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Peer Review File

Single-cell transcriptomics of bronchoalveolar lavage during PRRSV infection with different virulence

Corresponding Author: Professor Jun-Mo Kim

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.
Editorial Note: Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data or to remove third-party material where no permission to publish could be obtained.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors conducted a large-scale single-cell analysis of bronchoalveolar lavage fluid, coupled with an examination of clinical outcomes associated with strains of varying PRRSV virulence levels (NA8 low, JA142 intermediate, and NA10 high). The investigation employed multiple techniques such as single-cell transcriptomics and flow cytometry. The authors found that highly virulent infections exhibit a rapid viral replication pace, early infiltration of lymphocytes into the lungs, and prompt macrophage loss. The authors claimed they have identified a specific anti-inflammatory M2-like macrophage (SPP1-CXCL14high macrophage) that contributes to recovery from intermediate virulent infections. Finally, the authors concluded that highly virulent infections accelerate bystander cell death regulated by exosomal microRNAs, ultimately leading to fatal outcomes. I commend the authors for taking on the challenge of generating and analyzing the first single-cell dataset of BALF in pigs with varying virulence levels of PRRSV. However, the current manuscript suffers from the lacking of description of the necessary details in the methods, insufficient interpretation of biological issues, less than ideal presentation of results, and writing that needs to be more polished. Therefore, I do not think this manuscript is suitable to be published in Nature Communications.

1. The single-cell clustering results don't seem convincing. According to the description in the methods section (Pages 23-24), the authors used R package Seurat (v4.3.0) to perform clustering and annotated cells with some canonical markers. However, more details on how they did the clustering and annotation process and the should be explained more clearly. If the authors performed the standard clustering pipeline of Seurat (eg. https://satijalab.org/seurat/articles/pbmc3k_tutorial.html), it is confusing to me that the cells are of low heterogeneity in Fig. 2a, at the same time unknown cells are scattered in the UMAP plots of Fig. 2a and Fig. 3a. First, although there is currently no single-cell BALF dataset on PRRSV-infected pigs, there are some published articles of single-cell BALF datasets (eg. PMID 32398875, and PMID 34051097). These immune cells should show differences on UMAP plots at least on the three-cell categorical level (not detailed cell types) that the authors defined in their article. Second, the unknown cells should be the single cell cluster(s) that cannot be annotated with known markers. How possibly can they be scattered in the UMAP clustering? Did the authors perform cell annotation on separate samples but only display the integrated clustering results by Harmony?

2. Although the authors attempted to discuss the changes of cell populations in different virulence groups, very limited investigations of molecular characteristics and discoveries of potential mechanisms in comparing different virulence groups are made. The authors provided GSVA results of different cell groups in Fig. 6, but it still remains unknown what important genes are included in this process and what caused this difference. Statistical tests in evaluating the differences should also be provided.

3. The velocity fields in Supplementary Fig. 11, Supplementary Fig. 13, and Supplementary Fig. 16 seems not reliable enough to draw conclusions. There are no clear patterns in the vector fields on UMAP. More importantly, from the distribution of cell Dpi (which I guess is day after infection, since no explanation of this abbreviation was provided), the cells are mixed up, which meant the inferred vector fields are not in accordance with real-time developmental patterns. Therefore, the vector fields that the authors presented cannot be biologically interpreted, which may be caused by over-correction by Harmony. The authors could try other methods to minimize the impact of batch effects when performing vector field analyses, such as scTour (PMID: 37353848).

4. In the time series analysis, especially in the result of Fig. 3e and Supplementary Fig. 10, the variance of some figures seems very large. Significance tests in corresponding dpi in line plots in should be made to further evaluate the changes, not different dpi of different virulence.

Other concerns:

1. Throughout the manuscript, do the authors mean the “control” group instead of the “negative” group? If not, control groups should be set to fully characterize the changes of cell populations in different virulence groups.

2. Page 3 line 20, “Arteriviridae” and “Nidovirales” should be italic.

3. The introduction should add more current knowledge of single-cell studies of BALF in animals (eg. Homo sapiens) with infections and add the current knowledge of miRNA regulations to emphasize the urgent need for the authors’ research.

4. It may be better for the authors to enhance the writing. It is very hard to comprehend why the authors studied single-cell compositions first but ended with conclusions of miRNAs.

5. Fig. 2d, Fig. 2f, Supplementary Fig.7a, Supplementary Fig.7c and Supplementary Fig.7d are missing discrete cell labels or continuous color bars to explain the meaning of different colors of cells, which made it hard to explain the meaning of the figures.

6. The labels in Supplementary Fig. 11a, c, e, g and Supplementary Fig. 30 are hard to read, which made it very hard to understand what information the figures provided.

7. To compare the gene expression characteristics in different virulence conditions, the dot plots in Fig. 2, Fig. 3 and Fig. 4 should be split by different virulence conditions to show the annotation accuracy. If possible, split feature plots should also be provided.

8. The authors only focused on the expression levels of cell communication-related receptor genes in Supplementary Fig. 18b, Supplementary Fig. 19b, Supplementary Fig. 20b, Supplementary Fig. 21b, Supplementary Fig. 22b, Supplementary Fig. 23b, Supplementary Fig. 24b, Supplementary Fig. 25b, Supplementary Fig. 26b, Supplementary Fig. 27b, Supplementary Fig. 28b, and Supplementary Fig. 29b. However, the cell communication levels of ligand-receptors should not only be inferred by the expression levels of receptor genes, but also the expression of ligand genes. Therefore, many methods to infer scRNA-seq can be applied to evaluate the communication strength, such as CellPhoneDB (PMID: 32103204) and CellChat (PMID: 33597522).

Reviewer #2

(Remarks to the Author)
This manuscript contains a comprehensive single cell RNA Seq analysis of BAL cells after infection of pigs with 3 different PRRSV strain that differ in virulence. The data is complemented by BAL fluid cytokine levels, flow cytometry, virological, clinical and pathological data. Overall, the data quality is very good with some weak points listed below.
It would be really great to understand the relationship between these different elements but for this the study design is not ideal. A main difficulty for the conclusions is that not all groups were followed to the recovery phase. Therefore, it is not possible to conclude on a possible role of the SSP1+ macrophage population that emerged at day 14 in the JA142 group. This time point is missing with the two other viruses. In addition, with only three animals per time point, it may be risky to draw conclusions. Certainly, it is not correct to state that the SSP1-CXCL14high macrophage contribute to recovery (Abstract) or that the data set reveals the mechanisms for lung recovery. The authors need to tune down their conclusions and acknowledge the fact that the work remains descriptive by nature. Having said that, it is fine to speculate and propose additional work that will be required to consolidate the findings.
I also miss pointing on the fact that looking in the BAL compartment only is unlikely to give data on the mechanisms of lung recovery. We need to understand how that data correlates to events in the lung and lymphoid tissue. I understand that this cannot be included in such a study that is already fully packed with data, but these points should not be ignored either.

Despite these critical points, this work is a fantastic resource for future work and will be very helpful for the design of future work.

Additional critiques
1. DC definition based on FCM are not precise enough. It is not possible to properly differentiate DC based on MHCII, CD163 and CD172a. For example MHCII+CD172a-CD163- can be B cells. Also the differentiation of MO and MoDC based on CD163 expression is not secured. Accordingly, Supp. Fig. 2 and the text needs to be adapted.
2. I am missing Figure legends directly under the Figs. And Supplementary Figs. This makes it troublesome to review.
3. In Fig 3 22 different macrophages cluster were identified but is remains unclear how this was done. Were these found in all pigs? Where they also found following infection.
The same applies to the DC. In the single cell data, cDC and pDC are defined. However, it is not explained how. Furthermore, this data cannot be linked to the FCM data.
4. Lines 585: please explain how the 21 genes were identified to annotate cell types. To me this seems very little genes
5. It is unclear to me how a cluster can be traced over time. If SPP1 macrophage “increase” how can this be differentiated

from a upregulation of SPP1 (or the other genes defining this cluster), eg induced by IFN.
It needs to certain that the defined macrophages are real clusters that can by clearly be identified as clusters in a time and treatment independent manner. The past has shown that defining new “subsets” basing on scRNA-Seq data can be problematic.
6. Similar remarks apply to the T cells. Is it really helpful to defined so many T cell subsets, in particular considering that they follow the same trend?
7. Also for the B cells/plasma cells. How helpful and accurate is it to defined so many clusters.
What are CD3E+ B cells? How sure can you be that these are not T cells?
8. In the Chapter on cytokines, chemokine and their receptors it is referred to a series of Supplementary Figures 18-29. This is not reader friendly has the evolution of the response cannot be appreciated. Please summarise the group-dependent changes over time for the most important responses.
9. Why are the pDC missing in the chapter on cytokines? They are the likely source for IFN-a. Can that be verified with this dataset?
10. Supplementary Fig. 30 is unreadable.

