nature portfolio

Peer Review Information

Journal: Nature Microbiology
Manuscript Title: Murine parainfluenza virus Persists in Lung Innate Immune Cells Sustaining Chronic Lung Pathology
Corresponding author name(s): Professor Carolina Lopez

Reviewer Comments & Decisions:

Decision Letter, initial version:

Message: 14th February 2024

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Professor Lopez,

Thank you for your patience while your manuscript "Murine Parainfluenza Virus Persists in Lung Innate Immune Cells Sustaining Chronic Lung Pathology" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the end of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, reviewer #1 wants you to clarify and/or improve your gating strategy to identify ILC2s and to isolate them for the transcriptome analysis, has issues with the 2 populations of cells for which you compare transcriptomes as they have different cell compositions, and asks whether you can determine the main cell type contributing to the lung pathology. Reviewer #2 thinks it is important that you present evidence of the presence of pulmonary pathology in WT mice 49 days post-infection, acknowledge that there is a significant difference in the weights between virus and mock infected mice at day 49, quantify your IF images, discuss the likely attenuation of rSeV-CeGFP-Cre virus, clarify your focus on immune cells, and present evidence that the depletion of cells still holds at day 49. Reviewer #3 would like you to present a wider discussion of some important questions raised by the work and how the model could be exploited to address them, rule out the possibility that the presence of virus neutralizing antibody in lung homogenates does not inactivate any virus that might be present, and address how SeV RNA is present in tdTOM+NP- cells.

Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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Nature Microbiology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision, even if a similar study has been accepted for publication at Nature Microbiology or published elsewhere (up to a maximum of 6 months).

In the meantime we hope that you find our referees' comments helpful.

Yours sincerely,

Senior Editor
Nature Microbiology

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Reviewer Expertise:

Referee #1: ILCs
Referee #2: Respiratory infections, Immunology
Referee #3: Parainfluenza, Immunology

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, Araujo Castro et al. describe that Sendai virus (Sev) persist in ILC2s and DCs of mouse lungs long after the clearance of the infection. The infected cells have changed their transcriptome towards a more type 2 inflammation signature compared to non-infected cells, and contribute to the overall damage of the lung tissue. Indeed, depletion of the persistently infected cells, ameliorates this damage in the lung. These findings are novel and very interesting for the field. I have the following questions and concerns:

- Can the authors show that the gating strategy used in Fig. S1 to identify ILC2s contains a pure ILC2 population? I am a bit concerned about the CD3 negative gate as some low CD3+ cells may be included. Using a more robust lineage including anti-TCRs, other myeloid markers such as FCeRI and excluding NK.1.1+ cells prior to the ILC2 gate would help to make the ILC2 gate more accurate. Also, CD90 and CD25 can be down modulated in previously activated ILC2s, therefore, ILC2 numbers carrying the virus may be underestimated.

- Could the authors show the gating strategy used to isolate ILC2s for the transcriptome analysis in Figure 3?

- Text line 153 and Fig. 3E: not showing Il1rl1 (ST2) in the figure.

- In Figure 5, the authors compare the transcriptome signature of previously infected immune cells (Td tomato+ NP-) versus immune cells with persistent infection (Td tomato+ NP+). The immune cells with persistent infection are macrophages, DCs and ILC2, while other immune cell types would have been infected and clear the virus. Therefore, the cell composition of Td tomato+ NP- cells is different than the Td tomato+ NP+ cells. This can affect the gene ontology analysis. Which immune cells are part of the Td tomato+ NP- compartment? How can the authors determine the contribution of the cell composition differences in both samples versus having cleared or not the infection in the analysis?

- Finally, the authors show that depletion of the persistently infected cells has a beneficial effect in the pathogenesis of the lung tissue. Could the authors determine what is the main cell type contributing to the lung pathology? Can the authors either deplete DCs or ILC2s in a similar manner to evaluate the contribution of each cell type?

