EMBO reports

Interferon-induced transmembrane protein 3 (IFITM3) limits lethality of SARS-CoV-2 in mice

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1st Editorial Decision **16th Dec 2022**

Dear Dr. Yount,

Thank you for the transfer of your research manuscript to EMBO reports. I now went through your manuscript and the referee reports from a venue outside EMBO press (attached again below). The referees indicate that these findings are of high interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. We will contact the previous journal (we have a portable peer review agreement) to obtain the referee identities. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures (up to 8) and EV figures. Please upload these as separate, individual files upon re-submission.

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http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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12) Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend.

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Title page - Abstract - Keywords - Introduction - Results & Discussion - Materials and Methods - Data availability section - Acknowledgements (including funding information) - Disclosure and Competing Interests Statement - References - Figure

legends - Expanded View Figure legends

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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Best,

Achim Breiling

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The antiviral protein IFITM3 inhibits endocytic entry by several enveloped viruses and prevents severe disease in a mouse model of influenza A virus infection. Several groups have reported opposing inhibitory and enhancing effects of IFITM3 on SARS-CoV-2 infection when using cell culture models. Furthermore, IFITM3 gene polymorphisms have been identified as risk factors for severe COVID-19 by some groups, but not by others. Zani et al investigated the physiological role of IFITM3 in a mouse model and uncovered a significant contribution in constraining SARS-CoV-2 pathogenesis. The use of mice to study SARS-CoV-2 pathogenesis has been a field-wide challenge, due to incompatibilities between the spike glycoprotein of the original SARS-CoV-2 strain and the mouse ortholog of its cognate receptor, ACE2. Several solutions have emerged, including transgenic human ACE2-expressing mice, viral vector-mediated expression of human ACE2 in mice, and mouse adapted SARS-CoV-2, which is genetically distinct, albeit by only several amino acids, from strains circulating in human populations. More recent variants of concern (e.g. gamma, omicron) that possess the N501Y mutation in the spike glycoprotein have also gained the ability to infect mice, but do not cause visible disease such as weight loss in wildtype animals. A major strength of this paper is the use of multiple mouse models to address the antiviral contributions of IFITM3. The authors primarily use mouse adapted SARS-CoV-2 to demonstrate a critical role for IFITM3 in controlling pulmonary viral titers, inflammation, and pathology. They also generated mice that are homozygous for IFITM3 knockout and hemizygous for the keratin18-driven human ACE2 transgene (K18-hACE2) to show that IFITM3 also restricts pathogenesis of the parental human isolate of the mouse-adapted SARS-CoV-2. An unfortunate byproduct of using two distinct models is reconciling phenotypic differences, such as higher viral titers in the lungs of mouse-adapted virus-infected IFITM3 KO mice but not IFITM3-KO-K18-hACE2 mice or enhanced cardiac dissemination in IFITM3-KO-K18-hACE2 mice but not in mouse-adapted virus-infected IFITM3 KO mice. These differences make it difficult to identify a common cause of death between the two models, but the contribution of IFITM3 to resistance to SARS-CoV-2 is clearly demonstrated regardless. This discrepancy also highlight a potential shortcoming of the K18-hACE2 mouse model, which has been documented by others for its abnormally high expression of human ACE2 in the brain and other tissues when compared to endogenous mouse ACE2 levels.

Over the past few years, the field has made a strong case for the importance of an intact interferon response in controlling SARS-CoV-2 pathogenesis. In addition, several groups have also highlighted location-specific detrimental effects of interferon on the mucosa. Zani et al expand on earlier findings on the importance of antiviral interferons by showing that whole body loss of a single effector protein that is downstream of interferon (and likely basally expressed prior to interferon exposure) is largely responsible for restricting SARS-CoV-2 pathogenesis in two mouse models of infection. These findings not only further fine tune our knowledge of interferons and SARS-CoV-2 infection but also may be important for interpreting the risk factor of IFITM3 polymorphisms in patient populations.

Overall, the manuscript is already quite strong, and the data are convincing and carefully described without overinterpretation. I only have minor suggestions that may improve the manuscript.