Reviewer #3

(Remarks to the Author)
The present paper examines, using a single cell transcriptomic analysis, the development of PRRSV infection in lungs of animals infected by three different isolates of presumed different virulence. The paper is interesting and may contribute to a better knowledge of the pathogenesis of this infection. In its present form it is a comprehensive examination of the changes of different cell populations (macrophages, T and B cells, etc.), examines the cytokines present in BALF, analyses the transcriptome of infected and bystander cells at a single cell level and focuses on exosome miRNA expression in the studied cases. This comprehensiveness results in the inclusion of so much information that the reader feels overwhelmed, making it very difficult to discern what is central to the proposed question and what is secondary.
The authors designed an experiment including 3 different strains that were considered to be of different virulence and they performed an experimental infection. In the design, the groups inoculated with the strains of high and low virulence (NA8 and NA10) only contained 6 animals while the negative control and the group of moderate virulence were composed of 12 animals. As a result, the high and low virulence groups were only followed up to 7 pi while the other two groups were followed until 21 pi. This is a problem because comparisons for all groups are only possible during those 7 days. Why this unequal distribution of groups? Looking at the data of those 7 days it is difficult to assess the different degrees of virulence, particularly between JA142 and NA8. For example, looking at the data of strain NA10 in Figure 1d, at 3 dpi the microscopic lesions of those animals were not significantly different than those of the controls despite that at 7 dpi the scores were very high, and the picture shows a severe interstitial pneumonia. In contrast, viral load in blood for NA10 was the highest for all strains. In contrast, the severity of microscopic lesions (based on the scores and pictures) of JA 142 at 14 and 21 pi seems to be approximately equal than that of NA10 at 7 pi. Is JA142 less virulent than NA10 or it results in an equally severe process but with a slower development (if so, why?). It cannot be known with this design. With this in mind, the interpretation that is made in Figure 8 (that is the corollary of the work) needs to be revised. I’m not sure that the comparison and the delimitation of the phases is so straightforward. This is critical point.
By the way, the authors used a commercial kit and produced a curve for correlating Ct with TCID using only the JA142. Any mismatch between the primer (or primers) sequences and the sequences of any of the strains may easily result in a drop of about 2-6 Cts. Why did the authors not build the titration curves using a series of dilutions of each strain? The differences in viral loads (viremia) at 7 pi were 1.6 log10 between the highest and the lowest. Can this be an artifact created by the way you calculated them? Differences in virulence are the basis of the study so, assessing of viral loads is critical. This must be corrected, or proof of the similar performance of the PCR for all three strains must be showed somewhere.
Besides, some interesting results need further discussion to better understand the model. For example, NA10 was inducing higher IFN signalling pathways in early (3 pi) infection than the other strains and had a relative low number of myeloid infected cells. In contrast, at 7 pi, the proportion of myeloid infected cells in JA142 was higher but with high dispersion of results. This would not be in favour of a different virulence between the strains.
In my opinion, the most meaningful comparison is between JA142 and the negative control if the aim is to determine the mechanisms involved in “recovery” of the lung. However, I insist, the results suggest that recovery of lung lesions is not that clear since the pictures and the microscopic scores at 14pi and 21pi for JA142 look similar (no statistical comparison provided). Removing of the data that include the other strains and simplifying the writing, in the sense of trying to produce a shorter paper, (the current version is 85 pages including 33 supplementary figures) would help to better understand the significance of the work.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed most of my comments to the previous version of the manuscript. The revised manuscript has

been improved to a certain extent. However, below are some more adjustments or clarifications needed to further support the authors' findings.

1. The data variation in the negative group of MRC1-NREP high macrophage cells and the NA8 group of MT1A high macrophage cells in Fig. 3E, as well as the JA142 group of ATII cells in Supplementary Fig. 17 are still very large. I'm very concerned about the reliability of the conclusion drawn by these figures (page 8, lines 152-154; page 9, lines 191-193; page 16, lines 388-392).

2. I still insist that the demonstration of quantitative evaluation of the communication strength using analytical tools or experimental verification is necessary to ensure the actual existence of cell communication within different cell types. Since the authors have previously evaluated the communication strength using CellChat, there are many ways to interpret the complex results meaningfully. For example, they can list all significantly predicted ligand-receptor pairs within a supplementary table or choose to visualize certain pairs of the predicted ligand-receptor pairs by functions provided by CellChat. I was quite surprised when they stated that they decided not to include them due to concision issues, as it is common practice in many articles to present ligand-receptor analysis results in this manner (as shown in PMID 37824652, PMID 38649488, and PMID 38480880). Moreover, CellChat could provide comparative analysis under two conditions, which made it meaningful to compare the altered probability of ligand-receptor pairs in different cell types, even by the circle plots.

3. In newly added violin plots of Fig. 6B, and Supplementary Fig. 30, are the minimum values of the gene expression levels zero?

Reviewer #2

(Remarks to the Author)
The authors have addressed my previous comments partially in a satisfactory manner. Nevertheless, I still believe that it is not possible to conclude that the SPP1-CXCL14+ macrophages were identified as "the potential key cell population that contributes to recovery" (stated in the abstract). If this population did not emerge in virulent infection, it might just be a matter of timing. For such a statement I would like to see data showing how the emergence of this population correlates with disease control. This would of course require an animal experiment with sufficient number of animals and a variability in the outcome after infection with one intermediate strain. Furthermore, recovery is most likely impossible without the adaptive immune system kicking in. Therefore naming this macrophage population "the potential key" is scientifically problematic.

Along the same line, the summary Figure 8 claims to have identified "the mechanisms of lung recovery" (Title of Figure 8). This is scientifically not correct and does not reflect the content of the Figure and the manuscript, which is descriptive in nature and not suited for this purpose.

As previously mentioned, the study has its merits as an important resource paper for future studies on porcine BALF, and as a manuscript helping to build new working hypotheses to understand PRRSV pathogenesis. This is probably important also for other respiratory infection and might also have be important for human lung infection. So once again, the authors should systematically check their manuscript if statements are scientifically justified and correct accordingly.

In addition, a language check is required.

Reviewer #3

(Remarks to the Author)
The author exhaustively answered most of the questions and made an effort to improve the readability of the manuscript. However, I still think that the lack of data for NA8 and NA10, although understandable, is a point. The authors sent a very elaborated answer to this question, presenting data that help clarifying the different degrees of virulence, I suggest that the authors should mention something on this on the discussion.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I appreciate the authors' responsiveness to the feedback. The manuscript is now acceptable in terms of the analysis. I have no further comments.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors conducted a large-scale single-cell analysis of bronchoalveolar lavage fluid, coupled with an examination of clinical outcomes associated with strains of varying PRRSV virulence levels (NA8 low, JA142 intermediate, and NA10 high). The investigation employed multiple techniques such as single-cell transcriptomics and flow cytometry. The authors found that highly virulent infections exhibit a rapid viral replication pace, early infiltration of lymphocytes into the lungs, and prompt macrophage loss. The authors claimed they have identified a specific anti-inflammatory M2-like macrophage (SPP1-CXCL14high macrophage) that contributes to recovery from intermediate virulent infections. Finally, the authors concluded that highly virulent infections accelerate bystander cell death regulated by exosomal microRNAs, ultimately leading to fatal outcomes. I commend the authors for taking on the challenge of generating and analyzing the first single-cell dataset of BALF in pigs with varying virulence levels of PRRSV. However, the current manuscript suffers from the lacking of description of the necessary details in the methods, insufficient interpretation of biological issues, less than ideal presentation of results, and writing that needs to be more polished. Therefore, I do not think this manuscript is suitable to be published in Nature Communications.

1. The single-cell clustering results don't seem convincing. According to the description in the methods section (Pages 23-24), the authors used R package Seurat (v4.3.0) to perform clustering and annotated cells with some canonical markers. However, more details on how they did the clustering and annotation process and the should be explained more clearly. If the authors performed the standard clustering pipeline of Seurat (eg. https://satijalab.org/seurat/articles/pbmc3k_tutorial.html), it is confusing to me that the cells are of low heterogeneity in Fig. 2a, at the same time unknown cells are scattered in the UMAP plots of Fig. 2a and Fig. 3a. First, although there is currently no single-cell BALF dataset on PRRSV-infected pigs, there are some published articles of single-cell BALF datasets (eg. PMID 32398875, and PMID 34051097). These immune cells should show differences on UMAP plots at least

on the three-cell categorical level (not detailed cell types) that the authors defined in their article. Second, the unknown cells should be the single cell cluster(s) that cannot be annotated with known markers. How possibly can they be scattered in the UMAP clustering? Did the authors perform cell annotation on separate samples but only display the integrated clustering results by Harmony?

