated animals with unrelated virus or pamp have similar CD11c memory and ex vivo cytokine response? While the Th1 vs Th2 phenotype in B6 and 129 is known, I think less is understood in the differences of Th1 vs Th2 in this more trained immunity model and could provide novel insights.

2) Mechanism. The authors suggest that the CD11c+ response drives the noted pathology, however, they don't really address this in vivo. 1) the authors should show by flowcytometry or another quantitative method if there are differences in the total number of CD11c+ cells in lung and BALF at 30 days post BA.1 infection, and what types of cells these CD11c+ cells are. As the authors note, CD11c is expressed on many different cell types, and thus their difference is the CD11c cells in lungs and BALF between K18 and 129S1 both in terms of number and phenotype. 2) Do CD11c cells have an effect in vivo? This could be easily performed with CD11c-DTR cross to K18 and 129S1, a homozygous-to-homozygous cross could allow for selective depletion prior to reinfection.

Minor

1) In line 47-47 the authors write that K18 and 129S1 mice have similar "levels of germinal center B-cells" however, there is no data presented in this manuscript with GC data? Please add data or remove as this is misleading.

2) In figure 3C total number of cells is not calculated/represented, and so stating "mice from both genetic backgrounds had similar numbers of SARS-CoV-2 spike-specific CD8+ T-cells and tissue resident memory CD8+ T-cells" is incorrect as they had similar percentages. It would be important to know total number of cells as this could have a significant impact on phenotype.

3) It would be important not just to show CD8 T_{RM} responses, but also CD4 responses and phenotype. E.g. is there a Th2 response in 129 mice and a Th1 response in K18 from the CD4 cells as well. It certainly seems like this may be the case based on cytokine data, however showing CD4 cytokine and phenotype in lung prior to after initial challenge would be important and may lead to different or additional important conclusions. I think that the conclusion that T cell responses are

not playing a role is unsubstantiated. Additionally, the author could perform CD4/CD8 depletion prior to second infection to assess role of T cells in their phenotype.

4) In figure 4 I am not sure why they chose LPS as the comparison as this is a known PAMP, but one that is related to bacterial infection rather than viral infection, PolyI:C or another RLR ligand would have been a better comparison. Additionally figure 4B is somewhat confusing, I think a PCA or other dimensionality reducing analysis may provide a clearer representation of the data. How are the Genes in 4B decided? Are those the top DEGs? Additionally looking at fig 4B it only seems like a few genes are differential between K18 and 129S1? The authors should address this more in the discussion.

Reviewer #2

(Remarks to the Author)

Summary: Singh et al examined the immune profiles in response to SARS-CoV-2 infection (BA.1) and heterotypic reinfection (B.1.351) in mice of two different genetic background to determine which genetics factors, if any, play a role in protection and/or pathology. They determined that both mouse strains, K18-hACE2 and 129S1, had similar induction of the adaptive immune response (roughly equivalent SARS-2 antibody titers and tissue resident T-cell counts) and similarly reduced lung viral loads. However, heterotypic reinfection with B.1.351 demonstrated severe morbidity (body weight loss, evident lung histopathology) in 129S1 mice whereas K18-hACE2 mice were protected. Cytokine profiling suggested a severe inflammatory profile (large increases in IFN γ and IL18) in reinfected 129S1. Ex vivo stimulation of BALF-isolated CD11c+ cells isolated post-BA.1 (30 DPI) demonstrated profoundly different cytokine profiles upon ex vivo stimulation with B.1.351 or LPS, showing that 'pre-infection' with BA.1 resulted in very different levels of priming of the lung myeloid cells in each mouse strain. This was further supported by transcriptomic profiling of the CD11c+ cells with very different cellular profiles and gene ontologies, with a robust adaptive immune profile in K18 whereas 129S1 exhibiting strong pro-inflammatory signatures. Overall, the large differences seen upon heterotypic SARS-2 reinfection in these two different genetic backgrounds demonstrates the role of altered lung myeloid compartment responsiveness in SARS-2 disease severity and/or protection.

Critiques:

Line 38: "he" should be "the"

Line 270: "wether" should be "whether", and capitalize "omicron"

Line 281: no Figure numbers listed

Line 290: no Figure numbers listed

Lines 291-293 and Supplementary Figure 1A: Authors state that 129S1 mice show higher presence of macrophages with eosinophilic cytoplasm for all groups irrespective of any prior infection and point to Sup Fig 1A. Not seeing this in Sup Fig 1A. Only the BA.1-B.1.351 129S1 are shown there. It may be better to state 'data not shown'. Sup Fig 1A does not show baseline mouse images for 129S1 mice.

Supplementary Figure 1A: Please label each of the image panels to make it more easy to interpret as the legend is somewhat difficult to follow.

Line 298: "propmted" is misspelled

Line 300-301: Not sure what the authors are referring to by "mock-infected animals in the BA.1 pre-infected K18-hACE2 mice". This seems to mean BA.1:mock but that is not shown in Supp Fig 1B. If BA.1:mock data exists for cytokine analyses, it is missing from the figure. This is shown in Figure 4 with the ex vivo stimulation experiments. Further, Supp Fig 1B text size on x-axis is too small and hard to decipher.

Line 302: Should be Supplementary Figure 1B? and capitalize "interestingly"

Figure 2: Please increase the size of the lines in the legend for panel 2B. Also, it may make more sense to move legends in 2B to bottom of panel 1A. Also, please add label to panel 2C heatmap legend.

Line 338: "wether" should be "whether"

Figure 3: Increase the size of the lines in the legend here as well. Difficult to see differences in line color due to small size.

