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An Organoid-derived Bronchioalveolar Model for SARS-CoV-2 Infection of Human Alveolar-type II-like Cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 14th Jul 2020

Dear Dr Haagmans,

Thank you for the submission of your manuscript (EMBOJ-2020-105912) to The EMBO Journal. Please accept my apologies for the delay with the peer-review of your work due to protracted referee input and detailed discussions in the team. Your manuscript has been initially sent to three reviewers and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your liquid-air interface tissue model and related results, although they also express a number of major issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. In particular, referee #1, a lung epithelial biologist, raises issues regarding the depth of characterization of cellular lineages and alveolar cell type identity in the culture (ref#1, pts 1,3), thus physiological relevance of your findings. Further, this referee emphasizes that heterogeneity in the model is not conclusively addressed (ref#1, pts 2,5). Referee #2 is concerned that the statistical analysis remains incomplete and quantification of variability between replicates not sufficient, which in his-her view weakens the impact of your results (ref#2, pt.1). Further, this reviewer agrees in that resemblance of your model to native tissue complexity needs corroboration (ref#2, pt 3), and differential response to SARS-CoV-2 infection is not conclusively explored (ref#2, pts 4,5). Referee #3 with core expertise in virology, points to incomplete evidence that the current model is useful for analysis of ARDS respiratory distress, and s/he requests complementary measurements to consolidate this aspect. In addition, the reviewers raise a number of points related to validating experiments and controls required, data presentation as well as discussion of the findings and relevant literature, which would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

In light of the extensive experimentation requested by the reviewers, I would appreciate if you could contact me during the next weeks via e.g. a video call to discuss your perspective on the comments and potential plan for revisions.

Please note that we generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

In this context I also want to point to our adjusted GTA. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted. Please contact us should you need additional time, and also if you see a paper with related content published elsewhere.

Please see below for additional instructions for preparing your revised manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Editor
The EMBO Journal

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Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

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When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/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 & Method) that follows the model below (see also https://www.embopress.org/page/journal/14602075/authorguide#availabilityofpublishedmaterial). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) 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])

Our journal also 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 https://www.embopress.org/page/journal/14602075/authorguide#referencesformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

We are piloting Structured Methods a new format for the Materials and Methods of articles published at EMBO Press. Adhering to this format is optional for research articles. However, considering the strong methodological aspect of your study, we would strongly encourage you to use it. Specifically, the Material and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points. More information on how to adhere to this format as well as downloadable templates (.doc or .xls) for the Reagents and Tools Table can be found in the author guidelines of our sister journal Molecular Systems Biology http://msb.embopress.org/authorguide#materialsandmethods. An example of a paper with Structured Methods can be found here: http://msb.embopress.org/content/14/7/e8071. We encourage you to be even more explicit in adding

details on the experimental procedures, as this should be valuable in ensuring reproducible application if the approach.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 12th Oct 2020.

Link Not Available

Referee #1:

The manuscript by Lamers et al. titled "SARS-CoV-2 Infects Alveolar Type I- and II-like Cells in an Organoid-derived Bronchioalveolar Model" focuses on the development of an in vitro model that has important implications for the current state of COVID-19 research. In this study, Lamers et al. established two 2D air-liquid interface models using: 1) small airway (based on a prior EMBO publication from this same group) and 2) alveolar epithelial cells differentiated from self-renewing human fetal lung bud tip (Sox2+/Sox9+) cells - with the support of feeder cells - which they termed "bronchioalveolar" cells. The authors showed the permissiveness of cells in both models to SARS-CoV-2 infection, specifically ciliated (small airway) and lineages the authors interpret to be alveolar type I- and type II-like cells (alveolar), and investigated host interferon signaling responses to the infection.

Major concerns:

The novel bronchioalveolar model presented in the manuscript and its goal of providing a physiologically relevant system for COVID-19 research is promising, especially the generation of air-liquid interface cultures that allow for virus infection experiments to be performed efficiently. The authors also have published expertise in generating airway-based cultures, and are able to use that model as a means to compare against the new bronchioalveolar model they have established. However, there are major concerns regarding: 1) characterization of the claimed cell lineages in the bronchioalveolar model, and 2) mechanisms of action that should be addressed in order for the readers to better understand the responses to SARS-CoV-2 in this particular model.