Our response: Thanks for the reviewer's kind concern. We generated large amounts of single-cell data (306,052 cells), so cells were initially broadly classified (Fig. 2a) and subsequently examined in detail following the re-integration of subpopulations (myeloid, lymphoid 1, and lymphoid 2) (Fig. 3a, 4a, 5a). During the process of determining cell types, we integrated adjacent clusters that exhibited similar features across all cells, resulting in the appearance of low heterogeneity. The unknown cell type is thought to have been visualized in a scattered form because unknown clusters were classified in re-integration of subpopulations and combined as one unknown cell type (because of only few cells). As the reviewer mentioned, we also expected three distinct categories (myeloid, lymphoid, and epithelial) in the UMAP, but some cells were connected among them. In this study, we observed numerous proliferating cells in BALF samples from weaned pigs. These cells shared similar proliferative characteristics, which is likely why some of them appeared connected. Additionally, only a few cells were identified as epithelial. UMAP clustering was performed four times: for all cells, myeloid, lymphoid 1, and lymphoid 2. We further described more information in the manuscript to avoid confusion (page 25, lines 604-607; lines 624-627).

2. Although the authors attempted to discuss the changes of cell populations in different virulence groups, very limited investigations of molecular characteristics and discoveries of potential mechanisms in comparing different virulence groups are made. The authors provided GSVA results of different cell groups in Fig. 6, but it still remains unknown what important genes are included in this process and what caused this difference. Statistical tests in evaluating the differences should also be provided.

Our response: Thank you for your helpful comment to improve qualities. As the reviewer commented, we included the key genes that exhibit differential expression with statistical test in various virulent

groups (Fig. 6b). We further described related methods and results in the manuscript (page 26, lines 630-633).

3. The velocity fields in Supplementary Fig. 11, Supplementary Fig. 13, and Supplementary Fig. 16 seems not reliable enough to draw conclusions. There are no clear patterns in the vector fields on UMAP. More importantly, from the distribution of cell Dpi (which I guess is day after infection, since no explanation of this abbreviation was provided), the cells are mixed up, which meant the inferred vector fields are not in accordance with real-time developmental patterns. Therefore, the vector fields that the authors presented cannot be biologically interpreted, which may be caused by over-correction by Harmony. The authors could try other methods to minimize the impact of batch effects when performing vector field analyses, such as scTour (PMID: 37353848).

Our response: We agreed with the reviewer's concern. In this study, we identified M2-like macrophages that might contribute to damage healing, anticipating macrophage polarization into M1 and M2 types. However, the differentiation path was somewhat indistinct. Additionally, these results do not significantly impact the study's interpretation. Consequently, we have excluded Supplementary Figures 11, 13, 14, and 16, as recommended by the reviewer.

4. In the time series analysis, especially in the result of Fig. 3e and Supplementary Fig. 10, the variance of some figures seems very large. Significance tests in corresponding dpi in line plots in should be made to further evaluate the changes, not different dpi of different virulence.

Our response: Thank you for your kind comment. As the reviewer commented, we have included the significance tests (Fig. 3e, Fig. 4d, Fig. 5d, Supplementary Fig. 12, Supplementary Fig. 14, and Supplementary Fig. 16). Additionally, the reason for comparing JA142 at 14 dpi and NA10 at 7 dpi was confirmed, as despite differences in virulence and dpi, these comparisons can highlight differences in the trajectories towards recovery or death (below of Supplementary Fig. 10, 11, 12).

Other concerns:

1. Throughout the manuscript, do the authors mean the “control” group instead of the “negative” group? If not, control groups should be set to fully characterize the changes of cell populations in different virulence groups.

Our response: Thanks for the kind comment. As ‘Animals and experimental design’ was written in the manuscript, we used 4 groups (negative, NA8, JA142, and NA10). Negative group was not challenged with PRRSV, but NA8, JA142, and NA10 groups were challenged with corresponding PRRSV. Cell populations that were identified in the negative group were also identified in the different virulence groups, but decreased proportions were indicated because of macrophage death by PRRSV infection.

2. Page 3 line 20, “Arteriviridae” and “Nidovirales” should be italic.

Our response: As the reviewer pointed out, we have revised “Arteriviridae” and “Nidovirales” to be italic (page 3, line 21).

3. The introduction should add more current knowledge of single-cell studies of BALF in animals (eg. Homo sapiens) with infections and add the current knowledge of miRNA regulations to emphasize the urgent need for the authors’ research.

Our response: As the reviewer kindly suggested, additional information of single-cell studies of BALF in animals with infection (e.g. SARS-CoV2) and current knowledge of miRNA regulations in infection (e.g. SARS-Cov2) have been added into the manuscript (page 3, lines 40-44).

4. It may be better for the authors to enhance the writing. It is very hard to comprehend why the authors studied single-cell compositions first but ended with conclusions of miRNAs.

Our response: As the reviewer kindly pointed out, the authors agree that the writing from the original manuscript was difficult for the readers to comprehend. The main reason the authors focused on miRNA from single-cell composition data is that certain cell signaling mechanism was suspected to be involved in PRRSV-induced pathogenesis in the lung, as somewhat excessive immune responses and cell deaths

were observed in time-serial manner although only a small proportion (< 5%) of cells were identified to be directly infected with the virus through viral tracking. Among potential mechanisms, the authors focused on the exosomes and their compartments, miRNA, as many viral infections, such as HIV and SARS-CoV2, have recently demonstrated the involvement of virus-induced exosomes during the pathogenesis. The manuscript has been revised thoroughly to enhance the understanding, mainly at the introduction and discussion sections.

5. Fig. 2d, Fig. 2f, Supplementary Fig.7a, Supplementary Fig.7c and Supplementary Fig.7d are missing discrete cell labels or continuous color bars to explain the meaning of different colors of cells, which made it hard to explain the meaning of the figures.

Our response: Thank you for your helpful comment. As the reviewer pointed out, we have added the labels (Fig. 2df, Fig 3bc, and Supplementary Fig. 8acd).

6. The labels in Supplementary Fig. 11a, c, e, g and Supplementary Fig. 30 are hard to read, which made it very hard to understand what information the figures provided.

Our response: Thank you for kind comment. As mentioned above, Supplementary Fig 11, 13, 14 and 16 (previous numbers) have been excluded as suggested.

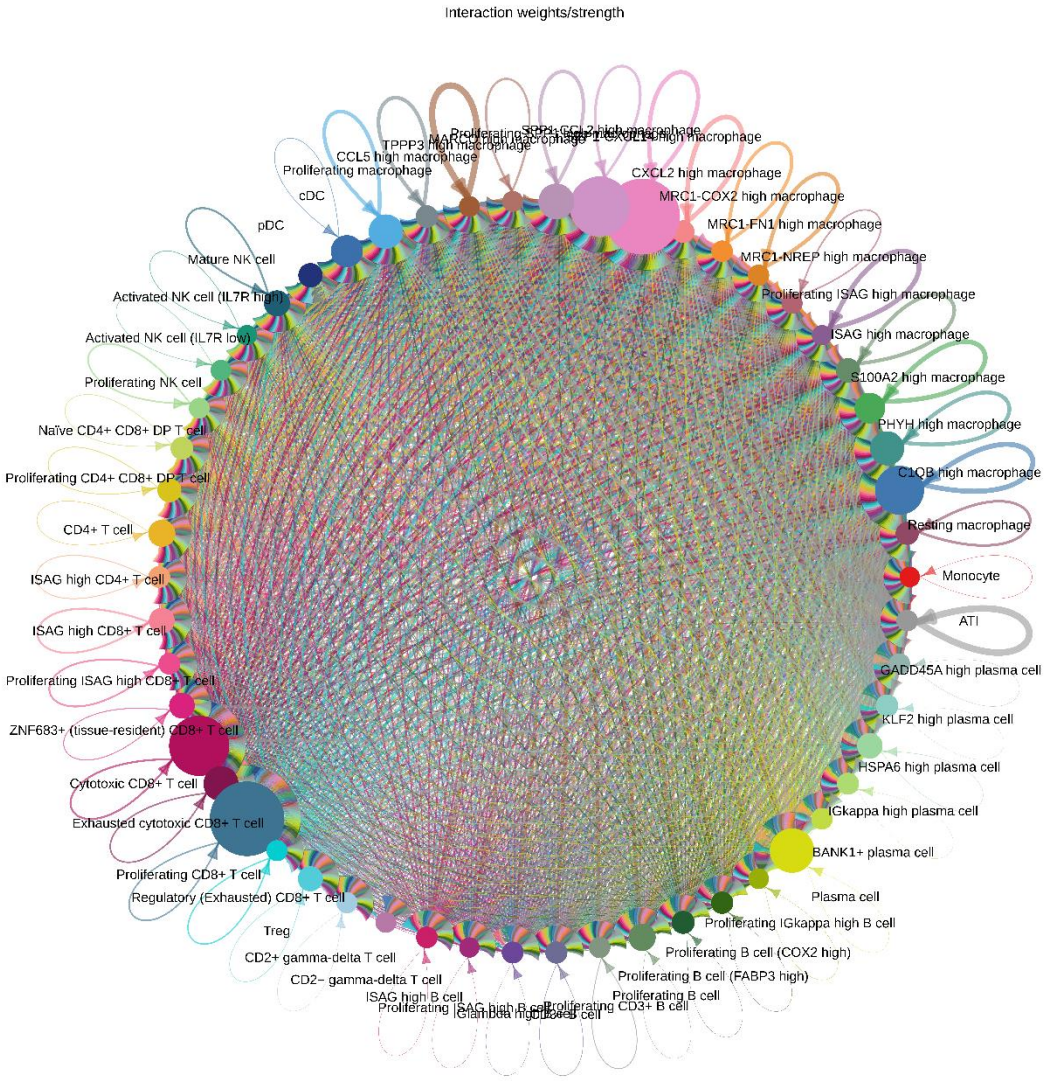
7. To compare the gene expression characteristics in different virulence conditions, the dot plots in Fig. 2, Fig. 3 and Fig. 4 should be split by different virulence conditions to show the annotation accuracy. If possible, split feature plots should also be provided.