Line 380: Were the numbers of CD11c+ cells isolated from BALF substantially different for the two mouse strains. I am not seeing any data regarding cell counts, etc.

Line 386-387: States that the levels of TNF-alpha was 2x higher in 129S1. Is this referring to the mock stimulated (i.e. mock stim, mock infected) or mock stimulated with prior BA.1 infection? I assume the latter but the text is not clear.

Section spanning lines 386-402 is confusing when trying to interpret Figure 4A and Supplementary Figure 3A. Further, Supp Fig 3A text size is too small to decipher with confidence.

Section spanning lines 403-439 lacks any intuitive flow and simply exhaustively lists what can be seen in Figure 4B. A lot of this is resolved in the discussion section, but it would be beneficial to provide the intuitive flow seen in the discussion section to this results section to provide better context and interpretation to the data.

Figure 4 legend states "Mock inoculated" mice were euthanized...I believe this should be "BA.1 inoculated".

Reviewer #3

(Remarks to the Author)

The manuscript by Singh and colleagues investigates the host characteristics which contribute to disease severity following SARS-CoV-2 infection using mouse models. Specifically host pre-existing immune and immune back grounds were investigated leveraging mice that had a previous SARS-CoV-2 infection as well as two different strains of mice to recapitulate various human genetic backgrounds. Specifically, K18-hACE2 (strain 2B6.Cg-K18-ACE2)2PrImn/J and

129S1 (strain 129S1/SvlmJ) were used. Mice were mock infected or infected with BA.1 or B.1.351 and left to recover until Day 30 post primary infection. On Day 31 post primary infection, mice received a secondary inoculation with Mock or B.1.351. Necropsies were performed 4 days after secondary infection for the analysis of histopathology, viral loads, and cytokine profiling. Serum, tissues, and BALF samples were also collected on Day 30 post primary infection for passive immunization studies, flow cytometry, and cell stimulation studies. K18-hACE2 mice with a previous BA.1 infection were protected during a B.1.351 challenge; however, disease seemed to be increased during the secondary challenge of 129S1 mice. These mice had cytokine profiles more similar to that of humans with severe SARS-CoV-2 infection (IL-10, IL-1beta, IL-18, and IFN-gamma). Adaptive immune responses (antibody levels and T cell numbers) were similar in both strains of mice, suggesting T or B cells to not be the mechanism of disease. This was complemented by similar decreases in virus levels in the lungs. Investigations into the responses of CD11c+ cells isolated from BALF suggested differences in the antiviral responses and proinflammatory responses as a potential driver of differential outcomes in the two mouse strains. Taken together, the data presented offers insight into the complexities of immune responses per host as well as per SARS-CoV-2 variant which will be of interest to the community.

1. The largest short coming of the manuscript was the terminology used when referring to the inoculation of the animals. Various terms and abbreviations were used such as reinfection, DPI, DPI2, DPI1, 1st infection, etc. It would be helpful for the authors to choose terminology, define the meaning, and be consistent in its use throughout the rest of the manuscript. Additionally, it does not make sense to call a BA.1 primary infection followed by a B.1.351 secondary challenge as a reinfection. It is not a reinfection since the first and second viruses are not the same and it is not known if the second virus will infect due as pre-existing immunity may protect against the virus from producing an active infection. Please be cautious with the language.
2. Please give rationale why mice would receive a BA.1 virus followed by a B.1.351 virus as this is not the natural occurrence of SARS-CoV-2 epidemic waves.
3. Please give rationale for mouse numbers used in the analysis per time point.
4. The text in many of the figures was small and hard to read. In particular, the text in Figures 1, 2 and supplementary Figures 1 and 3 should be increased.
5. Please put a legend for the graphs in both Figure 2 Ai, Figure 2 Aiii, Figure 2 Bi, and Figure 2 Biii. It was very confusing to only have the legend in the B parts of this figure.
6. Please give rationale as to why the study was terminated 4 days after the secondary inoculation when the height of pathology would most likely be observed between day 6 and 8.
7. There were several grammatical and spelling errors throughout the manuscript not limited to the following:
 - a. Line 38 "he" should be "the"
 - b. Line 107 – should Dr. Andy Pekosz be Dr. Andrew Pekosz
 - c. In the section on Animal Experiments line 131-132 please state the time point at which the serum is collected
 - d. Line 201 "lungs" should be "lung"
 - e. Line 302 "interesting" should be capitalized
 - f. Line 306 the comma after the bracket should be replaced with a period
 - g. Is "raise" being used correctly in Line 306 - possibly should be rise or increase
 - h. Line 311 "mice" replace with "mouse"

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Significant improvement in this resubmission, no additional comments.

Reviewer #2

(Remarks to the Author)

Summary: The revised manuscript has addressed my original concerns and has additionally provided a influenza H1N1 pre-infection data comparison (H1N1:B1.351), which interestingly showed opposing phenotypes in K18 and 129S1 mice as compared to BA1 pre-infection. The manuscript is substantially improved from the original with detailed, descriptive discussion of the proposed model that the bronchoalveolar CD11c+ compartment is driving distinct "trained immunity" in the two mouse strains. Overall, the manuscript has a wealth of data that will be of broad interest to the field.

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infection data comparison (H1N1:B1.351), which interestingly showed opposing phenotypes in K18 and 129S1 mice as compared to BA1 pre-infection. The manuscript is substantially improved from the original with detailed, descriptive discussion of the proposed model that the bronchoalveolar CD11c+ compartment is driving distinct "trained immunity" in the two mouse strains. Overall, the manuscript has a wealth of data that will be of broad interest to the field.

Reviewer #3

(Remarks to the Author)

The authors have sufficiently responded to many of my comments. Two comments were discussed in the response file but not added to the manuscript from what I can see. Can these comment please be addressed within the manuscript for clarity and transparency of the study? I have given details on these below.