Content:
1. While the data clearly shows that loss of IFITM3 renders mice highly susceptible to both mouse-adapted and human isolates of SARS-CoV-2, there are phenotypic dissimilarities between the two models, specifically a lack of difference in lung titers and a significant increase in cardiac dissemination in the K18 model, but not the MA10 model. The K18 mice probably succumbed due to increased infection of the vasculature that contributes to lethal inflammation, but the presence of the hACE2 transgene escalates viral replication to saturation in the lung and thus masks a titer difference at day 3. Given the many different animal models that have been developed to study SARS-CoV-2, I think the manuscript would benefit from a sentence or two that help the reader understand why some readouts between the MA10 and K18 models differ. Shuai et al (PMID:34689086) have a nice comparison of mACE2 and hACE2 expression that might be of interest.
2. In line 179, the authors state that increased viral antigen in the lungs of KO mice 'likely represents shedding of necrotic, highly

necrotic cells into the bronchioles.' This made me curious if the authors have looked at published single cell datasets that describe basal IFITM3 levels in mouse lungs. Is there enrichment in cell types lining the vasculature when compared to other cells in the airways? Any information on where IFITM3 may be highly expressed or induced would be interesting for interpreting the pathology and RNA-Seq data.
3. In line 221, the authors cite studies that suggest IFITM3 contributes to feedback inhibition of the type I interferon response. Were sequencing studies performed for lungs of uninfected WT and IFITM3 KO mice, either for this project or previously published work? While not a necessary experiment for publication, this data would provide some insight into whether mice succumb due to the absence of IFITM3 as an antiviral effector or as a regulator of inflammation, if basal interferon signaling is affected by IFITM3.
4. Based on published literature on the interferon response and SARS-CoV-2 infection in mouse models, can the authors speculate as to whether basal or interferon-induced IFITM3 is important for protection? Is there published data on MA10 infection of mice deficient in IFN signaling?

Presentation:
1. It is unclear how many animals are used in figure 1 weight loss curves or in the titering experiments in panels D and G where virus was undetectable. Could the authors add animal numbers below the X axis to clarify this for the reader?
2. Skull is spelled 'skull,' not 'scull,' - in the figure legend for figure 1
3. The lung sections in figure 2 could benefit from larger arrows or different colors. The black arrows are sometimes difficult to see amongst the dark nucleocapsid staining.

Referee #2:

The manuscript by Zani et al. examines the role of IFITM3 in SARS-CoV-2 pathogenesis. Prior studies have found a conflicting role for IFITM3 in SARS-CoV-2 infection. These studies, performed in vitro, have shown both inhibitory impact and augmentation of infection. In this study, Zani et al. use mouse models of to demonstrate a key role for IFITM3 in protection from SARS-CoV-2 infection. Leveraging previously generated IFITM3-/- mice, the authors show use a mouse adapted SARS-CoV-2 strain to show increased weight loss, lethality, and viral titers in IFITM3-/- mice. They also show evidence of disseminated disease to the heart/brain/spleen in a subset of animals. They further demonstrated this finding in the HACE2 expressing mice crossed to IFITM3-/-. Using the original SARS-CoV-2 WA1 strain, they found similar increase in weight loss and lethality, although viral titers were less impressive. They also found disseminated disease in the heart of these IFITM3-/- compared to control. They subsequently show more disseminated disease in the lungs of IFITM3-/- mice using histopathology and RNA expression analysis shows a clear distinction compared to control. Overall, the work clearly demonstrates a key role for IFITM3 in control of SARS-CoV-2 in vivo.

No significant modification are required; however, the following points are suggestions for further clarification or improvement of the manuscript.

1. The histopathology findings that are presented are convincing, however it is unclear if all the animals have a similar profile. Scoring by a trained pathologist would improve the manuscript.
2. In a similar vein, adding histopathology from an earlier timepoints would provide some insights into how quickly the change in antigen staining occurs. Day 2 or Day 3 antigen staining could/should be included.
3. The authors measured IL6 showing a clear difference. Is there a link to IFITM3 and IL6? Also, is there a reason that other inflammatory cytokines were not surveyed (IL1 for example).
4. For fig 1 D, can the authors comment on if the animals that have titers in the heart/brain/spleen come from the same animal.
5. The authors report high titers in the hearts of hACE2/IFITM3KO mice; is there any signs of myocarditis. Were histology sections of the heart considered. The authors might consider a few comment on if this mouse could be used as a model myocarditis associated with COVID19.
6. Is there numerical value that can be added to fig 2g. It is difficult to orient what the values of the expression are in this figure.
7. The authors should reference PMID:23919993 when discussing activation of coagulation pathways and coronavirus disease.