Reviewer #2 (Remarks to the Author):

The paper by Castro and colleagues addresses the highly topical question of the persistence

of viral antigens/PAMPs in the lung long after the acute infection has resolved, and the associated pathology. Specifically, the authors show that at 49 days post-infection mice infected with SeV have viral products persisting in lung macrophages, ILC2s and DCs – despite the absence of infectious virus. Importantly the depletion of these 'persistently infected' cells (or their descendants) reduced pulmonary pathology.
Overall this paper is well written and I congratulate the authors on using advanced molecular techniques to answer complex questions in a very complex in vivo model.
However, I believe the following comments must be addressed:
The central premise of this manuscript is that the persistence of viral antigen/RNA is associated with pulmonary pathology. However, the presence of pulmonary pathology in WT mice 49 days post-infection is not actually shown (although I acknowledge this is shown later in the context of cell depletion in recombinant mice). Perhaps this could somewhat be inferred from Figure 1E but in the absence of mock images it is hard to hell the specificity of staining like Krt5. Similarly, whilst I agree that there are transcriptional differences in cells at this later timepoint this is not necessarily indicative of lung disease. The authors need to perform something simple like H&E or show some sort of physiological dysfuncton(e.g. reduced blood oxygen staturation) at 49 dpi to support their conclusions
Line 84: "All mice fully recovered their weights by Day 35 post-infection" This is somewhat misleading – I cannot see any mention in the text of the fact that at day 49 there is a significant difference in the weights between virus and mock infected mice – this is not a problem, in fact it also helps provide evidence that there is persistent pathology at the later timepoint post-infection – but it needs to be acknowledged
All immunofluorescence shown in the manuscript should be coupled with image quantification, rather than simply the display of representative images – which can be very subjective
The mice infected with rSeV-CeGFP-Cre loose significantly less weight than mice infected with the WT virus – which is overlooked by the sentence on Line 172 - this suggests that there is a significant attenuation of the virus – the authors should discuss this and its implications for their conclusions
I am confused by the proportion of tdTomato+ CD45+ cells – from the data in Figure 4 it appears that the majority of infected cells at day 49 are non-immune; the question then arises as to why the initial investigation focussed on immune cells – I believe the justification was that majority of NP+ cells were in the alveolus but were these in the lumen of the alveolus? If so, how do the authors reconcile the results?
I appreciate that DT successfully depletes infected cells in the acute model – however I believe that it is essential to confirm that this still holds true when the depletion occurs at 21/22 dpi and then the mice are euthanised at day 49. In particular, given that that the initial findings focussed on ILC2s, macrophages and DCs – given the observation that the majority of the infected cells in this model appears to be in CD45- cells so I am wondering if the phenotype is in fact driven by a loss of epithelial cells
All statistics were performed with the assumption that data was normally distributed – how was this confirmed?

Minor points
The authors speculate in the discussion that RNA translation is still in place during the persistent phase of the infection – I am wondering if translation is really happening or whether its just the persistence of viral antigen – if it is translation how is that happening in terms of what viral proteins are mediating it?

Line 403 'injected' should be replaced with inoculated

Reviewer #3 (Remarks to the Author):

As well as causing acute infections, many RNA viruses can also establish prolonged or persistent infections. However, the molecular events that lead to the establishment of persistent RNA virus infections and their consequences for both the virus and host are in general are poorly understood as this is a difficult and under investigated area of virus research. Part of the reason for this is that there are very few good animal models to investigate persistent RNA virus infections. In the manuscript submitted by Caroline Lopez the authors report on the development of an elegant mouse model that can be used to investigate persistent infections caused by the respiratory murine paramyxovirus, Sendai virus (SeV). They have used this model to convincingly show that even at late times after infection (49 days) large numbers (~2%) of immune cells, primarily macrophages, type 2 innate lymphocytes and dendritic cells, found in the lungs of infected animals contain virus proteins and RNA. Importantly they show that at late times post infection that infected immune cells, or cells derived from these cells, are primarily responsible for chronic lung disease. Other important conclusions can also be gleaned from the work, for example that a significant number of cells that were originally infected with SeV at early times post infection clear the virus by non-lytic means. The work presented is of high quality and in general the data presented supports the conclusions drawn. The work is important as it highlights how persistent RNA virus infections can influence the development of chronic lung disease and provides a model as to how such infections may be better managed.

Specific comments.