Comment 3. Please give rationale for mouse numbers used in the analysis per time point.

Author's Response: All experiments were performed with at least 4-5 mice per condition, per mice strain. In this and previous studies, we found this number of biological replicates sufficient to provide statistical strength to the results observed. Therefore, and guided by the recommendations of the Institutional Animal Care and Use Committee of our institution as well as the principle of the 3R's, we selected these mouse numbers.

New Comment in Reply: The mouse numbers per analysis were not added to each figure, in the materials and methods, or addressed at all in the main body of the manuscript. It is important to report the numbers of mice that were included in each analysis for transparency. Please add the mouse numbers used to generate the data points.

Comment 6. Please give rationale as to why the study was terminated 4 days after the secondary inoculation when the height of pathology would most likely be observed between day 6 and 8.

Author's Response: We thank the reviewer for this important question Infection of mice with B.1.351 variant (and other SARS-CoV-2 variants) has been consistently shown to peak around 3-5 dpi, both in terms of bodyweight loss and in lung viral titers. We refer the reviewer to some of the available manuscripts in the literature that present these results (PMID: 34289384, 35264719, 35062015).

New Comment in Reply: The rationale for choosing day 4 post infection/inoculation for immunopathological analysis was not included in the revised version of the manuscript. Additionally, the references referred to by the authors in the response do not investigate pathology on day 4 post infection. In Martinez 2021, pathology was investigated on day 5. In Yasui 2022, pathology was investigated on day 7. In Halfmann 2022, pathology for B.1.351 was not investigated. Please add a rationale to the manuscript as to why day 4 post infection was chosen for analysis. Importantly, lung viral titers do not often coincide with maximum pathology. Additionally, body weight was still dropping in the Martinez report on day 4 post infection and weight loss maximum on day 4 did not coincide with pathology on day 7 of the Yasui report. Please give a rationale for the choice of day 4 post infection so that the reader can place the analysis in context with the literature.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the manuscript titled “Host immune responses associated with SARS-CoV-2 Omicron infection result in protection or pathology during reinfection depending on mouse genetic background.” Singh [et.al.](#) describe the different pathologies in K18 and 129S1 mice cause by reinfection by different SARS-CoV-2 variants. The authors find an interesting phenotype that Omicron (BA.1) preinfection, while providing similar antiviral protection against B.1.351 secondary infection, leads to enhanced lung pathologies in mice of 129S1 background. The authors hypothesize that the differential responses are due to CD11c+ cells which they show have persistent inflammatory signatures after 30 days after infection in 129S1 mice that is not present in K18. The authors thus show that ex vivo CD11c+ cells from either K18 or 129S1 mice respond differently from either mock or pre-infected animals when treated with LPS. Overall, the manuscript is well written, and experiments well done.

We thank the reviewer for taking the time and effort to review our manuscript as well as the kind words about our work and all the suggestions made below, that greatly contributed to enhance the scientific strength of the findings presented in the manuscript. Point-by point answers to all the comments and suggestions are included below.

The manuscript is deficient in a few key areas outlined below.

Major issues

1) Broad applicability of model/phenotype. While the authors acknowledge that “pre” infection with BA.1 and then reinfection with B.1.351 a contrived experimental model they don’t address how this phenotype may be broadly applicable or just an observation of the model?

We do agree with the reviewer that infection with BA.1 followed by B.1.351 does not match chronological events observed in society. However, as we mentioned in the discussion and justification of the research, we wanted to investigate if an infection with a SARS-CoV-2 variant that induces a mild disease phenotype, like BA.1, can protect against subsequent infection with a more severe SARS-CoV-2 strain. B.1.351 is more severe in mice compared to currently circulating Omicron variants, and therefore was chosen as a secondary challenge virus. This would reflect the potential emergence of a more virulent future variant in the human population.

While this point was made clear in the discussion of the manuscript, we decided to emphasize it and clarify it earlier in the manuscript, and now the initial results section has been modified with that purpose, now reading:

“Here, we tested whether mice that went through Omicron BA.1 infection would be protected from severe morbidity during B.1.351 reinfection. The selection of these two SARS-CoV-2 variants was based on our previous results demonstrating that BA.1 infection is mild and causes

no morbidity in mice whereas B.1.351 infection can cause severe morbidity in mouse models and is lethal in the K18-hACE2 mice model 13. Thus, while this infection regime contradicted chronological emergence of the variants, it allowed us to evaluate protection against severe infection conferred by mild disease-causing variant.”

- The study seems to suggest some sort of difference in trained immunity between K18 and 129S1 mice.

We want to thank the reviewer for suggesting that our observations may indicate a different induction of trained immunity based on genetic background. While we do agree with the statement, since trained immunity is associated with epigenetic changes, which we did not address in this work, we prefer not to use the terminology of “trained immunity” per se. Still, the potential implications of our results in trained immunity are addressed in the discussion section. Further investigation is currently being carried out in our laboratory to characterize several aspects of trained immunity in the context of influenza and SARS-CoV-2 infections, and will be communicated in the near future as a separate manuscript.

- Is this trained immunity pathogen specific, ie does pre infection with another respiratory virus such as Flu or just intranasal PAMP (polyIC or other) also lead to a similar exacerbation on pathology with B 1.351 challenge.