1. Cell lineage characterization of 3D and 2D models: The authors state that the 2D bronchioalveolar model is the first model to establish permissiveness of alveolar epithelial cells (types I and II) to SARS-CoV-2 infection. While the data indicating successful infection is convincingly presented, it is not clear that cells deserving of the terms type 1 and type 2 alveolar epithelial cells are present. Since this is the central title of this manuscript, it is particularly important to convincingly phenotype the cells being differentiated from fetal bud tip cells in this model. Therefore, a much more in-depth characterization of both the 3D and 2D models should be performed using conventional markers (e.g. SFTPC, AGER etc, more below) to convince the skeptical reader that type 2 and type 1 cells have been produced. Notably, no positive controls are included to allow interpretations of what the gene expression levels in this model system mean.

For example, the identity of cells claimed to be type 2 cells is very much in doubt. The key ATII marker, SFTPC, is missing from the data presented in the manuscript. Can the authors please include or comment on SFTPC readouts, especially since the lack of it on heatmaps and data is surprising? Importantly, most surfactants other than SFTPC are less specific as several are also expressed in airway epithelia either during development, injury, or in some adult settings.

Similarly, comparison to primary "fresh" lung controls is critical for the paper, as several figures (ex. Fig. 2H mass spec of surfactant proteins) are difficult to interpret if not shown beside primary controls.

2. Uncertainty regarding heterogeneity of "bronchioalveolar" cells: The heterogeneous nature of the "bronchioalveolar" cell cultures produced detracts from the ability to interpret the responses of the cultured cells on a population level. Conventionally lung epithelia are viewed as either airway or alveolar in lineage hierarchy, thus it would help to understand the system if the authors viewed these lineages separately, as is convention in the field with more quantitative profiles of their cells. For example, it is unclear what percentage of cells are basal, PNEC, type I, or type II cells in the bronchioalveolar culture, and upon looking at Fig. EV4 where there is wide-spread TP63 staining, there is additional concern that this is a very heterogeneous and predominantly basal cell culture, so it is essential to quantify and classify the different cell types in the bronchioalveolar culture. This is especially critical when looking at bulk-seq data where population-level interpretations are made. It is also unclear how consistent or variable these frequencies and cultures are across different wells or donors (ex. looking at heatmaps in Fig. 3B, there is variation between donors). Since the bulk-seq analyses were performed on two donors (two replicates each), the authors should consider increasing their sample size to be able to make stronger conclusions from their data.

3. Claimed production of type 1 cells: The authors use the marker HTI-56 to label ATI cells, but mentioned that this is also expressed in developing basal cells. In addition, another ATI marker used, HOPX, is non-specific as it is expressed in human ATII cells as well (in contrast to mice where Hopx is more specific for AT1 cells), and PDPN is expressed in basal cells. For the authors to claim that the bronchioalveolar model has "ATI-like cells", please consider using more biologically relevant ATI markers, such as AGER, in the analyses. The authors should also consider using different EM images (Fig. 5), as the ATI-like cells labeled here have microvilli, which are not characteristic of ATI cells. Please look at Ewald Weibel's classic ATI EM images for reference (Weibel, AJRCCM, 2015).

4. Host cell entry factors: The authors touch on the crucial role of a host entry factor (ACE2) in the text and referenced other studies on ACE2 expression in the primary lung, but the level of expression of host cell entry factors in the 2D bronchioalveolar model is missing. Protein level (immunofluorescence staining) and transcript level (qPCR) data would strengthen this manuscript significantly in showing that the bronchioalveolar cells do express certain host cell entry factors, and also may bring up further discussion on whether there is any correlation between entry factor expression and permissiveness to SARS-CoV-2 infection (ex. co-staining of ACE2 and NP).

5. Validation of bulk-seq: The authors have generated several key datasets comparing the small airway culture, the bronchioalveolar culture, and SARS-CoV-2 infected cultures. Validation of these datasets is necessary, and the authors should consider both transcript and protein validation (ex. RT-qPCR, immunofluorescence staining experiments, ELISAs) to confirm the host responses that have been highlighted in the bulk-seq.