Referee #3:

This study by Zani et al investigates an important topic of the role that the IFN-inducible protein IFITM3 plays in COVID19. The fact that the development of anti-IFN auto-antibody responses associate with severe COVID19 in humans demonstrates the possible importance of IFN in immune protection. As an IFN-inducible protein that has been shown to restrict the replication of a number of viruses, IFITM3 has the potential to play an important role in this response. However, data generated in vitro has demonstrated that the role that IFITM3 plays in SARS-CoV-2 replication is complicated and somewhat paradoxical. Herein, the authors attempt to gain clarity on this important topic, using mouse models of SARS-CoV-2 infection to understand how IFITM3 influences COVID19 pathogenesis in vivo.

The authors take the excellent approach of studying two separate IFITM3-deficient mouse models of SARS-CoV-2 infection to enable investigation of how IFITM3 impacts SARS-CoV-2 infection. Using the mouse-adapted SARS-CoV-2 infection model, the authors provide robust evidence that in this model IFITM3 protects the host from virus-induced weight loss and death, and limits SARS-CoV-2 replication, including spread within the lung and also to the heart. They also clearly show an accompanying increase in virus-induced inflammation, with increased cellular recruitment into the lungs and heightened chemokine expression in the absence of IFITM3. This supports the conclusion that IFITM3 exerts antiviral functionality in SARS-CoV-2 infection in vivo and in the absence of IFITM3, SARS-CoV-2 replicates and spreads widely, triggering more inflammation, which is analogous to reports in other respiratory infections such as influenza.

Data from the hACE-2 model also to some degree replicates the findings seen in the mouse-adapted SARS-CoV-2 model. This model also demonstrates robust protection afforded by IFITM3 from virus induced disease, with all IFITM3-deficient mice succumbing to infection by day 5. Moreover, extra-pulmonary spread of SARS-CoV-2 is controlled by IFITM3.

However, some of the findings do not fit well with data derived from the mouse adapted SARS-CoV-2 model. For example, the impact of IFITM3 on virus replication in the lungs appeared not to be dramatically altered by IFITM3, although the authors suggest that differences in lung virus loads between WT and IFITM3 deficient mice are significant. Furthermore, it is clear that in the mouse adapted SARS-CoV-2 model that IFITM3 limits virus spread and cellular inflammation in the lung. However, it is unclear whether the same is true here. This is important as IFITM3 is known to exert anti-inflammatory effects independently of antiviral functionality, which may be important. Also, the data from the two models may suggest that the ability of IFITM3 to limit extrapulmonary spread may be vital in the ability of IFITM3 to limit virus-induced death rather than local effects in the lung per se. Thus, although the study clearly shows in two mouse models that IFITM3 protects the host from SARS-CoV-2 disease and death, as per the conclusions of the paper, the data presented does not currently provide clear evidence as to how this occurs. Given the importance of IFN in protecting humans from COVID19 and the induction of anti-IFN antibody responses in individuals with severe outcomes, understanding better how IFN-inducible proteins like IFITM3 act in protective responses to SARS-CoV-2 could be beneficial clinically. Thus, further dissection by the authors of the similarities and differences between the two mouse models of SARS-CoV-2 infection may be very insightful in understanding how this IFN-induced protein protects the host from SARS-CoV-2 induced disease.

This is an interesting study and the dual approach of studying both the mouse-adapted SARS-CoV-2 model and the hACE-2 model is excellent. However, currently, the somewhat superficial analysis of the hACE-2 model makes the dual approach both a strength and a weakness. Further analysis of data from this model will provide important insight regarding how IFITM3 protects from SARS-CoV-2 induced death.

1. Central to this is providing convincing evidence whether or not IFITM3 restricts SARS-CoV-2 replication in the lungs. Data presented in Fig 1F was not particularly convincing. Was this the best of the two experiments and are the differences between the two groups significant? How clear was the difference in lung PFU in the other experiment? Do you observe the same pulmonary dissemination in the hACE-2 model as you see in the mouse-adapted SARS-CoV-2 model?
2. The authors discuss the anti-inflammatory role of IFITM3. Do the expression multiple pro-inflammatory cytokines elevate in the absence of IFITM3? The authors should extend their analysis of IL-6 to both models and incorporate multiple cytokines including TNF-alpa and IL-1b. Heightened pulmonary cytokine responses in both models but only dramatic differences in lung PFU in the mouse-adapted model may provide evidence for an important anti-inflammatory role for IFITM3 in dictating disease outcome. H+E and CD45+ analysis of lungs from the hACE-2 mice would further support such a conclusion.
3. It is clear in both models that extracellular spread of SARS-CoV-2 to the heart in IFITM3-deficient mice correlates with death. It would be useful to explore this further. Although I appreciate that it might not be possible to perform studies of cardiac dysfunction of SARS-CoV-2-infected mice (as per the group's excellent PNAS study), but some sense of whether IFITM3 protects from heart fibrosis and inflammation could be very informative, along with the suggested work above to understand better how IFITM3 protects from death.
4. Minor point - Figure 1I. The CD45+ stain for infected wt vs IFITM3-/- mice, although dramatic, does not appear particularly representative of the quantified data on the right-hand side i.e., there is a massive difference in infiltrates in the picture but only a moderate difference @d5 based on the data depicted in the graph.