We want to thank the reviewer for this excellent question. We agree this is an important topic to address in the manuscript and additional experimental work has been included with that purpose. We infected mice with influenza H1N1 virus (NC99, A/New Caledonia/20/99) and reinfected them 30 days later with SARS-CoV-2 B.1.351 (same experimental layout to the BA.1:B.1.351 reinfection). Production of the virus and mice infection are described in the Methods, sections: “Cells and Viruses” and “Animal Experiments”. Results from this experiment have been combined with the initial experimental work and are presented now in Figure 2 and associated text. Interestingly, results are quite different from what was observed with BA.1 pre-exposure. 129S1 mice pre-challenged with NC99 showed a similar reduction in B.1.351 viral titers to BA.1 pre-challenged mice, but instead of increased disease severity during secondary infection they showed reduced severity. On the contrary, K18-hACE2 mice pre-exposed with NC99 presented similar B.1.351 infection severity and lung titers to naive mice infected with B.1.351. While we consider that this information is relevant and it is worth including in the manuscript there’s a fundamental difference in the model, as NC99 infection, unlike BA.1 infection, is extremely severe in 129S1 mice, and to a lesser extent, also severe in K18-hACE2, even at very low infection doses (100 PFU). We addressed this in the revised manuscript.

Do pretreated animals with unrelated virus or pamp have similar CD11c memory and ex vivo cytokine response?

For this question we want to refer to the following publication from Aegerter *et al.* (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6983324/>) in which it was shown that alveolar macrophages from mice that were sub-lethally challenged with influenza virus have altered cytokine and chemokine responses upon subsequent restimulation with LPS. We have added this information in the discussion.

While the Th1 vs Th2 phenotype in B6 and 129 is known, I think less is understood in the differences of Th1 vs Th2 in this more trained immunity model and could provide novel insights.

To address this excellent question, we have performed a T-cell depletion study in which 129S1 mice that were exposed to BA.1 were rechallenged with B.1.351 after depletion of T-cells performed 72h and 24h before rechallenging. This data has been now combined with previous results and is presented in Fig 3 and associated text. Briefly, although B.1.351 infection severity showed no significant differences due to T-cell depletion, the reduction in viral titers observed in BA.1:B.1.351 mice at 4 dpi was lost after T-cell depletion, suggesting an important role of adaptive immunity, and particularly T cells in protection through controlling of virus replication. The T-cell depletion correlated with a loss of both Type 1 and Type 2 cytokines in this group.

2) Mechanism. The authors suggest that the CD11c⁺ response drives the noted pathology, however, they don't really address this *in vivo*. The authors should show by flow cytometry or another quantitative method if there are difference in the total number of CD11c⁺ cell in lung and BALF at 30 days post BA.1 infection, and what types of cells these CD11c⁺ cells are. As the authors note, CD11c is expressed on many different cell types, and thus are their difference is the CD11c cells in lungs and BALF between k18 and 129S1 both in terms of number and phenotype.

To address this excellent question, we have performed flow cytometry to immune-phenotype the CD11c⁺ compartment of naïve, NC99 and BA1-exposed K18 and 129S1 mice, both at an early timepoint (7dpi) as well as the time in which reinfection was performed in the other experiments (30dpi). The data are now presented in the new Figure 4 of the manuscript and associated text. Briefly, Overall CD11c⁺ cells were significantly increased in BA.1-challenged k18-hACE2 mice at 30 dpi. FlowSOM and UMAP analysis of the CD11c⁺ compartment revealed a predominance of alveolar macrophages in mock groups, representing between 83-93% of CD11c⁺ cells in 129S1 mice and 60-76% in K18-hACE2. As expected, NC99 infection led to an extreme reduction of MHCII⁺ alveolar macrophages at 7 dpi in both strains (13.1% in 129S1 and 16.4% in K18-hACE2) and a strong recruitment of inflammatory monocytes (as characterized by Ly6c expression) to replace the macrophages lost. While BA.1 infection led to some alveolar macrophage loss, this was very limited compared to NC99 infection. Infiltrating inflammatory monocytes were more abundant in K18-hACE2 than 129S1 at 7 dpi (16.3% and 9.45% on average, respectively), difference that increased even more at 30 dpi (20.3% and 1.87% on average, respectively). Other CD11c⁺ immune cells, including different subpopulations of dendritic cells revealed less

remarkable differences in BA.1 challenged mice, and increased levels were mainly associated with NC99 challenge.

Do CD11c cells have an effect in vivo? This could be easily performed with CD11c-DTR cross to K18 and 129S1, a homozygous-to-homozygous cross could allow for selective depletion prior to reinfection.

This is an excellent question, however, from previous depletion studies with CD11c-DTR mice as well as with clodronate-based depletion methods, and after consulting multiple experts in the field, we reasoned that depletion by itself will trigger host responses that affect the outcome of reinfection. For example, the group of Bart Lambrecht has shown that upon sublethal influenza infection, CD11c+ DCs in the lung of influenza-exposed mice are responsible for induction and maintenance of inducible bronchus associated lymphoid tissue (iBALT)¹. Depleting CD11c cells resulted in loss of iBALT, a tertiary lymphoid organ that correlates with protection during reinfection². Moreover, depletion of CD11c+ cells and the associated cell death will induce innate immune responses that will affect reinfection as well, thereby not allowing a simple readout to estimate the contribution of virus exposed alveolar macrophages and DCs to the observed protection/disease phenotype in 129S1 and K18-hACE2 mice. Moreover, since some of our observations are linked to mouse models with different genetic backgrounds, crossing CD11c-DTR mice with 129S1 or K18-hACE2 mice would require multiple backcrosses. Therefore, we have not performed CD11c-DTR depletion studies at this point.

Minor

1) In line 47-47 the authors write that K18 and 129S1 mice have similar “levels of germinal center B-cells” however, there is no data presented in this manuscript with GC data? Please add data or remove as this is misleading.