1. Whilst I fully appreciate that the manuscript is concerned with investigating persistent infections and chronic lung disease, a wider discussion of some important questions raised by the work, and how the model could be exploited to address them, would make the paper more accessible to a broader audience. Examples that could be briefly discussed are: i) why is there a failure of the immune (CTL) response to clear the SeV +ve cells, ii) what is the mechanism(s) that results in the non-lytic clearance of SeV from some infected cells, iii) are immune cells out-with the lungs "persistently" infected (NP+ve or SeV RNA +ve) and, if so, is chronic inflammation observed in other tissues (In this regard, would adoptive transfer experiments of tdTOM+ cells to uninfected animals be informative?), iv) given Dr Lopez's interest in the role of defective virus genomes (DVGs) in inducing innate immune responses, what, if any, is thought to be their possible role in chronic inflammation/lung disease.
2. Before the authors can conclude no infectious virus is produced at late time post infection, they need to rule out the possibility that the presence of virus neutralizing antibody in lung homogenates does not inactivate any virus that might be present. Indeed, the extensive NP fluorescence in Figure 2, panel A, suggests that NP was being, or had recently been, synthesised within these NP+ve cells, indicating that virus transcription and replication may still have been occurring within these cells at late time post infection. Infectious centre assays on individual cell populations may also prove instructive in answering this question.

3. It is difficult to understand how SeV RNA is present in tdTOM+NP- cells. Perhaps, tdTOM+NP- cells were weakly NP+ve but was below the level of detection in the assay used. This point should be addressed.
4. In figure 2, panel E it should be stated at what days p.i. the samples were analysed. What does "% of parent" mean.
5. Given the wide diversity of diseases caused by paramyxoviruses, in several places throughout the manuscript (e.g. lines 328, 349, 370), rather than use the term paramyxovirus, the authors should use the term SeV as their findings are unlikely to related to all paramyxoviruses.

Author Rebuttal to Initial comments

Point by point response

We thank the reviewers for their constructive reviews. We have addressed all the concerns resulting in a much-improved report. All revisions are marked in burgundy font in the "marked" copy of the manuscript. Below a point-by-point response to the concerns:

Reviewer #1 (Remarks to the Author):

In this manuscript, Araujo Castro et al. describe that Sendai virus (Sev) persist in ILC2s and DCs of mouse lungs long after the clearance of the infection. The infected cells have changed their transcriptome towards a more type 2 inflammation signature compared to non-infected cells, and contribute to the overall damage of the lung tissue. Indeed, depletion of the persistently infected cells, ameliorates this damage in the lung. These findings are novel and very interesting for the field. I have the following questions and concerns:

- Can the authors show that the gating strategy used in Fig. S1 to identify ILC2s contains a pure ILC2 population? I am a bit concerned about the CD3 negative gate as some low CD3+ cells may be included. Using a more robust lineage including anti-TCRs, other myeloid markers such as FCeRI and excluding NK.1.1+ cells prior to the ILC2 gate would help to make the ILC2 gate more accurate. Also, CD90 and CD25 can be down modulated in previously activated ILC2s, therefore, ILC2 numbers carrying the virus may be underestimated.

- We appreciate the suggestion to improve resolution on the ILC2 gate. We updated the gating strategy in **Figure S1A** and pre-gated in CD11b, CD11c, F4/80 myeloid markers as well as B220 and NK1.1 prior to the ILC2 gate. The frequencies did not significantly change, but all data presented in **Figure 2** was reanalyzed this way. Our initial focus for the characterization was not specifically ILC2s and we also wanted in the same panel to analyze other cell types infiltrating the lungs. For the sorting experiment then yes, we included a more robust lineage exclusion gate (previously validated and published) with

the markers suggested by the reviewer (**Figure S1B**) in a system optimized to sort ILC2s (sm13 IL13 reporter mice).

- We thank the reviewer for the comment regarding potential underestimation of ILC2 numbers. As mentioned above we have added a more stringent lineage exclusion gate for the following ILC2 sorting and RNAseq experiments. The resulting transcriptomic profile gave us satisfactory resolution in terms of expression of ILC2 hallmark genes, such as GATA3, RORa, and Klrg1, as well as other genes important to ILC2 biology (Il5, Il13, Areg).

- Could the authors show the gating strategy used to isolate ILC2s for the transcriptome analysis in Figure 3?