Reviewer #1:

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1. While the data clearly shows that loss of IFITM3 renders mice highly susceptible to both mouse-adapted and human isolates of SARS-CoV-2, there are phenotypic dissimilarities between the two models, specifically a lack of difference in lung titers and a significant increase in cardiac dissemination in the K18 model, but not the MA10 model. The K18 mice probably succumbed due to increased infection of the vasculature that contributes to lethal inflammation, but the presence of the hACE2 transgene escalates viral replication to saturation in the lung and thus masks a titer difference at day 3. Given the many different animal models that have been developed to study SARS-CoV-2, I think the manuscript would benefit from a sentence or two that help the reader understand why some readouts between the MA10 and K18 models differ. Shuai et al (PMID:34689086) have a nice comparison of mACE2 and hACE2 expression that might be of interest.

We have added new data directly comparing lung histology of viral antigen staining for the two models (Figure 2D). These results show that viral infection in the K18-hACE2 model is indeed near saturation as the reviewer suggested. We have also added text in the Discussion further commenting on the limitations of the K18-hACE2 model for the reasons outlined above.

2. In line 179, the authors state that increased viral antigen in the lungs of KO mice 'likely represents shedding of necrotic, highly necrotic cells into the bronchioles.' This made me curious if the authors have looked at published single cell datasets that describe basal IFITM3 levels in mouse lungs. Is there enrichment in cell types lining the vasculature when compared to other cells in the airways? Any information on where IFITM3 may be highly expressed or induced would be interesting for interpreting the pathology and RNA-Seq data.

We analyzed published scRNA-seq data from naïve and SARS-CoV-2-infected mice for expression of *Ifitm3* and have included these data as Figure 1A. *Ifitm* is widely expressed and is upregulated as expected following infection.

3. In line 221, the authors cite studies that suggest IFITM3 contributes to feedback inhibition of the type I interferon response. Were sequencing studies performed for lungs of uninfected WT and IFITM3 KO mice, either for this project or previously published work? While not a necessary experiment for publication, this data would provide some insight into whether mice succumb due to the absence of IFITM3 as an antiviral effector or as a regulator of inflammation, if basal interferon signaling is affected by IFITM3.

We previously performed sequencing of non-infected WT and IFITM3 KO lungs as part of a larger analysis of influenza virus-infected mice and did not observe baseline differences between WT and KO lungs in interferon or interferon-regulatory pathways. Data included in our current study clearly show that SARS-CoV-2 replication is increased in the absence of IFITM3, and that this is accompanied by increased inflammation. Though the increased viral replication almost certainly triggers enhanced inflammation, we also included a mention of IFITM3's reported feedback inhibition of inflammation since this is a possible secondary effect of IFITM3 that could be occurring. Our study does not distinguish the relative contributions of these IFITM3 mechanisms, but overall establishes that IFITM3 is protective *in vivo* by limiting SARS-CoV-2 replication inflammatory pathology.

4. Based on published literature on the interferon response and SARS-CoV-2 infection in mouse models, can the authors speculate as to whether basal or interferon-induced IFITM3 is important for protection? Is there published data on MA10 infection of mice deficient in IFN signaling?

We have added new data of IFNAR KO mice infected with SARS-CoV-2 (Figure 1C). We observed more pronounced illness in IFNAR KO mice compared to WT mice, although these mice recovered from infection in contrast to IFITM3 KO mice, which succumb to infection. Given the more severe phenotype of the IFITM3 KO mice, these results suggest that basal IFITM3, or IFITM3 induced by other cytokines (e.g., type III IFN), is important for limiting SARS-CoV-2 pathology.

Presentation

1. It is unclear how many animals are used in figure 1 weight loss curves or in the titering experiments in panels D and G where virus was undetectable. Could the authors add animal numbers below the X axis to clarify this for the reader?