We agree with the reviewer on this point, and it is now removed from the abstract so to avoid confusion. The sentence reads now:

“Interestingly, the enhanced pathology observed in 129S1 mice upon reinfection cannot be attributed to a less efficient induction of adaptive immune responses to the initial BA.1 infection, as both K18-hACE2 and 129S1 mice exhibited similar B and T cell responses at 30 DPI against BA.1, with similar anti-BA.1 or B.1.351 spike-specific ELISA binding titers and SARS-CoV-2-Spike specific tissue-resident T-cells.”

2) In figure 3C total number of cells is not calculated/represented, and so stating “mice from both genetic backgrounds had similar numbers of SARS-CoV-2 spike-specific CD8+ T-cells and tissue resident memory CD8+ T-cells” is incorrect as they had similar percentages. It would be important to know total number of cells as this could have a significant impact on phenotype.

We thank the reviewer for the careful revision of the manuscript. We agree that total number of T cells could have a significant impact on phenotype. When cell numbers are considered, similar to the outcome when percentage is considered, no additional differences between

strains were detected. Therefore, we have modified the text to clarify that proportions are presented in the figure.

3) It would be important not just to show CD8 T_{rm} responses, but also CD4 responses and phenotype. E.g. is there a Th2 response in 129 mice and a Th1 response in K18 from the CD4 cells as well. It certainly seems like this may be the case based on cytokine data, however showing CD4 cytokine and phenotype in lung prior to after initial challenge would be important and may lead to different or additional important conclusions. I think that the conclusion that T cell responses are not playing a role is unsubstantiated. Additionally, the author could perform CD4/CD8 depletion prior to second infection to assess role of T cells in their phenotype.

We agree with the reviewer on this excellent point. We have performed T-cell depletion studies as highlighted in a previous question of this reviewer, now results included in Figure 3. We concluded that T-cells are not a major driver of the phenotype in K18-hACE2 mice, and in 129S1 mice are needed to reduce B.1.351 lung titers at 4 dpi in BA.1 pre-challenged mice. As hypothesized by the reviewer T-cell depletion in BA.1:B.1.351 129S1 mice led to a reduction of Th2 cytokines that were elevated in the Mock:B.1.351 even after T-cell depletion, but also a reduction of Th1 cytokines was observed.

4) In figure 4 I am not sure why they chose LPS as the comparison as this is a known PAMP, but one that is related to bacterial infection rather than viral infection, PolyI:C or another RLR ligand would have been a better comparison.

While we agree with the reviewer that other stimuli such as PolyI:C or another RLR ligand could be an interesting and maybe more focused choice for stimuli, much of the current knowledge on monocyte-macrophage trained immunity comes from stimulation experiments with LPS. We bring the attention of the reviewer to excellent recent publications making use of it (PMID: 35856089, 37615937, 27863248). Thus, we believe that using this stimuli we and others we'll be able to contextualize our results much better and add to an already solid pool of scientific literature.

Additionally figure 4B is somewhat confusing, I think a PCA or other dimensionality reducing analysis may provide a clearer representation of the data. How are the Genes in 4B decided? Are those the top DEGs? Additionally looking at fig 4B it only seems like a few genes are differential between K18 and 129S1? The authors should address this more in the discussion.

We agree with the reviewer that the Sankey plot in Figure 4B might be take some extra effort to interpret, but we believe is a valuable visualization to represent how relevant genes to the immune response against SARS-CoV-2 present widely different expression levels in the uninfected state and how that expression is differentially modified depending on genetic background by BA.1. Selection criteria in this plot were a combination of a differential

expression due to BA.1 infection (Differentially expressed genes with a $\log_2(\text{Fold change}) > |1|$) as well as relevance in the immune response. This is now clarified in the text associated to the figure. We have also included an additional plot which shows the Top 100 up and down regulated genes by BA.1 in CD11c+ cells from 129S.1 and K18-hACE2 mice.

Reviewer #2 (Remarks to the Author):

Summary: Singh et al examined the immune profiles in response to SARS-CoV-2 infection (BA.1) and heterotypic reinfection (B.1.351) in mice of two different genetic background to determine which genetics factors, if any, play a role in protection and/or pathology. They determined that both mouse strains, K18-hACE2 and 129S1, had similar induction of the adaptive immune response (roughly equivalent SARS-2 antibody titers and tissue resident T-cell counts) and similarly reduced lung viral loads. However, heterotypic reinfection with B.1.351 demonstrated severe morbidity (body weight loss, evident lung histopathology) in 129S1 mice whereas K18-hACE2 mice were protected. Cytokine profiling suggested a severe inflammatory profile (large increases in IFN γ and IL18) in reinfected 129S1. Ex vivo stimulation of BALF-isolated CD11c+ cells isolated post-BA.1 (30 DPI) demonstrated profoundly different cytokine profiles upon ex vivo stimulation with B.1.351 or LPS, showing that 'pre-infection' with BA.1 resulted in very different levels of priming of the lung myeloid cells in each mouse strain. This was further supported by transcriptomic profiling of the CD11c+ cells with very different cellular profiles and gene ontologies, with a robust adaptive immune profile in K18 whereas 129S1 exhibiting strong pro-inflammatory signatures. Overall, the large differences seen upon heterotypic SARS-2 reinfection in these two different genetic backgrounds demonstrates the role of altered lung myeloid compartment responsiveness in SARS-2 disease severity and/or protection.

We thank the reviewer for taking the time to review our manuscript and for all the comments and suggestions provided. We proceed now to answer point-by-point to the reviewer comments

Critiques:

Line 38: "he" should be "the" - [Corrected](#)

Line 270: "wether" should be "whether", and capitalize "omicron" - [Modified](#)

Line 281: no Figure numbers listed.