Our response: Thanks for great suggestion. We have added the dot plots in different virulence conditions (Supplementary Fig. 7, Supplementary Fig. 11, Supplementary Fig. 13, and Supplementary Fig. 15).

8. The authors only focused on the expression levels of cell communication-related receptor genes in Supplementary Fig. 18b, Supplementary Fig. 19b, Supplementary Fig. 20b, Supplementary Fig. 21b,

Supplementary Fig. 22b, Supplementary Fig. 23b, Supplementary Fig. 24b, Supplementary Fig. 25b, Supplementary Fig. 26b, Supplementary Fig. 27b, Supplementary Fig. 28b, and Supplementary Fig. 29b. However, the cell communication levels of ligand-receptors should not only be inferred by the expression levels of receptor genes, but also the expression of ligand genes. Therefore, many methods to infer scRNA-seq can be applied to evaluate the communication strength, such as CellPhoneDB (PMID: 32103204) and CellChat (PMID: 33597522).

Our response: Thank you for the kind concerns. The expression of ligand genes (cytokines and chemokines) were shown in Supplementary Fig. 18a, Supplementary Fig. 19a, Supplementary Fig. 20a, Supplementary Fig. 21a, Supplementary Fig. 22a, Supplementary Fig. 23a, Supplementary Fig. 24a, Supplementary Fig. 25a, Supplementary Fig. 26a, Supplementary Fig. 27a, Supplementary Fig. 28a, and Supplementary Fig. 29a. We have also added the word ligands to avoid confusion. Given the vast amount of data produced, it was determined that including as much information as possible about BALF cells, which were being analyzed for the first time, was beneficial. In addition, we previously evaluated the communication strength using CellChat. However, the results were too complex to interpret meaningfully, so we decided not to include them. Our previous results (JA142 dpi 14) are presented below.



Reviewer #2 (Remarks to the Author):

This manuscript contains a comprehensive single cell RNA Seq analysis of BAL cells after infection of pigs with 3 different PRRSV strain that differ in virulence. The data is complemented by BAL fluid cytokine levels, flow cytometry, virological, clinical and pathological data. Overall, the data quality is very good with some weak points listed below.

It would be really great to understand the relationship between these different elements but for this the study design is not ideal. A main difficulty for the conclusions is that not all groups were followed to the recovery phase. Therefore, it is not possible to conclude on a possible role of the SSP1+ macrophage

population that emerged at day 14 in the JA142 group. This time point is missing with the two other viruses. In addition, with only three animals per time point, it may be risky to draw conclusions. Certainly, it is not correct to state that the SSP1-CXCL14high macrophage contribute to recovery (Abstract) or that the data set reveals the mechanisms for lung recovery. The authors need to tune down their conclusions and acknowledge the fact that the work remains descriptive by nature. Having said that, it is fine to speculate and propose additional work that will be required to consolidate the findings.

Our response: We appreciate the kind review. In general, PRRSV shows unique virus-induced immune response that is distinct from other viral infections (Figure 1) [1].

[Redacted]

Figure 1. Immune response to PRRSV infection. Borrowed from Lunney et al. [1]

Unlike other viral infections, PRRSV directly infects the porcine alveolar macrophages (PAMs), inducing dysregulated and delayed cell-mediated immune (CMI) responses as well as adaptive immune responses [1]. In our previous study investigating host-pathogen interactions in 75 piglets

infected with PRRSV-JA142 and 25 control pigs [2], we confirmed that the highest viremia and lung viral loads were detected between 3 and 10 days post challenge (dpc), followed by gradual virus clearance.

[Redacted]

Figure 2. Association of PRRSV-JA142 viral loads with the growth rate of pigs. Borrowed from Nazki et al. [2]

More importantly, it was confirmed that the pre-existing alveolar macrophage population ($CD163^+$ cells, Figure 3) significantly decreased at the time of peak viral load (10 dpc), but gradually recovered after the peak damage timepoint. Other studies have showed that no more than 2% of lung PAMs were directly infected with PRRSV antigen, as confirmed by IHC of the lung sections [3] or flow cytometry of BALF cells [4,5]. Furthermore, Chaudhari et al. showed that PRRSV directly infected cells and uninfected bystanders, separated by green fluorescent protein (GFP) expressing JA142, showed significantly different expression of negative immune regulators and T cell exhaustion markers [5]. Therefore, before designing the current study, the main questions were as follows:

Main questions

1) Even with a low percentage of directly infected PAMs (~2% of total PAMs), what causes a high loss of PAMs (up to 80%) during the infection in the lung? (signaling between infected and bystander cells?)
2) Why are recovered PAMs unlikely to be infected by pre-infected PRRSV (evidenced by decreased viremia and lung viral load after peak damage point) and how does the virus clearance occur in the lung even before major CMI or adaptive immune responses are induced in the host? (transition of PAM phenotypes is the major cause?)

[Redacted]

Figure 3. Fluctuations in the frequencies of lung macrophage and dendritic cell phenotypes

during infection. Borrowed from Nazki et al. [2]

In addition, PRRSV is one of the RNA viruses with the highest mutation rates, producing multiple genetic lineages with varying levels of virulence [6]. The two PRRSV strains used in this study (JB15-N-M8-GN; NA8 and JB15-N-PJ10-GN; NA10) are Korean PRRSV2 isolates that were confirmed to be highly virulent (NA10) and low virulent (NA8) based on our previous animal experiments (Figure 4) [7]. The highly virulent strain NA10 induced mortality within 10-12 dpc [7], which was confirmed by additional animal experiments (data not shown). This raises another important question:

Main questions

3) What virological or immunological processes are involved in the different virulence induced by different PRRSV strains?

[Redacted]

Figure 4. Results of a meta-analysis of seven variables obtained from pig experiments. Under Review [7]. (A) Three variables (viremia AUC, nasal viral load AUC and lung viral loads) made up the "transmission" indicator. (B) Four variables (high fever rate, microscopic lung lesion score, ADWG and mortality rate) made up the "clinical signs" indicator. The data from the overall PRRSV-2 infected group and the data from individual infected groups were compared, and the SMDs were calculated for each strain-specific variable. The meta-analysis was performed with the “meta” and “netmeta” packages in R software. The results were investigated using a random effect model with

95% confidential intervals (CIs) to account for the variation among the eleven challenged PRRSV-2 strains. The color of the square in the graph indicates the lineage to which PRRSV belongs (blue: lineage 1, red: lineage 5, dark green: Korean lineage A, light green: Korean lineage B, and dark yellow: Korean lineage C).

Together, the authors designed the animal study to find the answers for the main questions, using multiple techniques including single-cell RNA sequencing (scRNAseq) to investigate the transition of PAM phenotypes complemented by BAL fluid cytokine levels, flow cytometry, virological, clinical and pathological data.

For grouping, the negative control and JA142 infection groups were set at 0, 3, 7, 14 and 21 dpi, while the NA10 group was inevitably set at 0, 3 and 7 dpi as the virus induces mortality at 10-12 dpi. Unfortunately, the NA8 group was also only set for 0, 3 and 7 dpi due to the limited resources and funding as scRNAseq costs are very high. For the same reason, the number of pigs (three animals) per timepoint was minimized. However, the virological, clinical and pathological data showed that three PRRSV strains showed significantly different virulence even with the minimized number of animals, which was sufficient for further analysis. Indeed, although the number of animals were minimized, the scRNAseq data generated in this study is still large (36 pigs, 306,052 cells) and unique (BALF cells with PRRSV infection) compared to other scRNAseq datasets (Table 1) [8-18].