Animal numbers have been incorporated into the figure legend.

2. Skull is spelled 'skull,' not 'scull,' - in the figure legend for figure 1.

We have corrected this typo throughout the text.

3. The lung sections in figure 2 could benefit from larger arrows or different colors. The black arrows are sometimes difficult to see amongst the dark nucleocapsid staining.

This is now Figure 1G and has been updated as requested.

Reviewer #2:

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1. The histopathology findings that are presented are convincing, however it is unclear if all the animals have a similar profile. Scoring by a trained pathologist would improve the manuscript.

Representative images are shown for day 3 and day 5 post infection in comparison to mock, with analysis performed on lung sections from multiple mice (n=4 per group). Unbiased analysis was performed in ImageJ using the same threshold parameters for every image to achieve the "Lung Consolidation" measurements presented in Figure 3B. This method calculates area of the lung that is inflamed/consolidated tissue versus open airspace.

2. In a similar vein, adding histopathology from an earlier timepoints would provide some insights into how quickly the change in antigen staining occurs. Day 2 or Day 3 antigen staining could/should be included.

We added virus titers from lungs at day 2 post infection. Additionally, viral antigen staining in Figure 1 is from day 2 post infection as this is the peak of viral titers. Day 2 imaging shows that lung damage is already starting in IFITM3 KO mice at this early time post infection as we note in the text.

3. The authors measured IL6 showing a clear difference. Is there a link to IFITM3 and IL6? Also, is there a reason that other inflammatory cytokines were not surveyed (IL1 for example).

We have added new ELISA data for TNFa and IL-1B. These data are now included in Figure 1E.

4. For fig 1 D, can the authors comment on if the animals that have titers in the heart/brain/spleen come from the same animal.

With the exception of one mouse that had virus in both the heart and brain, positive samples were from separate animals. We now make note of this in the Results text.

5. The authors report high titers in the hearts of hACE2/IFITM3KO mice; is there any signs of myocarditis. Were histology sections of the heart considered. The authors might consider a few comment on if this mouse could be used as a model myocarditis associated with COVID19.

We have added text in the Discussion discussing the utility of IFITM3 KO mice as a model of COVID-19-associated cardiac pathology. However, much like the limitations of the K18-hACE2 model for studying relevant lung pathology, the overexpression of the virus receptor makes it unlikely that this model would provide information on relevant mechanisms of virus pathogenesis in the heart. While we do not believe this model is useful for studying virus tropism or pathological mechanisms, it was informative in confirming that IFITM3 plays a protective role during SARS-CoV-2 infection *in vivo* and that IFITM3 limits extrapulmonary virus dissemination to the heart.

6. Is there numerical value that can be added to fig 2g. It is difficult to orient what the values of the expression are in this figure.

This figure (now Figure 5D) was generated from relative minimum and maximum expression values of specific subsets of genes, rather than a specific numerical value. The figure has been clarified by the addition of a "Relative Expression" axis label. This output was provided by the Rosalind.bio RNAseq analysis service.

7. The authors should reference PMID:23919993 when discussing activation of coagulation pathways and coronavirus disease.

This citation has been incorporated as requested.

Reviewer #3:

This study by Zani et al investigates an important topic of the role that the IFN-inducible protein IFITM3 plays in COVID19. The fact that the development of anti-IFN auto-antibody responses associate with severe COVID19 in humans demonstrates the possible importance of IFN in immune protection. As an IFN-inducible protein that has been shown to restrict the replication of a number of viruses, IFITM3 has the potential to play an important role in this response. However, data generated in vitro has demonstrated that the role that IFITM3 plays in SARS-CoV-2 replication is complicated and somewhat paradoxical. Herein, the authors attempt to gain clarity on this important topic, using mouse models of SARS-CoV-2 infection to understand how IFITM3 influences COVID19 pathogenesis in vivo.

The authors take the excellent approach of studying two separate IFITM3-deficient mouse models of SARS-CoV-2 infection to enable investigation of how IFITM3 impacts SARS-CoV-2 infection. Using the mouse-adapted SARS-CoV-2 infection model, the authors provide robust evidence that in this model IFITM3 protects the host from virus-induced weight loss and death, and limits SARS-CoV-2 replication, including spread within the lung and also to the heart. They also clearly show an accompanying increase in virus-induced inflammation, with increased cellular recruitment into the lungs and heightened chemokine expression in the absence of IFITM3. This supports the conclusion that IFITM3 exerts antiviral functionality in SARS-CoV-2 infection in vivo and in the absence of IFITM3, SARS-CoV-2 replicates and spreads widely, triggering more inflammation, which is analogous to reports in other respiratory infections such as influenza.