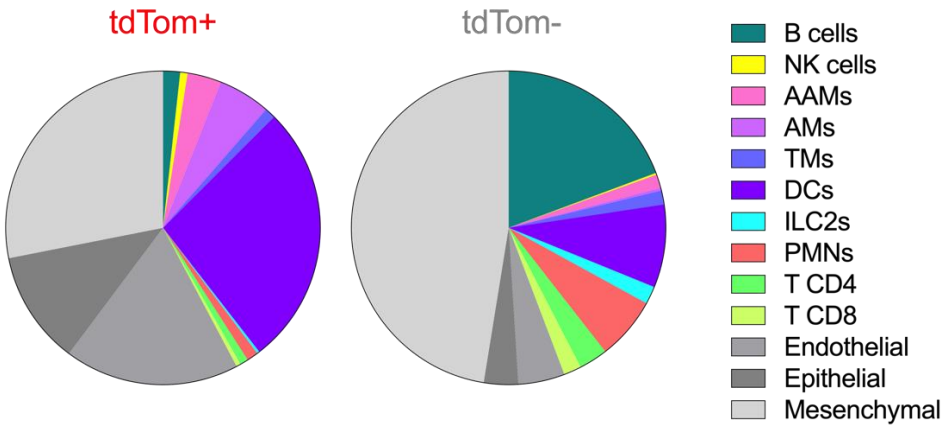
- We included a gating strategy panel in **Figure S1** for the sorting/transcriptome experiment.

- Text line 153 and Fig. 3E: not showing Il1rl1 (ST2) in the figure.

- We thank the reviewer for catching this mistake. It is now corrected in the manuscript.

- In Figure 5, the authors compare the transcriptome signature of previously infected immune cells (Td tomato+ NP-) versus immune cells with persistent infection (Td tomato+ NP+). The immune cells with persistent infection are macrophages, DCs and ILC2, while other immune cell types would have been infected and clear the virus. Therefore, the cell composition of Td tomato+ NP- cells is different than the Td tomato+ NP+ cells. This can affect the gene ontology analysis. Which immune cells are part of the Td tomato+ NP- compartment? How can the authors determine the contribution of the cell composition differences in both samples versus having cleared or not the infection in the analysis?

- This is a good point. In the RNAseq tdTom/NP experiment, the transcriptomes of tdTom+NP- and tdTom+NP+ are compared against Mock and tdTom- cells ("Negative") present in the infected lung. Cells from the same infected lung microenvironment that had not interacted with the virus (tdTom- or "Negative' cells) showed no altered transcriptomes in comparison with Mock. Because of this, we decided that the fairest comparison was between tdTom+ and tdTom- present in the same environment. Moreover, we confirmed by flow cytometry that in the tdTom- population, we have good representation of the cells of interest (macrophages, DCs and ILC2s). However, as the reviewer suggest, the percentage of the different cell types in these groups varies (see pie charts included below). To complement these studies, we now added new data showing sorting on tdTom and specific cell types (**Figure 4 I-J**). This new data set gets to the same conclusion but it is certainly more specific.



- Finally, the authors show that depletion of the persistently infected cells has a beneficial effect in the pathogenesis of the lung tissue. Could the authors determine what is the main cell type contributing to the lung pathology? Can the authors either deplete DCs or ILC2s in a similar manner to evaluate the contribution of each cell type?

- We thank the reviewer for the suggestion. Based on our characterization of SeV persistently infected cells (**Figure 2**), we sorted the most frequent SeV NP-expressing cell types (ILC2s, macrophages, and DCs) and relevant for SeV pathogenesis from infected tdTomato reporter mice. We then analyzed the transcriptomes of tdTom^{+} ILC2s, macrophages, and DCs in comparison with their tdTom^{-} counterparts. This approach allowed us to demonstrate that ILC2s and macrophages that were infected and are still present in the lungs in the chronic phase of infection have long-lasting transcriptional changes that associate with the maintenance of the chronic lung pathology. These results are now displayed in **Figure 4** and **Figure S4**. We agree that depletion of ILC2 and other cell populations would ultimately demonstrate the contribution of different cell populations and we are gearing up to perform these experiments. However, these are complex experiments that require dose adaptations and a plethora of proper controls to ensure that the course of infection is not affected and that the animals survive. We hope that the reviewer understands that these experiments are beyond the scope of this publication.