6. References: There are key historical studies and labs that have pioneered the protocols for

establishing both proximal and distal lung organoid cultures that the authors did not include in the references. Relevant works on iPSC-derived alveolar epithelial cell models including type 2 cells (Briana Dye et al., eLife, 2015) (Anjali Jacob et al., Cell Stem Cell, 2017) (Yuki Yamamoto et al., Nat Methods, 2017) (Ana Luisa Rodrigues Toste de Carvalho et al., Development, 2019) and the concept of bronchioalveolar stem cells (BASCs) (Carla Kim et al., Cell, 2015) should be discussed and compared because of the overlapping terminology.

Minor concerns:

1. The interpretation of Ki67 staining has some caveats: the authors show in Fig. EV4 that Ki67+ cells are not NP+, but that may be due to infected cells reducing their proliferative capacity. Consider doing an infection time course to better understand the balance between proliferation kinetics and viral infection.

2. Initial bud tip culture characterization would benefit from more quantitative characterization. For example, Fig. 2 shows Sox2 and Sox9 staining but lacks quantitation of percentage of Sox2+/Sox9+ cells in the system, and subsequently, how many cells are differentiated to their respective cell types - flow cytometry data would be useful to include.

3. Can the authors explain their rationale for choosing JAK/STAT signaling as the focus of the host responses? Consider using an unbiased approach to analyzing the SARS-CoV-2 infection bulk-seq to provide a better rationale for investigating this particular pathway. A time course would be helpful, especially in Fig. 6A, showing changes in pSTAT1 (which may come up earlier as well). An additional bioinformatics panel highlighting JAK/STAT signaling (heatmap or Hallmark GSEA) would also strengthen the manuscript.

4. Fig. 3A: Gene names are very small, the authors should consider enlarging the relevant gene names for each culture system.

5. Fig. 4E,F: Can the authors consider including more immunofluorescence staining of NP at the lower MOI or earlier time point to show the spread of the virus?

6. Fig. 5: Missing or mislabeled legend notes on 5F, no scale bar distance mentioned. Virions are difficult to see, can the authors consider using arrows to point out specific virions?

Referee #2:

Summary
Lamers et al. (2020) developed an in vitro platform to generate airway and alveolar cells from human lung bud tip progenitor organoids. They used this model to study SARS-CoV-2 infection and identify target cell types and gene expression changes upon infection. Although a very interesting and important study, the present manuscript lacks extensive quantitative and statistical analysis to further validate suggested infection signatures and gene expression changes they observed in brochioalveolar cells upon SARS-CoV-2 infection. These analyses are critical to fully support their conclusions that susceptibility to SARS-CoV-2 infection differs between cell types and that there are regional differences in gene expression upon infection.

Major comments

- The authors did not assess variation between biological replicates in cell type complexity and composition of organoid-derived bronchioalveolar tissues, which confounds the interpretation of whether this model is robust enough to model in vivo lung complexity. The authors should provide quantification of proportion of bronchiolar and alveolar cell types in their 2D bronchioalveolar model using cell type specific markers. They should also assess variation in cell type proportion between biological replicates and donor-to-donor variation to further address this point.
- The gene expression differences the authors reported between airway-like and bronchioalveolar 2D organoids is based on 2 biological replicates. The authors should provide additional gene expression data to confirm these results with qPCR of relevant cell type markers.
- The authors suggest that there are differences in the susceptibility to SARS-CoV-2 infection between cell types yet they do not provide quantification of infection levels for each cell type. For example, based on the data in Figure 1, they suggest goblet cells can get infected yet to a lower extent compared to ciliated cells and AT2 cells, but they do not provide quantitative evidence for this and estimation of variability between replicates. The authors should quantity and compare levels of SARS-CoV-2 infection in each cell type using cell type-specific markers to support this conclusion.
- It seems that in the bronchioalveolar 2D model, AT1 and AT2 cells are the main targets of SARS-CoV-2. Please, provide quantification of infection levels for these cell types and compare to airway cells present in 2D bronchioalveolar tissues they generate from bud tip progenitors. Are the levels of infection of ciliated cells in bronchioalveolar tissues similar to the levels observed in airway epithelial cell cultures? This is critical to determine whether this model recapitulates the gradient of SARS-CoV-2 infectivity observed in primary human lung tissues reported in Hou et al., Cell, 2020 (doi: 10.1016/j.cell.2020.05.042).
- The authors suggest that there are regional differences in virus-induced cytokine expression when they compare airway-like and bronchioalveolar cells. As their RNA-seq analysis is statistically underpowered with only 2 replicates, they should confirm these results with an orthogonal assay such as qPCR or ELISA to measure regional differences in gene expression upon SARS-CoV-2 infection.
- In the discussion, the authors suggest their model is the only organoid alveolar-like model available to study SARS-CoV-2 infection. This is not completely accurate as lung organoids derived from human pluripotent stem cells were recently employed to study SARS-CoV-2 infection in alveolar cells (Han et al., 2020: https://doi.org/10.1101/2020.05.05.079095). These models recapitulate similar aspects of in vivo lung physiology and can be used to follow SARS-CoV-2 infection in alveolar cells. The authors should discuss why this model offers advantages over these other established organoid models that have been used to study productive SARS-CoV-2 infection.