Data from the hACE-2 model also to some degree replicates the findings seen in the mouse-adapted SARS-CoV-2 model. This model also demonstrates robust protection afforded by IFITM3 from virus induced disease, with all IFITM3-deficient mice succumbing to infection by day 5. Moreover, extra-pulmonary spread of SARS-CoV-2 is controlled by IFITM3.

However, some of the findings do not fit well with data derived from the mouse adapted SARS-CoV-2 model. For example, the impact of IFITM3 on virus replication in the lungs appeared not to be dramatically altered by IFITM3, although the authors suggest that differences in lung virus loads between WT and IFITM3 deficient mice are significant. Furthermore, it is clear that in the mouse adapted SARS-CoV-2 model that IFITM3 limits virus spread and cellular inflammation in the lung. However, it is unclear whether the same is true here. This is important as IFITM3 is known to exert anti-inflammatory effects independently of antiviral functionality, which may be important. Also, the data from the two models may suggest that the ability of IFITM3 to limit extrapulmonary spread may be vital in the ability of IFITM3 to limit virus-induced death rather than local effects in the lung per se. Thus, although the study clearly shows in two mouse models that IFITM3 protects the host from SARS-CoV-2 disease and death, as per the conclusions of the paper, the data presented does not currently provide clear evidence as to how this occurs. Given the importance of IFN in protecting humans from COVID19 and the induction of anti-IFN antibody responses in individuals with severe outcomes, understanding better how IFN-inducible proteins like IFITM3 act in protective responses to SARS-CoV-2 could be beneficial clinically. Thus, further dissection by the authors of the similarities and differences between

the two mouse models of SARS-CoV-2 infection may be very insightful in understanding how this IFN-induced protein protects the host from SARS-CoV-2 induced disease.

This is an interesting study and the dual approach of studying both the mouse-adapted SARS-CoV-2 model and the hACE-2 model is excellent. However, currently, the somewhat superficial analysis of the hACE-2 model makes the dual approach both a strength and a weakness. Further analysis of data from this model will provide important insight regarding how IFITM3 protects from SARS-CoV-2 induced death.

1. Central to this is providing convincing evidence whether or not IFITM3 restricts SARS-CoV-2 replication in the lungs. Data presented in Fig 1F was not particularly convincing. Was this the best of the two experiments and are the differences between the two groups significant? How clear was the difference in lung PFU in the other experiment? Do you observe the same pulmonary dissemination in the hACE-2 model as you see in the mouse-adapted SARS-CoV-2 model?

Lung titer data from K18-hACE2 mice (now Figure 2B) is representative of two independent experiments, one of which showed statistically significant increases in IFITM3 KO animals. We suggest in the Discussion that a limitation of the K18-hACE2 model is the ubiquitous cellular expression of the virus receptor that appears to allow widespread infection throughout the lung, which causes a near-saturating infection and likely explain why the overall effect of IFITM3 on lung viral burden was blunted. To illustrate this point, we added new data directly comparing lung histology of viral antigen staining in the two mouse models (Figure 2D).

2. The authors discuss the anti-inflammatory role of IFITM3. Do the expression multiple pro-inflammatory cytokines elevate in the absence of IFITM3? The authors should extend their analysis of IL-6 to both models and incorporate multiple cytokines including TNF-alpa and IL-1b. Heightened pulmonary cytokine responses in both models but only dramatic differences in lung PFU in the mouse-adapted model may provide evidence for an important anti-inflammatory role for IFITM3 in dictating disease outcome. H+E and CD45+ analysis of lungs from the hACE-2 mice would further support such a conclusion.

We have added additional ELISA data for TNFa and IL-1B. These data are included in Figure 1E. We did not perform additional analysis of samples from K18-hACE2 mice because of the limitations of this model as outlined above and because we have not maintained this mouse colony due to its narrow utility.

3. It is clear in both models that extracellular spread of SARS-CoV-2 to the heart in IFITM3-deficient mice correlates with death. It would be useful to explore this further. Although I appreciate that it might not be possible to perform studies of cardiac dysfunction of SARS-CoV-2-infected mice (as per the group's excellent PNAS study), but some sense of whether IFITM3 protects from heart fibrosis and inflammation could be very informative, along with the suggested work above to understand better how IFITM3 protects from death.