[We thank the reviewer for the careful reading of the manuscript. The figure correct number was 2A \(i\) and B \(i\), now added to the manuscript.](#)

Line 290: no Figure numbers listed

[The figure number is added now. Figure 2A \(iii\) and B \(iii\)](#)

Lines 291-293 and Supplementary Figure 1A: Authors state that 129S1 mice show higher presence of macrophages with eosinophilic cytoplasm for all groups irrespective of any prior infection and point to Sup Fig 1A. Not seeing this in Sup Fig 1A. Only the BA.1-B.1.351 129S1 are shown there. It may be better to state 'data not shown'. Sup Fig 1A does not show baseline mouse images for 129S1 mice.

We thank the reviewer for this comment. Supplementary Figure 1 A(iv) presents an image from 129S1 mice pre-challenged with BA.1 and subsequently infected with B.1.351 to show the macrophages with eosinophilic cytoplasm as a hallmark of 129S1 lung inflammation. As this observation occurs regardless of the pre-exposure with BA.1 or not, the image is representative of both Mock:B.1.351 group and BA.1:B.1.351. Indeed, no 129S1 baseline image is shown but the text in those lines only refers to infected mice. Still, we agree with the reviewer that the text could lead to confusion, and has been now clarified to:

“On closer examination, B.1.351-challenged 129S1 mice show higher presence of macrophages with eosinophilic cytoplasm; this was true for all 129S1 mice irrespectively of the BA.1 any prior infection or not, unveiling itself as a distinct hallmark of 129S1 mice inflammatory response [supplementary figure 1A].”

Supplementary Figure 1A: Please label each of the image panels to make it more easy to interpret as the legend is somewhat difficult to follow. Labels are included in each image panel, from A(i) to A(iv). Each image is independently described in the legend for clarification to the reader

Line 298: "propmted" is misspelled
Corrected

Line 300-301: Not sure what the authors are referring to by "mock-infected animals in the BA.1 pre-infected K18-hACE2 mice". This seems to mean BA.1:mock but that is not shown in Supp Fig 1B. If BA.1:mock data exists for cytokine analyses, it is missing from the figure. This is shown in Figure 4 with the ex vivo stimulation experiments. Further, Supp Fig 1B text size on x-axis is too small and hard to decipher.

We thank the reviewer for the careful correction of the manuscript. The text was indeed confusing and led to the conclusion that a BA.1:Mock group (that did not existed) was analyzed. Text has been re-written now for easier interpretation. It now reads:

“On the other hand, levels of these cytokine/chemokines in the BA.1:B.1.351 group were found to be similar to those in the Mock:Mock K18-hACE2 animals.”

INCREASE FONT SIZES

Line 302: Should be Supplementary Figure 1B? and capitalize "interestingly"

The reviewer is correct, modified to 1B and interestingly is now capitalized

Figure 2: Please increase the size of the lines in the legend for panel 2B. Also, it may make more sense to move legends in 2B to bottom of panel 1A. Also, please add label to panel 2C heatmap legend.

Figure 2 has been now modified to include new data from H1N1 pre-challenged mice (we refer to Reviewer #1 comments for further information). Additionally, all the formatting suggestions from the reviewer have been included in this new figure

Line 338: "wether" should be "whether"

Corrected

Figure 3: Increase the size of the lines in the legend here as well. Difficult to see differences in line color due to small size.

Now modified

Line 380: Were the numbers of CD11C+ cells isolated from BALF substantially different for the two mouse strains. I am not seeing any data regarding cell counts, etc.

We have addressed this by performing flow cytometry to do in depth characterization of the CD11c+ compartment in bronchoalveolar fluid. All the information is included now in figure 4 and associated text. We refer to our answers to reviewer 1 for in depth explanation of the new data.

Line 386-387: States that the levels of TNF-alpha was 2x higher in 129S1. Is this referring to the mock stimulated (i.e. mock stim, mock infected) or mock stimulated with prior BA.1 infection? I assume the latter but the text is not clear.

The text refers to the mock infected group, that is then stimulated with either nothing, B.1.351 or LPS. BA.1 challenged mice are also discussed in the following phrases.

Section spanning lines 386-402 is confusing when trying to interpret Figure 4A and Supplementary Figure 3A. Further, Supp Fig 3A text size is too small to decipher with confidence.

We thank the reviewer for this comment. We agree that the text in this section was hard to interpret and has now been modified for easier reading:

"The levels of TNF- α were high in mock groups of both models, being almost 2 times higher in 129S1 mice than in K18-hACE2 mice. These TNF- α levels in both mouse models mock-challenged, dropped when stimulated with the B.1.351 variant, where the 129S1 group showed \sim 10 times reduction, while K18-hACE2 only \sim 2 times reduction. When comparing mock-infected groups to BA.1-infected groups, TNF- α levels were lower in BA.1-infected mice, with a \sim 5 times reduction in K18-hACE2 and \sim 2 times reduction in 129S1 [Figure 4A, supplementary figure 3A]. The levels of MIP-2- α were also elevated and were similar in the mock groups of both models (\sim 1500 pg/ml), however only in 129S1 these levels

dropped ~5-fold when stimulated with B.1.351 variant [supplementary data]. Conversely, in BA.1 infected groups, the level of MIP-2- α was lower in the K18-hACE2 group compared to the K18-hACE2 mock infected group as well as the 129S1 BA.1 infected group [Figure 4A, supplementary figure 3A]. Surprisingly, we observed that IFN- γ , IL-17A, and IL-22 was only induced by LPS stimulation in BA.1 infected K18-hACE2. IL-13 and IL-18 were high in 129S1 mock group and were upregulated in the BA.1 infected group. However, these levels were reduced with B.1.351 stimulation, whereas LPS boosted production in all groups. BA.1 infected 129S1 CD11c+ BALF cells also showed higher levels of Eotaxin when compared to other groups [Figure 4A, supplementary data].”