Minor comments
- In Figure 1B, why is the TCID50 value at time 0 hrs post infection above background levels? In all other virus titer quantification panels such as in Figure 1 and Figure 4, this value is at background levels at 0 hpi, as expected. This confounds the comparison of virus titer quantification at different MOI.
- The authors used mass spectrometry to identify various surfactant proteins but they only used one marker, HTII-280, to identify ATII cells by immunostaining. They should also provide immunostaining data for surfactant proteins to further validate these results.
- The gene labels in the heatmaps in Figure 3 and Figure 6 are too small. Please, provide clear legible labels for these figures.

Referee #3:

The manuscript by Lamer and van der Vaart et. al. describes the establishment of a novel primary bronchioloalveolar cell culture model that is comprised out of a mixture of bronchial cell types and Type I and II-like alveolar epithelial cells which can be utilized to investigate virus - host interactions during respiratory virus infections. This model facilitates the potential investigation of mechanisms that drive virus-induced acute respiratory distress (ARDS) in an authentic environment. The authors performed a detailed characterization of the bronchioloalveolar cell culture model and supplemented this with SARS-CoV-2 infection studies to demonstrate that the Type I and II-like epithelial cell culture model are susceptible to virus infection. The model is interesting and would be useful for a broad audience working lung development and infectious diseases, although the authors should complement the current manuscript with some additional experiments, to clearly demonstrate the full potential of the novel cell culture model in the context of a respiratory virus infection.

Major:
The manuscript, is largely framed around the clinical picture that is been observed been among patients diagnoses with COVID-19, namely Acute Respiratory Distress Syndrome (ARDS) and the need of a model that allows the investigation of mechanistic aspects driving virus-induced ARDS, which currently cannot be addressed by other cell culture models, as they do not recapitulate the natural environment. The authors suggest that the novel bronchioloalveolar cell culture model could be used to address this, however only demonstrate that it is susceptible to SARS-CoV-2. Therefore, and regrettably, the authors do not demonstrate the full potential of the model by including additional experimental data on some of the hallmarks of ARDS, to confirm that the new model is indeed suitable to address these mechanistic aspects. Therefore, the reviewer would strongly encourage the authors to include some additional experimental data, such as the measurement of Trans Epithelial Electrical Resistance (TEER) and/or accumulation of fluids in the apical compartment during SARS-CoV-2 infection.

Minor:
Line 96 - 99; "ciliated cells were the main target" Please provide some quantitative data to support this.

Line 130 - 136; based on the limit number of donors used the authors should be careful with their interpretations, especially with terms as "significantly different".

Line 150 - 151; the authors mention that the donor-to-donor variation is limited, while there is approximately 1-log variation visible between donors at a MOI 0.01 (Figure 4 and EV5), nonetheless with the limit number of donors used such statement should be supported by providing data from additional donors.

Line 152; The results demonstrated that SARS-CoV-2 infects both ATI-like and ATII-like cells, but is unclear whether this corresponds with the expression of ACE-2 and TMPRSS2, or that the virus uses an alternative receptor. It would therefore be interesting to assesses whether viral entry overlaps with the corresponding receptor expression in these cells. Furthermore, it would be interestingly to assess whether this is similar or distinct from SARS-CoV?