We have added text in the Discussion discussing the utility of IFITM3 KO mice as a model of cardiac dysfunction in SARS-CoV-2 infection. We are in the process of establishing standard operating

procedures for cardiac readouts in our BSL3 biocontainment facility, but this is a separate, ongoing major project in the laboratory and is beyond the scope of the current study.

4. Minor point - Figure 1I. The CD45+ stain for infected wt vs IFITM3-/- mice, although dramatic, does not appear particularly representative of the quantified data on the right-hand side i.e., there is a massive difference in infiltrates in the picture but only a moderate difference @d5 based on the data depicted in the graph.

We have updated and expanded the CD45 images (now Figure 4) to better represent the quantification data.

1st Revision - Editorial Decision 24th Jan 2023

Dear Dr. Yount,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports. Referee #2 has some comments, requests and suggestions to improve the manuscript, I ask you to address in a final revise manuscript. Please also provide a final p-b-p-response addressing these remaining points.

Moreover, I have these editorial requests I also ask you to address:

- I would suggest a slightly modified title:
Interferon-induced transmembrane protein IFITM3 limits lethality of SARS-CoV-2 in mice

- Please provide the abstract written in present tense throughout.

- We plan to publish your manuscript in the Report format (as you also indicated during submission), as there are not more than 5 main figures. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors:
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- Please order the manuscript sections like this (using these names as headings):
Title page - Abstract - Key Words - Introduction - Results & Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

- Please add scale bars of similar style and thickness to the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Several images presently do not have scale bars.

- The arrows or arrowheads in panel 1G are rather small and hard to see (i.e. the red arrowheads). Could this be improved?

- Figure 5 has not the correct format (is too high/long). Please consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

- Appendix Tables 1 and 2 are datasets. Please upload these as original excel files datasets using the names 'Dataset EV1' and 'Dataset EV2'. Please add a title and a legend to the first TAB of the excel file and change the callouts to these items using 'Dataset EV1' and 'Dataset EV2'. Finally, please remove the Appendix file.

- Please provide a fully completed author checklist, providing information in column D (select responses using the pull down menue).

- Please remove the referee token from the data availability section and make sure the data are public latest upon publication of the study.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text and comments. Please use the attached file as basis for further revisions and provide your final manuscript file with track changes, in order that we can see any modifications done.

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- a short, two-sentence summary of the manuscript (not more than 35 words).
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Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

I am content that the authors have addressed my comments where technically feasible. This is an interesting paper that reports some interesting findings regarding the role of IFITM3 in SARS-CoV-2 infection. The data are robust and any limitations with the models used are discussed.

Referee #2:

The analysis of the published scRNA-seq data (Figure 1A) shows that the most significant upregulation of IFITM3 appears to occur in myeloid cells, including resident alveolar macrophages and recruited monocytes. Interestingly, it appears that few epithelial cells express IFITM3 at baseline or after SARS-CoV-2 infection. This is surprising because of the strong viral antigen staining that extends outside of the bronchioles in IFITM3 KO mice. Could infected IFITM3 KO macrophages and monocytes be contributing to the dissemination beyond the bronchioles and the higher viral burden in whole lung? Could inflammasome activation in macrophages (PMID: 35483404) or pyroptotic death of infected monocytes (PMID: 35385861) be contributing to the hyperinflammatory state of the lung? Data from the Tabula Muris scRNA-Seq database also indicates high expression of IFITM3 in lung endothelial cells (https://tabula-muris.ds.czbiohub.org/), which might also provide a hint of how IFITM3 prevents viral dissemination. The additional findings of Zani et al open up an exciting door into future studies of how IFITM3 helps constrain SARS-CoV-2 pathogenesis.

The manuscript should be published after minor revisions to the newly added scRNA-seq analysis, which is all for the sake of clarity.
1. Fix the figure legend for figure 1A - currently it reads that the 1A is IFITM3 expression in nasopharyngeal swabs from COVID-19 patients.
2. In figure legend or methods, please indicate what each population is (e.g. infl mo = inflammatory monocytes, non clas mo = non-classical monocytes, etc)
3. In methods, please briefly indicate some info about the analyzed dataset, such as mouse strain, time point after SARS-CoV-2 infection, and what strain of SARS-CoV-2 was used.
4. The authors indicate on line 110 that IFITM3 is expressed in epithelial cells in mock infected mice and in line 112 that expression increases in epithelial cells with SARS-CoV-2 infection. However, it is unclear what proportion of epithelial cells express IFITM3 at baseline and it does not appear that expression goes up in epithelial cells with infection. The manuscript that the original dataset is derived from indicates that the frequency of epithelial cells decrease with infection, which may explain why IFITM3 expression does not appear to increase in epithelial cells. Could the authors please add the percentage of each subset with greater than 0 for IFITM3 expression? Perhaps as a small table to the right of the violin plots?