Section spanning lines 403-439 lacks any intuitive flow and simply lists what can be seen in Figure 4B. A lot of this is resolved in the discussion section, but it would be beneficial to provide the intuitive flow seen in the discussion section to this results section to provide better context and interpretation to the data.

We thank the reviewer for this comment. Upon revision of the text, we agree with the reviewer that it was hard to read and follow intuitively. We have rearranged the section and added some information we believe would be valuable for the readers. Additionally, one more panel has been included in the figure to provide more relevant information on the differential gene expression of CD11c+ cells after BA.1 challenge

Figure 4 legend states "Mock inoculated" mice were euthanized...I believe this should be "BA.1 inoculated".

The reviewer is absolutely correct on this point. Thank you for the careful reading. Now modified.

Reviewer #3 (Remarks to the Author):

The manuscript by Singh and colleagues investigates the host characteristics which contribute to disease severity following SARS-CoV-2 infection using mouse models. Specifically host pre-existing immune and immune back grounds were investigated leveraging mice that had a previous SARS-CoV-2 infection as well as two different strains of mice to recapitulate various human genetic backgrounds. Specifically, K18-hACE2 (strain 2B6.Cg-Tg(K18-ACE2)2PrImn/J) and 129S1 (strain 129S1/SvlmJ) were used. Mice were mock infected or infected with BA.1 or B.1.351 and left to recover until Day 30 post primary infection. On Day 31 post primary infection, mice received a secondary inoculation with Mock or B.1.351. Necropsies were performed 4 days after secondary infection for the analysis of histopathology, viral loads, and cytokine profiling. Serum, tissues, and BALF samples were also collected on Day 30 post primary infection for passive immunization studies, flow cytometry, and cell stimulation studies. K18-hACE2 mice with a previous BA.1 infection were protected during a B.1.351 challenge; however, disease seemed to be increased during the secondary challenge of 129S1 mice. These mice had cytokine profiles more similar to that of humans with severe SARS-CoV-2 infection (IL-10, IL-1 β , IL-18, and IFN- γ). Adaptive immune responses (antibody levels and T cell numbers) were similar in both strains of

mice, suggesting T or B cells to not be the mechanism of disease. This was complemented by similar decreases in virus levels in the lungs. Investigations into the responses of CD11c+ cells isolated from BALF suggested differences in the antiviral responses and proinflammatory responses as a potential driver of differential outcomes in the two mouse strains. Taken together, the data presented offers insight into the complexities of immune responses per host as well as per SARS-CoV-2 variant which will be of interest to the community.

We thank the reviewer for taking the time to review our paper and for all the comments and suggestions provided that will most certainly improve the scientific quality of the work here presented. We proceed now to answer point-by-point to the reviewer comments

1. The largest shortcoming of the manuscript was the terminology used when referring to the inoculation of the animals. Various terms and abbreviations were used such as reinfection, DPI, DPI2, DPI1, 1st infection, etc. It would be helpful for the authors to choose terminology, define the meaning, and be consistent in its use throughout the rest of the manuscript.

We agree with the reviewer that terminology in the manuscript could get confusing so we have modified the manuscript to avoid the use of concepts such as DPI, DPI2, DPI1. Figure 1 where the experimental layout is presented has been improved. Additionally, as more experimental work has been performed, and more complexity is added to the manuscript we have defined some concepts and maintained them through the manuscript:

- Primary infection to define the first infection of naive mice
- Secondary infection to define an infection in mice already previously challenged
- Reinfection when primary and secondary infections are by a SARS-CoV-2 variants
- Only DPI are used to define days post infection, regardless of primary or secondary infection. All secondary infections are performed 31 days after the primary infection, so there was indeed no need to overcomplicate the terminology.

Additionally, it does not make sense to call a BA.1 primary infection followed by a B.1.351 secondary challenge as a reinfection. It is not a reinfection since the first and second viruses are not the same and it is not known if the second virus will infect due as pre-existing immunity may protect against the virus from producing an active infection. Please be cautious with the language.

While we do agree with the reviewer that a primary BA.1 infection and a secondary B.1.351 infection do not represent a “reinfection” in the strict sense of the term, as they are different variants and probably “secondary infection” or “heterotypic reinfection” is a more accurate term, “reinfection” is routinely used to describe these SARS-CoV-2 secondary infection by institutions such as CDC ([What Is COVID-19 Reinfection?](#)). Therefore, we prefer to use the terminology commonly used by the community. Still, as the reviewer brings up this valid point,

this is now clarified in the text to avoid any confusion.

2. Please give rationale why mice would receive a BA.1 virus followed by a B.1.351 virus as this is not the natural occurrence of SARS-CoV-2 epidemic waves.

We thank the reviewer for this comment. We agree that this point needs to be clarified and it is now in the manuscript. We refer the reviewer to our answer to the first comment of Reviewer #1 for this where it is now clarified.

3. Please give rationale for mouse numbers used in the analysis per time point.

All experiments were performed with at least 4-5 mice per condition, per mice strain. In this and previous studies, we found this number of biological replicates sufficient to provide statistical strength to the results observed. Therefore, and guided by the recommendations of the Institutional Animal Care and Use Committee of our institution as well as the principle of the 3R's, we selected these mouse numbers

4. The text in many of the figures was small and hard to read. In particular, the text in Figures 1, 2 and supplementary Figures 1 and 3 should be increased.