Line 198 - 212; the authors performed mRNA sequencing analysis, that provides interesting data, however based on the limit number of donors used the authors should be careful with their interpretations of their RNA-seq data and statements, as differential gene expression analysis should be performed on at least 3 independent biological replicates. Moreover, there is a lot of variation between donors, and therefore, inclusion of additional donors would be recommended.

Line 92 (Figure 1 / EV1D); From the figure legend it is unclear whether the data is from independent biological replicates/preparations from multiple donors, or technical replicates (e.g. 3 - 4 inserts from on donor/preparation infected at the same time)? Furthermore, it is unclear whether images are representative for multiple donors. This comment also applies to other figures.

Line 129: "data not shown", please include data.

Line 463: AdDF++ abbreviation should be included earlier in the M&M.

Please change "live" virus titer to "infectious" virus titer, as it is questionable if a virus is alive.

How was the MOI calculated, as there is no information given regarding the number of cells in each culture?

Line 642, please clarify why the mapping data on SARS-CoV-2 omitted from the manuscript?

Certain aspects of the M&M can be reduced, as they are already published elsewhere (Lamers et.al 2020).

Figure 4A-D; please combine MOI data from different donors.

Figure 1 and 4; why is the titer at 0 h h.i for a MOI 0.1 so markedly different?

Line 159: "data not shown", please include data.

Referee #1:

The manuscript by Lamers et al. titled "SARS-CoV-2 Infects Alveolar Type I- and II-like Cells in an Organoid-derived Bronchioalveolar Model" focuses on the development of an in vitro model that has important implications for the current state of COVID-19 research. In this study, Lamers et al. established two 2D air-liquid interface models using: 1) small airway (based on a prior EMBO publication from this same group) and 2) alveolar epithelial cells differentiated from self-renewing human fetal lung bud tip (Sox2+/Sox9+) cells - with the support of feeder cells - which they termed "bronchioalveolar" cells. The authors showed the permissiveness of cells in both models to SARS-CoV-2 infection, specifically ciliated (small airway) and lineages the authors interpret to be alveolar type I- and type II-like cells (alveolar), and investigated host interferon signaling responses to the infection.

Major concerns:

The novel bronchioalveolar model presented in the manuscript and its goal of providing a physiologically relevant system for COVID-19 research is promising, especially the generation of air-liquid interface cultures that allow for virus infection experiments to be performed efficiently. The authors also have published expertise in generating airway-based cultures, and are able to use that model as a means to compare against the new bronchioalveolar model they have established. However, there are major concerns regarding: 1) characterization of the claimed cell lineages in the bronchioalveolar model, and 2) mechanisms of action that should be addressed in order for the readers to better understand the responses to SARS-CoV-2 in this particular model.

1. Cell lineage characterization of 3D and 2D models: The authors state that the 2D bronchioalveolar model is the first model to establish permissiveness of alveolar epithelial cells (types I and II) to SARS-CoV-2 infection. While the data indicating successful infection is convincingly presented, it is not clear that cells deserving of the terms type 1 and type 2 alveolar epithelial cells are present. Since this is the central title of this manuscript, it is particularly important to convincingly phenotype the cells being differentiated from fetal bud tip cells in this model. Therefore, a much more in-depth characterization of both the 3D and 2D models should be performed using conventional markers (e.g. SFTPC, AGER etc, more below) to convince the skeptical reader that type 2 and type 1 cells have been produced. Notably, no positive controls are included to allow interpretations of what the gene expression levels in this model system mean.
For example, the identity of cells claimed to be type 2 cells is very much in doubt. The key ATII marker, SFTPC, is missing from the data presented in the manuscript. Can the authors please include or comment on SFTPC readouts, especially since the lack of it on heatmaps and data is surprising? Importantly, most surfactants other than SFTPC are less specific as several are also expressed in airway epithelia either during development, injury, or in some adult settings.

Similarly, comparison to primary "fresh" lung controls is critical for the paper, as several figures (ex. Fig. 2H mass spec of surfactant proteins) are difficult to interpret if not shown beside primary controls.