Table 1. scRNAseq studies with samples from pigs

Sample type	**# pigs**	**Age of pigs**	**Generated scRNAseq data**	**Pathogen**	**Reference**
BALF cells	**36**	**4~7 week old**	**306,052 cells**	**PRRSV**	**This study**
Embryo	10	-	70,201 cells	-	Cai et al., 2023 [8]
Testis	4	7, 30, 60, 90 day old	36,852 cells	-	Zhang et al., 2022 [9]

ileum	2	7 week old	31,983 cells	-	Wiarda et al., 2022 [10]
PBMC	2	7 week old	28,810 cells	-	Herrera-Uribe et al., 2021 [11]
Testis	1	150 day old	16,966 cells	-	Zhang et al., 2021 [12]
Ovary	1	210 day old	16,400 cells	-	Chen et al., 2023 [13]
lung tissue	1	3 month old	13,580 cells	-	Zhang et al., 2021 [14]
Uterine	24	6 month old	13,544 cells	-	Han et al., 2021 [15]
Jejunum	8	3 day old	12,774 cells	PEDV	Fan et al., 2023 [16]
Uterine	6	Gilts	12,415 cells	-	Tian et al., 2022 [17]
lung tissue	2	3.5 month old	11,452 cells	-	Raredon et al., 2019 [18]

Nevertheless, the authors admit the need to tone down the conclusions as kindly suggested, and the manuscript has been revised.

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I also miss pointing on the fact that looking in the BAL compartment only is unlikely to give data on the mechanisms of lung recovery. We need to understand how that data correlates to events in the lung and lymphoid tissue. I understand that this cannot be included in such a study that is already fully packed with data, but these points should not be ignored either. Despite these critical points, this work is a fantastic resource for future work and will be very helpful for the design of future work.

Our response: Thank you for your kind concerns. As mentioned above, PRRSV infection is unique compared to the other viral infection as the virus is cleared before CMI or adaptive immunity are involved, which highlights the local immune response within the lung is critical for lung recovery. However, lymphoid tissues should have been involved during the lung recovery and should not be ignored. Although this study focused on the local immune response against PRRSV infection within the lung, lymphoid tissues and immune cells (lymph nodes and PBMC) were also collected during the

experiment and will be analyzed in future study.

Additional critiques

1. DC definition based on FCM are not precise enough. It is not possible to properly differentiate DC based on MHCII, CD163 and CD172a. For example MHCII+CD172a-CD163- can be B cells. Also the differentiation of MO and MoDC based on CD163 expression is not secured. Accordingly, Supp. Fig. 2 and the text needs to be adapted.

Our response: As the reviewer kindly suggested, Supplementary Fig. 2 and the text has been adapted based on the gating strategy, not differentiated cell names.

2. I am missing Figure legends directly under the Figs. And Supplementary Figs. This makes it troublesome to review.

Our response: Sorry for the inconvenience. The information was described in "supplementary materials" word file. In addition, we added the legends under the whole figures.

3. In Fig 3 22 different macrophages cluster were identified but is remains unclear how this was done. Were these found in all pigs? Where they also found following infection.

Our response: Thank you for the helpful comments. As described in the manuscript, we initially classified the cells into three categories (myeloid, lymphoid, and epithelial) comprising seven cell types (macrophage, DC, NK cell, T cell, B cell, ATI, and ATII) based on the expression levels of 21 genes (page 25, lines 603-604). Subsequently, the myeloid category, which includes macrophages and DCs, was re-integrated and further classified into 23 sub-cell types using the expression levels of 47 genes (page 25, lines 614-619). All genes included those previously recognized as useful for macrophage classification and differentially expressed genes among clusters in this study. To clarify how we separated, we have made a table highlighting the key distinguishing features (page 25, lines 627-628; Supplementary Table 8). Sub-cell types of macrophages were identified in most pigs, in all groups with the exception of PDCD1LG2^{high} macrophages (only three pigs at NA10 7 dpi) (Fig 3e and

Supplementary Fig. 12).

The same applies to the DC. In the single cell data, cDC and pDC are defined. However, it is not explained how. Furthermore, this data cannot be linked to the FCM data.

Our response: Sorry for the confusion. As we mentioned above, we have provided key distinguishing features in Supplementary Table 8. As for FCM data,

4. Lines 585: please explain how the 21 genes were identified to annotate cell types. To me this seems very little genes

Our response: Thank you for your kind concerns. First, 21 genes were used to separate into 7 cell types (macrophage, DC, NK cell, T cell, B cell, ATI, and ATII) in three cell categories (myeloid, lymphoid, and epithelial). Subsequently, 47 (for macrophage and DC), 31 (for NK and T cells), and 24 (for B cells) genes were employed to separate into sub-cell types. To clarify how to separate, we have constructed a table highlighting the key distinguishing features (Supplementary Table 8). All genes comprised both those known as markers and those specifically identified genes (through differential expression analysis among clusters) in this study.

5. It is unclear to me how a cluster can be traced over time. If SPP1 macrophage "increase" how can this be differentiated from a upregulation of SPP1 (or the other genes defining this cluster), eg induced by IFN.

It needs to certain that the defined macrophages are real clusters that can by clearly be identified as clusters in a time and treatment independent manner. The past has shown that defining new "subsets" basing on scRNA-Seq data can be problematic.

Our response: We agree with your kind concerns. In the previous study in which human PBMC were largely investigated through scRNA-seq, the study group identified ISAG high T cells, "IFN signaling-associated gene (ISAG)" high T cells with remarked expression of genes such as ISG15, MX1, MX2, IRF7 and OAS1 [1]. In the same manner, not only ISAG high $CD4^+$ and $CD8^+$ T cells (Figure 4) but

also ISAG high macrophages (Figure 3) were newly identified during the PRRSV infection in this study. The highest population of ISAG high T cells were detected during the lung peak-damage timepoint for both NA10 (7 dpi) and JA142 (14 dpi), while the highest population of ISAG high macrophages were detected before the peak-damage timepoint for JA142 (7 dpi). Remarkedly, NA10 infected pigs showed decreased population of ISAG high macrophage at 7 dpi, most probably due to faster disease progression and cell death compared to the JA142 infection. In JA142 infection, we observed that the population of this cell type decreased over time post-infection, while SPP1 macrophage "increased" at the same time, in which the population could be clearly distinguished from ISAG high macrophages.

In previous researches studying BALF cell population dynamics through scRNA-seq in severe COVID-19 infection, it was demonstrated that monocyte/macrophage population and monocyte-derived macrophage population expressed high levels of SPP1, in which the $SPP1^{+}$ macrophage populations expressed well-known tissue-remodeling or fibrosis markers TGFB1, LGMN and CCL18 as well as high expression of CD163 [2, 3]. SPP1 high macrophages were also identified in idiopathic pulmonary fibrosis (IPF) model [4], and lung adenocarcinoma model in which tumor-associated macrophages (TAMs) produced SPP1 [5].

In PRRSV infection, PRRSV primarily infects macrophages and cause enormous cell deaths through either direct infection (< 5%) or bystander deaths, inducing modulation of the cell population at the lung peak-damage timepoint in which the proportion of macrophages is low in total BALF cells. However, the proportion of macrophage is "refilled" after the peak-damage timepoint, which is most probably due to monocyte-derived macrophage infiltration into the lung as like the infiltration of $CD163^{+}$ monocyte-derived macrophages expressing SPP1 in COVID-19 [6]. Therefore, although the origin of SPP high macrophage could not be traced in this study, it is highly plausible that the defined macrophages are real clusters that can clearly be identified as clusters in a time and treatment independent manner.

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6. Similar remarks apply to the T cells. Is it really helpful to defined so many T cell subsets, in particular considering that they follow the same trend?

Our response: Thank you for the kind concerns. Although very few T cells exist in the lungs, when PRRSV is infected, T cells flow into the lungs after PRRSV infection. As mentioned above, in this study, since we produced a large number of scRNA-seq data, we distinguished cells that exhibit several expression characteristics although they are rare cells. These results are thought to be helpful for future research regarding T cells after PRRSV infection.

7. Also for the B cells/plasma cells. How helpful and accurate is it to defined so many clusters. What are CD3E+ B cells? How sure can you be that these are not T cells?

Our response: Thank you for the kind concerns. Although B cells are typically scarce in the lungs, they, like T cells, migrate into the lungs during PRRSV infection. As mentioned above, in this study, since we produced a large number of scRNA-seq data, we distinguished cells that exhibit several expression characteristics although they are rare cells. Therefore, these results are thought to be helpful for future research regarding B cells after PRRSV infection. Additionally, CD3+ B cells are not few in number, so they are marked, but we determined that CD3+ B cells are likely to be doublets. To avoid any confusion, we have added "doublet possibility" in Fig. 5 and Supplementary Table 8.

8. In the Chapter on cytokines, chemokine and their receptors it is referred to a series of Supplementary Figures 18-29. This is not reader friendly has the evolution of the response cannot be appreciated. Please summarise the group-dependent changes over time for the most important responses.

Our response: Thanks for the helpful comments. Given the extensive scRNA-seq data generated from BALF samples of both negative and PRRSV-infected pigs, we aimed to provide comprehensive information. Since PRRSV induces immune evasion mechanisms, such as IFN-a suppression, information on the presence or absence of various cytokines, chemokines, and their receptors was deemed crucial. Unfortunately, this was complicated and difficult to show evolution of the all responses because of various factors, including virulence types, time points, and the large number of cell types involved. However, as the reviewer suggested, we selected $ISAG^{high}$ and $SPP1\text{-}CXCL14^{high}$ macrophages, which may play crucial roles in the immune response during PRRSV infection, and time-serially indicated some gene expression levels that were observed different patterns between the NA10 and JA142 groups (page 11, lines 237-248; Supplementary Fig. 30).