Figures have been now modified to improve readability. We apologize to the reviewer for any inconvenience this may have caused in the review of the manuscript

5. Please put a legend for the graphs in both Figure 2 Ai, Figure 2 Aiii, Figure 2 Bi, and Figure 2 Biii. It was very confusing to only have the legend in the B parts of this figure.

Legends are now included in all the panels of the figure to avoid potential confusions.

6. Please give rationale as to why the study was terminated 4 days after the secondary inoculation when the height of pathology would most likely be observed between day 6 and 8.

We thank the reviewer for this important question Infection of mice with B.1.351 variant (and other SARS-CoV-2 variants) has been consistently shown to peak around 3-5 dpi, both in terms of bodyweight loss and in lung viral titers. We refer the reviewer to some of the available manuscripts in the literature that present these results (PMID: 34289384, 35264719, 35062015)

7. There were several grammatical and spelling errors throughout the manuscript not limited to the following:

- a. Line 38 "he" should be "the"
- b. Line 107 – should Dr. Andy Pekosz be Dr. Andrew Pekosz
- c. In the section on Animal Experiments line 131-132 please state the time point at which the serum is collected
- d. Line 201 "lungs" should be "lung"
- e. Line 302 "interesting" should be capitalized
- f. Line 306 the comma after the bracket should be replaced with a period

- g. Is “raise” being used correctly in Line 306 - possibly should be rise or increase
- h. Line 311 “mice” replace with “mouse”

These and other grammatical errors have been addressed. We thank the reviewer for the careful revision of our manuscript

Point by point response to the reviewer's comments.

We want to thank the editors and reviewers for their help in making the manuscript better.

We have incorporated all responses as discussed in the previous point-by-point response letter in the manuscript and have provided a clear rationale regarding the timing for pathological analysis as well as a discussion of potential limitations associated with this analysis to address the remaining concerns raised by reviewer #3.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Significant improvement in this resubmission, no additional comments.

Answer: We thank Reviewer #1 for their positive answer.

Reviewer #2 (Remarks to the Author):

Summary: The revised manuscript has addressed my original concerns and has additionally provided a influenza H1N1 pre-infection data comparison (H1N1:B1.351), which interestingly showed opposing phenotypes in K18 and 129S1 mice as compared to BA1 pre-infection. The manuscript is substantially improved from the original with detailed, descriptive discussion of the proposed model that the bronchoalveolar CD11c+ compartment is driving distinct "trained immunity" in the two mouse strains. Overall, the manuscript has a wealth of data that will be of broad interest to the field.

Answer: We thank Reviewer #2 for their positive answer.

Reviewer #3 (Remarks to the Author):

The authors have sufficiently responded to many of my comments. Two comments were discussed in the response file but not added to the manuscript from what I can see. Can these comment please be addressed within the manuscript for clarity and transparency of the study? I have given details on these below.

Comment 3. Please give rationale for mouse numbers used in the analysis per time point.

Author's Response: All experiments were performed with at least 4-5 mice per condition, per mice strain. In this and previous studies, we found this number of biological replicates sufficient to provide statistical strength to the results observed. Therefore, and guided by the recommendations of the Institutional Animal Care and Use Committee of our institution as well as the principle of the 3R's, we selected these mouse numbers.

New Comment in Reply: The mouse numbers per analysis were not added to each figure, in the materials and methods, or addressed at all in the main body of the manuscript. It is important to report the numbers of mice that were included in each analysis for transparency. Please add the mouse numbers used to generate the data points.

Answer: If this was not mentioned before, we have now added the mouse numbers to each figure legend, which also mentions the statistical tests that were performed with the data represented in those figures. We have also mentioned in the Methods section at line 201 that mouse numbers for each analysis can be found in each figure legend. Where relevant, mouse numbers are also mentioned in the main text.

Comment 6. Please give rationale as to why the study was terminated 4 days after the secondary inoculation when the height of pathology would most likely be observed between day 6 and 8.

Author's Response: We thank the reviewer for this important question. Infection of mice with B.1.351 variant (and other SARS-CoV-2 variants) has been consistently shown to peak around 3-5 dpi, both in terms of bodyweight loss and in lung viral titers. We refer the reviewer to some of the available manuscripts in the literature that present these results (PMID: 34289384, 35264719, 35062015).

New Comment in Reply: The rationale for choosing day 4 post infection/inoculation for immunopathological analysis was not included in the revised version of the manuscript. Additionally, the references referred to by the authors in the response do not investigate pathology on day 4 post infection. In Martinez 2021, pathology was investigated on day 5. In Yasui 2022, pathology was investigated on day 7. In Halfmann 2022, pathology for B.1.351 was not investigated. Please add a rationale to the manuscript as to why day 4 post infection was chosen for analysis. Importantly, lung viral titers do not often coincide with maximum pathology. Additionally, body weight was still dropping in the Martinez report on day 4 post infection and weight loss maximum on day 4 did not coincide with pathology on day 7 of the Yasui report. Please give a rationale for the choice of day 4 post infection so that the reader can place the analysis in context with the literature.

Answer: We thank the reviewer for these constructive remarks. We meant that in our experiments the peak of virus replication is around 3-5 dpi. In 129S1 mice, this typically also coincides also with peak body weight loss. This however does typically indeed not coincide with maximum pathology, as it might be too early as the reviewer suggests. Because virus titer and body weight loss are an important read out for our experiments, we have chosen to terminate the experiment at 4 dpi. This, in our opinion, gave the best window to look at virus titer and allowed to use body weight loss as primary read-outs for protection, or lack thereof. As mentioned above, this most likely is too early to observe maximum pathology. We have mentioned this now in the manuscript at line 377 in the results section and have repeated this as a limitation of this study at line 701 in the discussion section.