A: Well taken. To further characterize the bronchioalveolar model, we have added immunofluorescent stainings and performed single cell RNA sequencing. Stainings for SFTPC and LPCAT1 as AT2 markers (Fig 2; Fig EV1) are now added. The single cell

sequencing analysis reveals a large alveolar-like cluster containing AT2-L cells that express surfactant proteins (SFTPA1, A2, B, D C) and LPCAT1 in addition to cells with a more AT1-L phenotype expressing AGER, CAV1, HOPX. SFTPC was detected at very low levels (or in some samples remained under the detection threshold) in the bulk sequencing analysis, but expression was found using the single cell sequencing analysis, albeit at lower levels than the other surfactant proteins. This explains its low levels in the bulk RNA sequencing data. Taken together, the new single cell data clearly reveal a cluster of alveolar-like cells with AT1-L and AT2-L characteristics.

2. Uncertainty regarding heterogeneity of "bronchioalveolar" cells: The heterogeneous nature of the "bronchioalveolar" cell cultures produced detracts from the ability to interpret the responses of the cultured cells on a population level. Conventionally lung epithelia are viewed as either airway or alveolar in lineage hierarchy, thus it would help to understand the system if the authors viewed these lineages separately, as is convention in the field with more quantitative profiles of their cells. For example, it is unclear what percentage of cells are basal, PNEC, type I, or type II cells in the bronchioalveolar culture, and upon looking at Fig. EV4 where there is wide-spread TP63 staining, there is additional concern that this is a very heterogeneous and predominantly basal cell culture, so it is essential to quantify and classify the different cell types in the bronchioalveolar culture. This is especially critical when looking at bulk-seq data where population-level interpretations are made.
It is also unclear how consistent or variable these frequencies and cultures are across different wells or donors (ex. looking at heatmaps in Fig. 3B, there is variation between donors). Since the bulk-seq analyses were performed on two donors (two replicates each), the authors should consider increasing their sample size to be able to make stronger conclusions from their data.

A: Percentages of alveolar cells, PNECs and basal cells cells are now calculated based on the single cell sequencing data. These are now indicated in the text. From these data we conclude that the cultures are predominantly alveolar. Basal cells can be locally enriched as revealed by confocal stainings. We also note (Fig EV1) that the mesenchyme from a specific donor (19wk post gestation) supported the specific outgrowth of alveolar cells. This donor mesenchyme donor was therefore used in the single cell sequencing experiment.

3. Claimed production of type 1 cells: The authors use the marker HTI-56 to label ATI cells, but mentioned that this is also expressed in developing basal cells. In addition, another ATI marker used, HOPX, is non-specific as it is expressed in human ATII cells as well (in contrast to mice where Hopx is more specific for AT1 cells), and PDPN is expressed in basal cells. For the authors to claim that the bronchioalveolar model has "ATI-like cells", please consider using more biologically relevant ATI markers, such as AGER, in the analyses. The authors should also consider using different EM images (Fig. 5), as the ATI-like cells labeled here have microvilli, which are not characteristic of ATI cells. Please look at Ewald Weibel's classic ATI EM images for reference (Weibel, AJRCCM, 2015).

A: Indeed, PDPN and HTI-56 appear to be aspecific markers in this system. To avoid confusion, we have removed the PDPN and HTI-56 stainings in Fig 2I and Fig EV4D (labels refer to initial submission) from the manuscript and confirmed the presence of AT1-L cells by single cell sequencing: we note a gradient of AT1 marker expression which is highest in one cluster within the alveolar-like cluster. We agree with this reviewer that the cell indicated in Fig 5 lacks some ultrastructural characteristics of an AT1 cell. This claim was removed from the figure. We do not make the claim that the cultures produce fully differentiated AT1 cells,

but we clearly show that cultures contain cells with AT1-L characteristics.

4. Host cell entry factors: The authors touch on the crucial role of a host entry factor (ACE2) in the text and referenced other studies on ACE2 expression in the primary lung, but the level of expression of host cell entry factors in the 2D bronchioalveolar model is missing. Protein level (immunofluorescence staining) and transcript level (qPCR) data would strengthen this manuscript significantly in showing that the bronchioalveolar cells do express certain host cell entry factors, and also may bring up further discussion on whether there is any correlation between entry factor expression and permissiveness to SARS-CoV-2 infection (ex. co-staining of ACE2 and NP).

A: We now include stainings of ACE2 and the entry-mediating protease TMPRSS2 (Fig 6) and show that these proteins are co-expressed in the same cells. In line with observations by others, ACE2 mRNA expression as quantified by single cell RNA sequencing was very low (Fig 6).