Reviewer #2 (Remarks to the Author):

The paper by Castro and colleagues addresses the highly topical question of the persistence of viral antigens/PAMPs in the lung long after the acute infection has resolved, and the associated pathology. Specifically, the authors show that at 49 days post-infection mice infected with SeV

have viral products persisting in lung macrophages, ILC2s and DCs – despite the absence of infectious virus. Importantly the depletion of these 'persistently infected' cells (or their descendants) reduced pulmonary pathology.
Overall this paper is well written and I congratulate the authors on using advanced molecular techniques to answer complex questions in a very complex in vivo model. However, I believe the following comments must be addressed:

The central premise of this manuscript is that the persistence of viral antigen/RNA is associated with pulmonary pathology. However, the presence of pulmonary pathology in WT mice 49 days post-infection is not actually shown (although I acknowledge this is shown later in the context of cell depletion in recombinant mice). Perhaps this could somewhat be inferred from Figure 1E but in the absence of mock images it is hard to hell the specificity of staining like Krt5. Similarly, whilst I agree that there are transcriptional differences in cells at this later timepoint this is not necessarily indicative of lung disease. The authors need to perform something simple like H&E or show some sort of physiological dysfuncton(e.g. reduced blood oxygen staturation) at 49 dpi to support their conclusions

- We thank the reviewer for the suggestions and agree that including a mock image as a negative control for **Figure 1** as well as correspondent H&E images from the initial SeV infection model was needed to strengthen our claims. We have now added these **to Figure 1** in panels **D and F**.

Line 84: "All mice fully recovered their weights by Day 35 post-infection" This is somewhat misleading – I cannot see any mention in the text of the fact that at day 49 there is a significant difference in the weights between virus and mock infected mice – this is not a problem, in fact it also helps provide evidence that there is persistent pathology at the later timepoint post-infection – but it needs to be acknowledged

- We apologize for the misinterpretation and thank the reviewer for the suggestion. We have now acknowledged the lingering weight difference seen in the persistently infected mice in the text (**Lines 85-88**).

All immunofluorescence shown in the manuscript should be coupled with image quantification, rather than simply the display of representative images – which can be very subjective

- In general, we agree with this statement and we included quantification of Krt5-stained area in the **Figure 1**. For **Figure 2** we chose to use spectral flow cytometry to quantify different SeV NP+ subpopulations given the complexity of markers necessary to define each cell subset, keeping the immunofluorescence panels as representative images only. The immunofluorescence images present in **Figure 6** are also quantified for Krt5

and Krt8.

The mice infected with rSeV-CeGFP-Cre loose significantly less weight than mice infected with the WT virus – which is overlooked by the sentence on Line 172 - this suggests that there is a significant attenuation of the virus – the authors should discuss this and its implications for their conclusions

- We thank the reviewer for noticing this discrepancy. We agree that SeVC$^{eGFP-Cre}$, which was generated in the Cantell strain backbone, is attenuated compared to SeV 52, as expected do to well described differences between the 52 and Cantell strains. Most importantly, the Cantell strain generated more immunostimulatory viral molecules (copy-back genomes). We have now revised this statement in **Lines 182-186**. Since we observed similar phenotypes with both strains (albeit with different intensities of disease) we are not concerned with the overall interpretation of the results.

I am confused by the proportion of tdTomato+ CD45+ cells – from the data in Figure 4 it appears that the majority of infected cells at day 49 are non-immune; the question then arises as to why the initial investigation focussed on immune cells – I believe the justification was that majority of NP+ cells were in the alveolus but were these in the lumen of the alveolus? If so, how do the authors reconcile the results?

- We are sorry for the confusion. As noted by the reviewer, the number of tdTom+CD45- cells at 49 dpi is higher than the number of tdTom+CD45+ cells. In addition, the proportion of CD45- cells in the tdTom+ cell compartment at 49 dpi is lower (averaging 57.6%) than at 3 dpi (averaging 87.4%) while the proportion of the CD45+ compartment increases at 49 dpi (average 38.6%). However, most of the tdTom+CD45- cells do not express viral antigen in the chronic phase of the infection most likely representing cells that survived the infection but cleared the virus, or non-infected daughter cells (see 3D lung reconstruction panels added to **Figure 4** for clarification). The actual source of persistent antigens (SeV NP+) are CD45+ cells from the tdTom+ compartment, as corroborated by the characterization of NP-expressing cells in **Figure 2** (no NP expression from the non-immune compartment). This can be further appreciated with the whole lung 3D reconstruction where the only eGFP-expressing cells (proxy for active viral replication) are in cellular infiltrates around terminal bronchioles and not in the airways (**new Figure 4B**) in our rSeV-C$^{eGFP-Cre}$ / tdTomato reporter mice system. Hence, we focused this work on the antigen expressing population.