9. Why are the pDC missing in the chapter on cytokines? They are the likely source for IFN-a. Can that be verified with this dataset?

Our response: Thank you for the reviewer's concern. We confirmed that pDCs had tiny population and especially were found after PRRSV infection (JA142 and NA10) (Supplementary Fig. 12). Additionally, to minimize errors due to a small number of cells, only pDC clusters with more than 20

cells were included in the dot plots. For these reasons, information on pDCs is available only at the following time points: negative 21 dpi (Supplementary Fig. 21), NA8 3 dpi (Supplementary Fig. 22), JA142 14 dpi (Supplementary Fig. 26), JA142 21 dpi (Supplementary Fig. 27), and NA10 7 dpi (Supplementary Fig. 29). Unfortunately, no gene expression of IFN-a in pDCs was observed, likely due to the immune evasion mechanisms of PRRSV that inhibit IFN-a production.

10. Supplementary Fig. 30 is unreadable.

Our response: Sorry for the inconvenience. I have additionally attached a high-resolution file (Supplementary Fig. 31; new number).

Reviewer #3 (Remarks to the Author):

The present paper examines, using a single cell transcriptomic analysis, the development of PRRSV infection in lungs of animals infected by three different isolates of presumed different virulence. The paper is interesting and may contribute to a better knowledge of the pathogenesis of this infection. In its present form it is a comprehensive examination of the changes of different cell populations (macrophages, T and B cells, etc.), examines the cytokines present in BALF, analyses the transcriptome of infected and bystander cells at a single cell level and focuses on exosome miRNA expression in the studied cases. This comprehensiveness results in the inclusion of so much information that the reader feels overwhelmed, making it very difficult to discern what is central to the proposed question and what is secondary.

Our response: We appreciate for the kind review. As kindly suggested, the present form of this manuscript seems quite over-descriptive, and the following information has been removed for better understanding: RNA velocity data (Supplementary Fig. 11, 13, 14 and 16; previous number) was removed.

The authors designed an experiment including 3 different strains that were considered to be of different virulence and they performed an experimental infection. In the design, the groups inoculated with the strains of high and low virulence (NA8 and NA10) only contained 6 animals while the negative control and the group of moderate virulence were composed of 12 animals. As a result, the high and low virulence groups were only followed up to 7 pi while the other two groups were followed until 21 pi. This is a problem because comparisons for all groups are only possible during those 7 days. Why this unequal distribution of groups? Looking at the data of those 7 days it is difficult to assess the different degrees of virulence, particularly between JA142 and NA8. For example, looking at the data of strain NA10 in Figure 1d, at 3 dpi the microscopic lesions of those animals were not significantly different than those of the controls despite that at 7 dpi the scores were very high, and the picture shows a severe interstitial pneumonia. In contrast, viral load in blood for NA10 was the highest for all strains. In

contrast, the severity of microscopic lesions (based on the scores and pictures) of JA 142 at 14 and 21 pi seems to be approximately equal than that of NA10 at 7 pi. Is JA142 less virulent than NA10 or it results in an equally severe process but with a slower development (if so, why?). It cannot be known with this design. With this in mind, the interpretation that is made in Figure 8 (that is the corollary of the work) needs to be revised. I'm not sure that the comparison and the delimitation of the phases is so straightforward. This is critical point.

Our response: In general, PRRSV is one of the RNA viruses with the highest mutation rates, producing multiple genetic lineages with varying levels of virulence [1]. For example, Chinese highly pathogenic PRRSV (HP-PRRSV) lineage induces high mortality (20~100%) with higher virus replication (with differences of more than 10^2 copies/ml compared to the reference strains) and immune responses compared to other lineages [1, 2]. Likewise, the two PRRSV strains used in this study (JB15-N-M8-GN; NA8 and JB15-N-PJ10-GN; NA10) are Korean PRRSV2 isolates that were confirmed to be highly virulent (NA10) and low virulent (NA8) based on our previous animal experiments (Figure 1 and 2) [3]. The highly virulent strain NA10 showed higher replication than other viruses and induced nearly 100% mortality within 10-12 dpc under experimental condition [3], which was confirmed by additional animal experiments (data not shown).

[Redacted]

Figure 1. The viral loads in sera obtained from inoculated pigs. Under Review [3]. The viral loads in the serum at 0, 3, 7, 14, 21 and 28 DPC were quantified by real-time reverse transcription-PCR (qRT-PCR). The raw viremia data for each group at each time point are shown with statistical differences at the bottom. The viral titers were calculated using the standard curve of the threshold number of cycles plotted against each of the known viral titers. The color of the line in the graph indicates the lineage to which PRRSV belongs (blue: lineage 1, red: lineage 5, dark green: Korean lineage A, light green: Korean lineage B, and dark yellow: Korean lineage C). The red and blue squares in the raw data table indicate the highest and lowest viral loads, respectively, at each time point. The data are presented as group means±SEM. Significant differences among the experimental groups are denoted by different letters ($p<0.05$, Tukey's test, two-way ANOVA).

[Redacted]

Figure 2. Results of a meta-analysis of seven variables obtained from pig experiments. Under Review [3]. (A) Three variables (viremia AUC, nasal viral load AUC and lung viral loads) made up the "transmission" indicator. (B) Four variables (high fever rate, microscopic lung lesion score, ADWG and mortality rate) made up the "clinical signs" indicator. The data from the overall PRRSV-2 infected group and the data from individual infected groups were compared, and the SMDs were calculated for each strain-specific variable. The meta-analysis was performed with the “meta” and “netmeta” packages in R software. The results were investigated using a random effect model with 95% confidential intervals (CIs) to account for the variation among the eleven challenged PRRSV-2 strains. The color of the square in the graph indicates the lineage to which PRRSV belongs (blue: lineage 1, red: lineage 5, dark green: Korean lineage A, light green: Korean lineage B, and dark yellow: Korean lineage C).

Although the criteria for “high virulence” of PRRSV is still vague, PRRSV strains exhibiting high mortality and higher replication are considered to be highly virulent [1] (Figure 3).

[Redacted]

Figure 3. Summary of the virulence correlates to define a virulent PRRSV strain. Borrowed from Ruedas-Torres et al. [1]

In our previous study investigating host-pathogen interactions in 75 piglets infected with PRRSV2 reference strain JA142 and 25 control pigs [4], we confirmed that the highest viremia and lung viral loads were detected between 3 and 10 days post challenge (dpc), followed by gradual virus clearance (without mortality), which was set as the intermediate virulence strain in the current study (Figure 4).

[Redacted]

Figure 4. Association of PRRSV-JA142 viral loads with the growth rate of pigs. Borrowed from Nazki et al. [4]

More importantly, our previous study has confirmed that the pre-existing alveolar macrophage population ($CD163^+$ cells, Figure 5) significantly decreased at the time of peak viral load (10 dpc), but gradually recovered after the peak damage timepoint [4], which was consistently observed from other "typical" and "recoverable (but wasting, due to decreased weight gain)" PRRSV infections under experimental conditions from our lab (data not shown).

[Redacted]

Figure 5. Fluctuations in the frequencies of lung macrophage and dendritic cell phenotypes during infection. Borrowed from Nazki et al. [4]

Other studies have showed that no more than 2% of lung PAMs were directly infected with PRRSV antigen, as confirmed by IHC of the lung sections [5] or flow cytometry of BALF cells [6,7]. Furthermore, Chaudhari et al. showed that PRRSV directly infected cells and uninfected bystanders,

separated by green fluorescent protein (GFP) expressing JA142, showed significantly different expression of negative immune regulators and T cell exhaustion markers [6].

Therefore, with the backgrounds mentioned above, the interaction between directly infected cells and bystander cells (mainly macrophages) as well as the phenotypic transition of immune cells during the lung infection and recovery (NA8 and JA142 strain) or irreversible mortality (NA10 strain) were the main points we have investigated.

In this study, the authors designed the animal study with using multiple techniques including single-cell RNA sequencing (scRNAseq) to investigate the transition of PAM phenotypes complemented by BAL fluid cytokine levels, flow cytometry, virological, clinical and pathological data.

For grouping, the negative control and JA142 infection groups were set at 0, 3, 7, 14 and 21 dpi, while the NA10 group was inevitably set at 0, 3 and 7 dpi as the virus induces nearly 100% mortality at 10-12 dpi. Unfortunately, the NA8 group was also only set for 0, 3 and 7 dpi due to the limited resources and funding as scRNAseq costs are very high. For the same reason, the number of pigs (three animals) per timepoint was minimized.

Despite unequal distribution of groups, virus replication (Figure 1b), pathological data (Figure 1c), and viral tracking data (Figure 2f) showed the different degrees in virulence between the viruses, complemented with previous experiments [3, 4].