Referee #3:

The authors have responded to all queries and suggestions with suitable modifications and answers.

Dear Dr. Breiling,

We are delighted to see that all three reviewers support publication of our work. Please find below a point -by-point response to the remaining minor suggestions of Reviewer 2. Additionally, as editorially requested, we have combined the Results and Discussion sections of our manuscript. Changes to the content of the manuscript have been highlighted in yellow while relocation of our discussion within the results has been highlighted in light blue.

We thank you for handling the review of our manuscript and look forward to publication in EMBO Reports.

Best regards,

Jacob Yount

Referee #2:

The analysis of the published scRNA-seq data (Figure 1A) shows that the most significant upregulation of IFITM3 appears to occur in myeloid cells, including resident alveolar macrophages and recruited monocytes. Interestingly, it appears that few epithelial cells express IFITM3 at baseline or after SARS-CoV-2 infection. This is surprising because of the strong viral antigen staining that extends outside of the bronchioles in IFITM3 KO mice. Could infected IFITM3 KO macrophages and monocytes be contributing to the dissemination beyond the bronchioles and the higher viral burden in whole lung? Could inflammasome activation in macrophages (PMID: 35483404) or pyroptotic death of infected monocytes (PMID: 35385861) be contributing to the hyperinflammatory state of the lung? Data from the Tabula Muris scRNA-Seq database also indicates high expression of IFITM3 in lung endothelial cells (https://tabula-muris.ds.czbiohub.org/), which might also provide a hint of how IFITM3 prevents viral dissemination. The additional findings of Zani et al open up an exciting door into future studies of how IFITM3 helps constrain SARS-CoV-2 pathogenesis.

The manuscript should be published after minor revisions to the newly added scRNA-seq analysis, which is all for the sake of clarity.

1. Fix the figure legend for figure 1A - currently it reads that the 1A is IFITM3 expression in nasopharyngeal swabs from COVID-19 patients.

This has been corrected in the figure legend.

2. In figure legend or methods, please indicate what each population is (e.g. infl mo = inflammatory monocytes, non clas mo = non-classical monocytes, etc)

These populations have been defined in the figure legend as requested.

3. In methods, please briefly indicate some info about the analyzed dataset, such as mouse strain, time point after SARS-CoV-2 infection, and what strain of SARS-CoV-2 was used.

This information has been added to the Materials and Methods section.

4. The authors indicate on line 110 that IFITM3 is expressed in epithelial cells in mock infected mice and in line 112 that expression increases in epithelial cells with SARS-CoV-2 infection. However, it is unclear what proportion of epithelial cells express IFITM3 at baseline and it does not appear that expression goes up in epithelial cells with infection. The manuscript that the original dataset is derived from indicates that the frequency of epithelial cells decrease with infection, which may explain why IFITM3 expression does not appear to increase in epithelial cells. Could the authors please add the percentage of each subset with greater than 0 for IFITM3 expression? Perhaps as a small table to the right of the violin plots?

We have added the following text in the Results and Discussion section to address this comment:

"…showed *Ifitm3* transcripts in epithelial cells and endothelial cells as well as immune cells, with prominent expression in monocytes, dendritic cells, and neutrophils (Fig 1A). Expression of *Ifitm3* in these cell populations was enhanced following infection (Fig 1A). For example, the percentage of cells in which *Ifitm3* expression was detected in mock vs SARS-CoV-2 infected samples increased from 3.8% to 20% in epithelial cells and 12.4% to 22.6% in endothelial cells."

2nd Revision - Editorial Decision 16th Feb 2023

Jacob Yount
The Ohio State University
Microbial Infection and Immunity
Columbus 43210
United States

Dear Dr. Yount,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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Corresponding Author Name: Jacob S. Yount	
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
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The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods, p. 9-10

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Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods, p. 9-10
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods, p. 9-10

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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods, p. 12
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, p. 12

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends, p. 16-17
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends, p. 16-17

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods, p. 10
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability, p. 12
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and Methods, p. 11