I appreciate that DT successfully depletes infected cells in the acute model – however I believe that it is essential to confirm that this still holds true when the depletion occurs at 21/22 dpi and then the mice are euthanised at day 49. In particular, given that that the initial findings focussed on ILC2s, macrophages and DCs – given the observation that the majority of the infected cells

in this model appears to be in CD45- cells so I am wondering if the phenotype is in fact driven by a loss of epithelial cells

- These are great points. We have now included in **Figure S5** representative panels and the correspondent data of DT-mediated ablation efficacy of SeV NP$^+$ cells also at 49 dpi, following the 21/22 dpi DT administration regime.
- We are currently studying the contribution of epithelial cells to the chronic pathology in a parallel project. At the moment, as explained above, we have focused on cells expressing viral antigen in the chronic phase of the infection. We do agree that the DT depletion experiment does not directly address whether antigen is required for disease. However, data shown in **Figures 4 and 5** of sorted tdTom+ cells segregated by cell type or viral antigen expression, respectively argues that viral presence in ILC2s and macrophages strongly associate with transcriptional profiles previously established to drive type 2 inflammation and chronic lung disease in this model.

All statistics were performed with the assumption that data was normally distributed – how was this confirmed?

- All statistical analysis was changed to non-parametrical tests throughout the entire manuscript.

Minor points
The authors speculate in the discussion that RNA translation is still in place during the persistent phase of the infection – I am wondering if translation is really happening or whether its just the persistence of viral antigen – if it is translation how is that happening in terms of what viral proteins are mediating it?

- Regrettably we do not have good means to fully address this interesting question at this point. However, we found viral RNA reads in the sorted populations as shown in **Figure S4B** and evidence of viral RNA based on RNA scope (**Figure 1**), suggesting that not only protein antigen remains.

Line 403 ‘injected’ should be replaced with inoculated

- The correction was made.

Reviewer #3 (Remarks to the Author):

As well as causing acute infections, many RNA viruses can also establish prolonged or persistent infections. However, the molecular events that lead to the establishment of persistent RNA virus infections and their consequences for both the virus and host are in general are

poorly understood as this is a difficult and under investigated area of virus research. Part of the reason for this is that there are very few good animal models to investigate persistent RNA virus infections. In the manuscript submitted by Caroline Lopez the authors report on the development of an elegant mouse model that can be used to investigate persistent infections caused by the respiratory murine paramyxovirus, Sendai virus (SeV). They have used this model to convincingly show that even at late times after infection (49 days) large numbers (~2%) of immune cells, primarily macrophages, type 2 innate lymphocytes and dendritic cells, found in the lungs of infected animals contain virus proteins and RNA. Importantly they show that at late times post infection that infected immune cells, or cells derived from these cells, are primarily responsible for chronic lung disease. Other important conclusions can also be gleaned from the work, for example that a significant number of cells that were originally infected with SeV at early times post infection clear the virus by non-lytic means. The work presented is of high quality and in general the data presented supports the conclusions drawn. The work is important as it highlights how persistent RNA virus infections can influence the development of chronic lung disease and provides a model as to how such infections may be better managed.

Specific comments.

1. Whilst I fully appreciate that the manuscript is concerned with investigating persistent infections and chronic lung disease, a wider discussion of some important questions raised by the work, and how the model could be exploited to address them, would make the paper more accessible to a broader audience. Examples that could be briefly discussed are: i) why is there a failure of the immune (CTL) response to clear the SeV +ve cells, ii) what is the mechanism(s) that results in the non-lytic clearance of SeV from some infected cells, iii) are immune cells out-with the lungs “persistently” infected (NP+ve or SeV RNA +ve) and, if so, is chronic inflammation observed in other tissues (In this regard, would adoptive transfer experiments of tdTOM+ cells to uninfected animals be informative?), iv) given Dr Lopez’s interest in the role of defective virus genomes (DVGs) in inducing innate immune responses, what, if any, is thought to be their possible role in chronic inflammation/lung disease.

- We thank the reviewer for the insightful suggestions. We have now expanded the discussion on some of these topics when we feel we have an educated discussion to provide. For example, our laboratory described that infection of human lung cell lines *in vitro* with SeV containing a high load of copy-back genomes result is persistent infections due to the stimulation of cell survival mechanisms. Although we are not ready to imply that this is a mechanism relevant in vivo, we have included this evidence as part of the discussion. Please l**ines 438-452** for the expanded discussion.