For the concerns of the degree of virulence with microscopic lesion data and viral load, which the reviewer provided as an example, the authors consider the different timepoint of the peak lung-damage timepoint (NA10; 7dpi, JA142; 14dpi) is the major difference between the high virulent and intermediate or low virulent PRRSV, which has been analyzed and discussed throughout the manuscript. The higher viremia of NA10 (Figure 1b) as well as the faster virus replication in the lung (Figure 1b and 2f) resulted in the faster loss of $CD163^{+}$ cells or normal PAMs (Figure 1e, 2d and 2g) with microscopic lesion of severe interstitial pneumonia (Figure 1c) at 7dpi. In contrast, JA142 showed less viremia, viral replication in the lung (Figure 1b and 2f) and slower peak lung-damage timepoint (Figure

1e, 2d and 2g) with microscopic lesion of severe interstitial pneumonia (Figure 1c) at 14 dpi. Although the microscopic lesions of JA142 at 14 and 21 dpi seems to be equal due to the infiltration of alveolar space with multiple types of immune cells, the composition of infiltrated cells are significantly different, in which infiltrated cells of 21 dpi contains more CD163+ cells (recover, confirmed by FCM and scRNAseq) (Figure 1e, 2d and 2g), or specifically more cells (e.g. C1QB high macrophage, PHYH high macrophage, S100A2 high macrophage) that composed most of the lung microenvironment before the progression of lung damage (recover, confirmed by scRNAseq) (Figure 3e).

However, genetic markers or viral proteins that critically differs the virulence of different PRRSVs have not been fully elucidated as PRRSV shows a high level of genetic heterogeneity [1, 8]. Discovering the genetic compartments may need virological techniques such as infectious clone manipulating viral genes between PRRSVs with different virulence, which is not under the scope of the current study. Rather, the authors have chosen the viruses with known virulence based on previous experiments [3, 4], and focused on the host immune response, specifically local immune response at the lung, to elucidate what immunological compartments determines the “fate” of the host, recovery or mortality (Figure 8).

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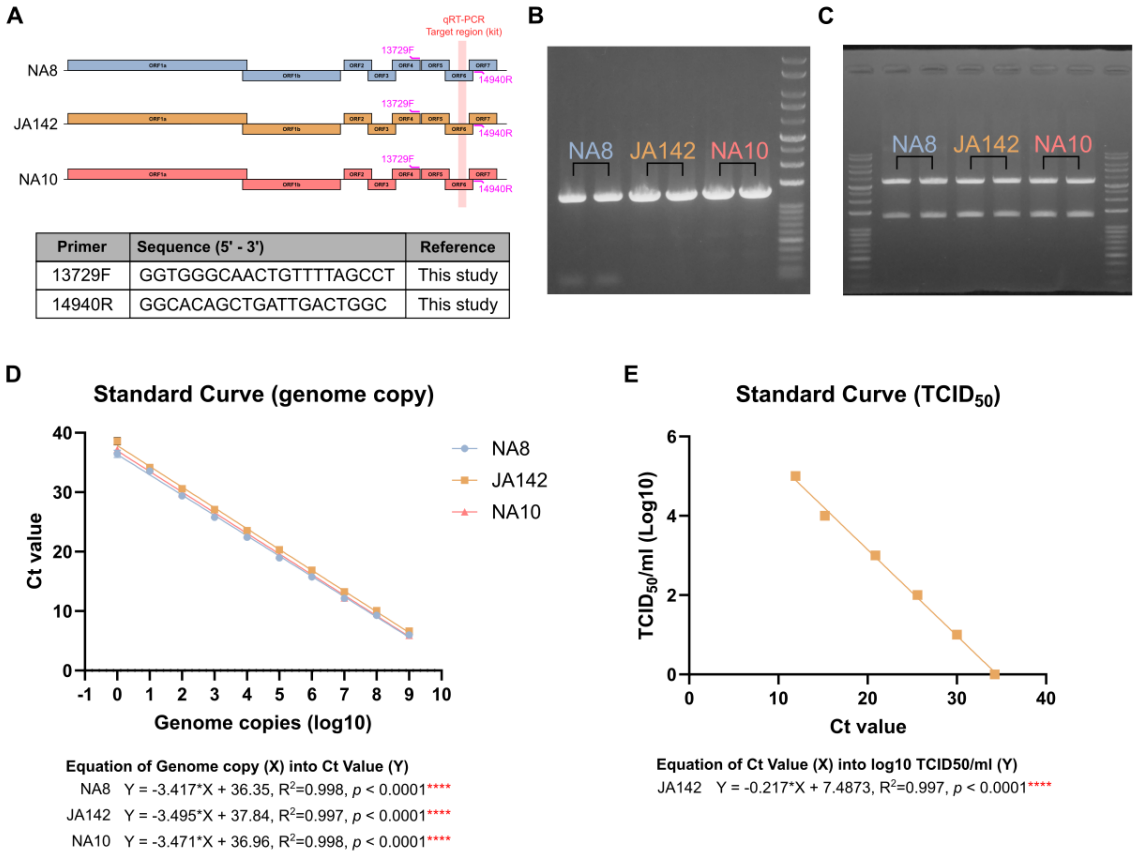
[7] Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallarés, F. J., Mateu, E., Carrasco, L., & Gómez-Laguna, J. (2019). Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells. ***Veterinary microbiology***, 235, 101-109.

[8] Kim, S. C., Moon, S. H., Jeong, C. G., Park, G. S., Park, J. Y., Jeoung, H. Y., ... & Kim, W. I. (2022). Whole-genome sequencing and genetic characteristics of representative porcine reproductive and respiratory syndrome virus (PRRSV) isolates in Korea. ***Virology Journal***, 19(1), 66.

By the way, the authors used a commercial kit and produced a curve for correlating Ct with TCID using only the JA142. Any mismatch between the primer (or primers) sequences and the sequences of any of the strains may easily result in a drop of about 2-6 Cts. Why did the authors not build the titration curves using a series of dilutions of each strain? The differences in viral loads (viremia) at 7 pi were 1.6 log10 between the highest and the lowest. Can this be an artifact created by the way you calculated them? Differences in virulence are the basis of the study so, assessing of viral loads is critical. This must be corrected, or proof of the similar performance of the PCR for all three strains must be showed somewhere.

Our response: Thank you for your helping comments. PRRSV shows high genetic heterogeneity with different virulence [1]. Highly virulent PRRSV-2, such as Chinese high pathogenic PRRSV (HP-PRRSV), induces higher viremia and tissue viral loads with differences of more than 10^2 copies/ml compared to the reference strains [1 ,2].

As the reviewer concerned, the authors have re-analyzed by 1) PCR and cloning of the qRT-PCR (kit) target region, 2) generating genome-copy based standard curve of Ct value with constructed plasmids (Figure 1).



Primer	Sequence (5' - 3')	Reference
13729F	GGTGGGCAACTGTTTTAGCCT	This study
14940R	GGCACAGCTGATTGACTGGC	This study

Figure 1. Standard curves for correlating Ct value with TCID. Incorporated in to the manuscript as Supplementary Figure 35. (A) Primers designed for cloning PRRSV ORF5 and ORF6 including the target of qRT-PCR kit. (B) Gel image of PCR products. All viruses have been tested in duplication. (C) Gel image of constructed TOPO-clones after cut with restriction enzymes (*XbaI* and *SacI*). (D) Standard curve correlating Ct value with genome copies based on constructed plasmids. (E) Standard curve correlating Ct value with TCID using JA142 [3].

As expected, generated standard curves of each virus correlating Ct value with genome copies were almost identical, which is most probably due to the target region of PRRSV qRT-PCR kit (ORF6, highly conserved among PRRSV-2). Therefore, using a curve for correlating Ct with TCID using only

the JA142 was considered to be more solid, as generating curves using a series of dilutions of each strain might be able to provide more biased result owing to issue of reproducibility and technical errors.

Reference

[1] Ruedas-Torres, I., Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Larenas-Muñoz, F., Pallarés, F. J., Carrasco, L., & Gomez-Laguna, J. (2021). The jigsaw of PRRSV virulence. ***Veterinary microbiology***, 260, 109168.

[2] Tong, G. Z., Zhou, Y. J., Hao, X. F., Tian, Z. J., An, T. Q., & Qiu, H. J. (2007). Highly pathogenic porcine reproductive and respiratory syndrome, China. ***Emerging infectious diseases***, 13(9), 1434.

[3] Nazki, S., Khatun, A., Jeong, C. G., Mattoo, S. U. S., Gu, S., Lee, S. I., ... & Kim, W. I. (2020). Evaluation of local and systemic immune responses in pigs experimentally challenged with porcine reproductive and respiratory syndrome virus. ***Veterinary Research***, 51, 1-19.

Besides, some interesting results need further discussion to better understand the model. For example, NA10 was inducing higher IFN signalling pathways in early (3 pi) infection than the other strains and had a relative low number of myeloid infected cells. In contrast, at 7 pi, the proportion of myeloid infected cells in JA142 was higher but with high dispersion of results. This would not be in favour of a different virulence between the strains.

Our response: We agree with your kind concerns. As newly written in the introduction section, PRRSV shows high genetic heterogeneity and various levels of virulence. However, so-called “high virulent” PRRSV such as Chinese high pathogenic PRRSV-2 (HP-PRRSV) or PRRSV1 subtype 3 Lena strain, induce higher viral loads and faster disease progression with earlier cell population modulation in the lung and often cause high mortality [1, 2, 3, 4]. As discussed above, we have previously confirmed the different virulence between the strains based on clinical outcomes through animal experiments.

Thanks to the reviewer, the authors re-checked the data, and it was confirmed that the bystander cells of the NA10 at 3 dpi and JA142 at 7 dpi, when the highest viral transcripts were detected through viral tracking (Fig. 2f), showed high enrichment of IFN signaling pathways in similar levels

(Supplementary Fig 31a). However, the pathway was more enriched at the peak damage timepoint of NA10 (7 dpi) compared to JA142 (14 dpi) in bystander macrophages (Supplementary Fig 31a). Phenotypically, higher concentrations of serum and BALF IFN-α were detected for the NA10-infected group when compared with the JA142 infected group at an earlier timepoint (Fig. 1b, Supplementary Fig. 3), and it is generally known that increased levels of IFN-α is correlated with the development of interstitial pneumonia in PRRSV infection [5, 6]. Therefore, earlier and higher induction of IFN signaling by NA10 infection (7 dpi) than that by JA142 infection (14 dpi) regardless of the number of myeloid infected cells suggest the different virulence between the strains that lead to different host immune responses and disease outcome. More details and references were added into the manuscript as kindly suggested (updated lines 380 - 389)

[1] Sánchez-Carvajal, J. et al. Time series transcriptomic analysis of bronchoalveolar lavage cells from piglets infected with virulent or low-virulent porcine reproductive and respiratory syndrome virus 1. Journal of virology 96, e01140-01121 (2022).

[2] Rodríguez-Gómez, I.M. et al. Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells. Veterinary microbiology 235, 101-109 (2019).

[3] Ruedas-Torres, I. et al. The jigsaw of PRRSV virulence. Veterinary microbiology 260, 109168 (2021).

[4] Tong, G.-Z. et al. Highly pathogenic porcine reproductive and respiratory syndrome, China. Emerging infectious diseases 13, 1434 (2007).

[5] Ruedas-Torres, I. et al. The scene of lung pathology during PRRSV-1 infection. Frontiers in veterinary science 11, 1330990 (2024).

[6] Liu Y, Shi W, Zhou E, Wang S, Hu S, Cai X, et al. Dynamic changes in inflammatory cytokines in pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Clinical and Vaccine Immunology. 2010;17(9):1439-45.

In my opinion, the most meaningful comparison is between JA142 and the negative control if the aim

is to determine the mechanisms involved in “recovery” of the lung. However, I insist, the results suggest that recovery of lung lesions is not that clear since the pictures and the microscopic scores at 14pi and 21pi for JA142 look similar (no statistical comparison provided). Removing of the data that include the other strains and simplifying the writing, in the sense of trying to produce a shorter paper, (the current version is 85 pages including 33 supplementary figures) would help to better understand the significance of the work.

Our response: Thanks for the kind comments. As mentioned above, although “recovery” process could not be determined from NA8 group due to the limited timepoint, the “fate” of the host after the peak lung-damage timepoint (NA10 at 7dpi and JA142 at 14dpi) are clearly different. Therefore, maintaining the data from different infections would expand our understanding of PRRSV infection. As the reviewer kindly suggested, we have eliminated RNA dynamics parts to simplify the manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed most of my comments to the previous version of the manuscript. The revised manuscript has been improved to a certain extent. However, below are some more adjustments or clarifications needed to further support the authors' findings.

1. The data variation in the negative group of MRC1-NREP high macrophage cells and the NA8 group of MT1A high macrophage cells in Fig. 3E, as well as the JA142 group of ATII cells in Supplementary Fig. 17 are still very large. I'm very concerned about the reliability of the conclusion drawn by these figures (page 8, lines 152-154; page 9, lines 191-193; page 16, lines 388-392).

Our response: Thank you for your kind comment. We have revised the sentences to include more appropriate information for clarity (page 8, lines 161-165; page 9, lines 202-204). In addition, except for SPP1-expressing macrophages, most macrophage populations (12 subtypes among 17 subtypes) tended to decrease after infection, so the sentence “all macrophage populations decreased” was revised to “most macrophage populations tended to decrease” (page 17, line 426).

2. I still insist that the demonstration of quantitative evaluation of the communication strength using analytical tools or experimental verification is necessary to ensure the actual existence of cell communication within different cell types. Since the authors have previously evaluated the communication strength using CellChat, there are many ways to interpret the complex results meaningfully. For example, they can list all significantly predicted ligand-receptor pairs within a supplementary table or choose to visualize certain pairs of the predicted ligand-receptor pairs by functions provided by CellChat. I was quite surprised when they stated that they decided not to include them due to concision issues, as it is common practice in many articles to present ligand-receptor analysis results in this manner (as shown in PMID 37824652, PMID 38649488, and PMID 38480880).

Moreover, CellChat could provide comparative analysis under two conditions, which made it meaningful to compare the altered probability of ligand-receptor pairs in different cell types, even by the circle plots.

Our response: Thank you for your constructive comment. As the reviewer suggested, we performed further analyses using CellChat for identifying cell-cell communication (page 11, lines 261-276; page 27, lines 686-690; Supplementary Fig. 31; Supplementary Fig. 32). The number of interactions was predicted using ligand-receptor pairs and functions were compared between JA142 14 dpi and NA10 7 dpi using interaction probability differences. Additionally, we focused on the SPP1-CXCL14^{high} macrophages that might contribute to anti-inflammation and identified differences in ligand-receptor pairs between JA142 14 dpi and NA10 7 dpi.

3. In newly added violin plots of Fig. 6B, and Supplementary Fig. 30, are the minimum values of the gene expression levels zero?

Our response: We have confirmed that the minimum values of the gene expression levels were zero. Since a large amount of macrophage data was produced and many macrophage sub-cell types were included in each gene, it is believed that the following results were obtained.

Reviewer #2 (Remarks to the Author):

The authors have addressed my previous comments partially in a satisfactory manner. Nevertheless, I still believe that it is not possible to conclude that the SPP1-CXCL14+ macrophages were identified as "the potential key cell population that contributes to recovery" (stated in the abstract). If this population did not emerge in virulent infection, it might just be a matter of timing. For such a statement I would like to see data showing how the emergence of this population correlates with disease control. This would of course require an animal experiment with sufficient number of animals and a variability in the outcome after infection with one intermediate strain. Furthermore, recovery is most likely impossible without the adaptive immune system kicking in. Therefore naming this macrophage population "the

potential key" is scientifically problematic.

Our response: We would like to thank the reviewer for their constructive comment. As they rightly observed, there is currently insufficient data to reach a definitive conclusion regarding the role of the SPP1-CXCL14^{high} macrophage, which has been tentatively named the potential key cell. Therefore, we have attempted to present more nuanced interpretation of the data in the abstract and the manuscript (page 2, lines 10-19; page 19, lines 472-475), suggesting that the SPP1-CXCL14^{high} cell may play a role in either the defense mechanism or the recovery process, but needs further investigation in the future.

Along the same line, the summary Figure 8 claims to have identified "the mechanisms of lung recovery" (Title of Figure 8). This is scientifically not correct and does not reflect the content of the Figure and the manuscript, which is descriptive in nature and not suited for this purpose.

Our response: As the reviewer suggested, the title of Figure 8 has been revised into “Figure 8. Illustration of chronological order of lung recovery and bystander cell death according to the virulence of PRRSV observed in this study”.

As previously mentioned, the study has its merits as an important resource paper for future studies on porcine BALF, and as a manuscript helping to build new working hypotheses to understand PRRSV pathogenesis. This is probably important also for other respiratory infection and might also have be important for human lung infection. So once again, the authors should systematically check their manuscript if statements are scientifically justified and correct accordingly.

Our response: Thank you for your kind comment. As the reviewer suggested, the whole manuscript has been systematically checked accordingly, and revised the conclusions (page 2, lines 19-21; page 19, lines 475-477).

In addition, a language check is required.

Our response: The manuscript has been checked for language errors, and revised. We attached the certificate of English correction.

Reviewer #3 (Remarks to the Author):

The author exhaustively answered most of the questions and made an effort to improve the readability of the manuscript. However, I still think that the lack of data for NA8 and NA10, although understandable, is a point. The authors sent a very elaborated answer to this question, presenting data that help clarifying the different degrees of virulence, I suggest that the authors should mention something on this on the discussion.

Our response: We would like to thank the reviewer for their constructive comment. As the reviewer suggested, more description and data for NA8 and NA10 has been added into the discussion section (first paragraph) to improve the readability of the manuscript (page 15, lines 348-362).