2. Before the authors can conclude no infectious virus is produced at late time post infection, they need to rule out the possibility that the presence of virus neutralizing antibody in lung homogenates does not inactivate any virus that might be present. Indeed, the extensive NP

fluorescence in Figure 2, panel A, suggests that NP was being, or had recently been, synthesised within these NP+ve cells, indicating that virus transcription and replication may still have been occurring within these cells at late time post infection. Infectious centre assays on individual cell populations may also prove instructive in answering this question.

- The reviewer is right in that the animals are likely having a robust neutralizing response at the chronic stage of infection. In fact, the use of immune competent animals is by design and a key differential in this study from other models using immunosuppressed models. We agree that the question of whether infectious virus is produced remains, and we have carefully revised the wording to reflect this fact. We would like to mention that we have been unable to see infectious particles even when trying to expand the virus in embryonated chicken eggs, which will expand from as low as one infectious unit. In preliminary experiments, we have however observed transmission of viral products when ILC2 or macrophages infected in vitro and stripped from left-over bound virus are exposed to susceptible cells and we are exploring the possibility that there is cell to cell transmission events occurring but no particle production. Additionally, SeV requires furin or a similar enzyme for productive infection (particle production) and to the best of our knowledge ILC2s and phagocytes do not express this enzyme, supporting the idea that particles may not be produced.

3. It is difficult to understand how SeV RNA is present in tdTOM+NP- cells. Perhaps, tdTOM+NP- cells were weakly NP+ve but was below the level of detection in the assay used. This point should be addressed.

- We acknowledged the possibility that some tdTom+ cells could have been excluded from the tdTom+NP+ gate due to low level of NP expression. We repeated the experiment after troubleshooting the SeV NP staining in simultaneous combination with detection of tdTom and eGFP, and got better resolution for separation of NP+ cells. We performed a new experiment isolating ILC2s, macrophages and dendritic cells from tdTom reporter mice infected with our recombinant rSeV-C$^{eGFP\text{-}Cre}$ virus. When we compared the transcriptomes of tdTom+ ILC2s, macrophages and DCs with their correspondent tdTom- counterparts, we were able to detect viral reads substantially only in cells that encountered the virus. This new dataset replaced the previous virus reads results and is now displayed in **Figure 4** and **Figure S4**.

4. In figure 2, panel E it should be stated at what days p.i. the samples were analysed. What does “% of parent” mean.

- We apologize for the confusion. Figure 2 now has an extra label indicating the timepoint. ‘% of parent’ means that the frequencies shown are the resulting % of SeV NP+ cells

within each specific cell subset, example, inside the ILC2 subset, an average of 15% ILC2s were positive for SeV NP. We replaced the axis titles to '% within cell population' to avoid misinterpretation.

5. Given the wide diversity of diseases caused by paramyxoviruses, in several places throughout the manuscript (e.g. lines 328, 349, 370), rather than use the term paramyxovirus, the authors should use the term SeV as their findings are unlikely to related to all paramyxoviruses.

- We acknowledge the reviewer's concern and as suggested, we replaced the generalist term 'paramyxovirus' to SeV in the manuscript when appropriated.

Decision Letter, first revision:

essage: Our ref: NMICROBIOL-23113030A

24th July 2024

Dear Dr. Lopez,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "Murine Parainfluenza Virus Persists in Lung Innate Immune Cells Sustaining Chronic Lung Pathology" (NMICROBIOL-23113030A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Microbiology's

editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Murine Parainfluenza Virus Persists in Lung Innate Immune Cells Sustaining Chronic Lung Pathology". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Microbiology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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If you have any further questions, please feel free to contact me.

Best regards,

Reviewer #1:
Remarks to the Author:
I appreciate the efforts of the authors to address my comments. I am satisfied with the additions and answers so I support this publication. Congratulations to the authors!

Reviewer #2:
None

Reviewer #3:
Remarks to the Author:
The authors are thanked for taking on board my comments/suggestions, which have now been addressed. I have no other remarks to make.

Final Decision Letter:

Message: 6th August 2024

Dear Carolina,

I am pleased to accept your Article "Murine parainfluenza virus Persists in Lung Innate Immune Cells Sustaining Chronic Lung Pathology" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Microbiology style. We look particularly carefully at the titles of all papers to ensure that they are relatively brief and understandable.

Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required. Once your paper has been scheduled for

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