5. Validation of bulk-seq: The authors have generated several key datasets comparing the small airway culture, the bronchioalveolar culture, and SARS-CoV-2 infected cultures. Validation of these datasets is necessary, and the authors should consider both transcript and protein validation (ex. RT-qPCR, immunofluorescence staining experiments, ELISAs) to confirm the host responses that have been highlighted in the bulk-seq.

A: We now complement the bulk-seq data with single cell RNA sequencing data (Fig 4). Both approaches indicate the presence of alveolar cells, basal cells and PNECs. Immunofluorescent stainings for alveolar cells (Fig 2, Fig EV1), PNECS (Appendix Fig S4) and basal cells (Fig 2, Fig EV1) are now included.

6. References: There are key historical studies and labs that have pioneered the protocols for establishing both proximal and distal lung organoid cultures that the authors did not include in the references. Relevant works on iPSC-derived alveolar epithelial cell models including type 2 cells (Briana Dye et al., eLife, 2015) (Anjali Jacob et al., Cell Stem Cell, 2017) (Yuki Yamamoto et al., Nat Methods, 2017) (Ana Luisa Rodrigues Toste de Carvalho et al., Development, 2019) and the concept of bronchioalveolar stem cells (BASCs) (Carla Kim et al., Cell, 2015) should be discussed and compared because of the overlapping terminology.

A: References for iPSC-derived cultures are added in the text. BASCs do indeed allow differentiation towards alveolar and bronchiolar cell types (similar to the sox2+/sox9+ fetal bipotent progenitor cells), but have so far only been identified in mice to our knowledge. It is currently unclear whether a progenitor cell, with characteristics of the fetal bipotent progenitor or BASC, is present in human lungs in adulthood. We have added a sentence on this topic to avoid confusion over the overlapping terminology: "In adult mice an additional cell type, the bronchioalveolar stem cell (BASC) (Kim et al., 2015), differentiates into both bronchiolar and alveolar cells, but in adult humans such bipotent progenitors have not been identified yet.".

Minor concerns:

1. The interpretation of Ki67 staining has some caveats: the authors show in Fig. EV4 that Ki67+ cells are not NP+, but that may be due to infected cells reducing their proliferative

capacity. Consider doing an infection time course to better understand the balance between proliferation kinetics and viral infection.

A: Well taken. We have rephrased the text to: "Using a marker for dividing cells (Ki67), we showed that infected cells were not dividing (Appendix Fig S4C)" to make it clear that we do not know whether the virus infects non-dividing cells or whether infection decreases proliferative capacity.

2. Initial bud tip culture characterization would benefit from more quantitative characterization. For example, Fig. 2 shows Sox2 and Sox9 staining but lacks quantitation of percentage of Sox2+/Sox9+ cells in the system, and subsequently, how many cells are differentiated to their respective cell types - flow cytometry data would be useful to include.

A: As shown in Fig 2A, the majority of cells is double-positive for Sox2 and Sox9. As the subject of this manuscript is the differentiated 2D air-liquid interface model, we have not characterized the lung bud tip culture further and refer to the original paper by Nikolic and colleagues (2017) who have also shown that nearly all cells in this culture are Sox2 Sox9 double-positive.

3. Can the authors explain their rationale for choosing JAK/STAT signaling as the focus of the host responses? Consider using an unbiased approach to analyzing the SARS-CoV-2 infection bulk-seq to provide a better rationale for investigating this particular pathway. A time course would be helpful, especially in Fig. 6A, showing changes in pSTAT1 (which may come up earlier as well). An additional bioinformatics panel highlighting JAK/STAT signaling (heatmap or Hallmark GSEA) would also strengthen the manuscript.

A: Well taken. The phosphorylation of STAT1 by JAK occurs after the binding of type I and type III interferons by their respective receptors. Hence the phosphylation of STAT1 is a marker for interferon I and III production. An unbiased analysis of the host response is now shown in Fig 6 B, D, E and F and also indicates activation of the type I/III interferon response (shown by upregulation of IFITM1, IRF9, OAS1, ISG15 etc).

4. Fig. 3A: Gene names are very small, the authors should consider enlarging the relevant gene names for each culture system.

A: Gene names are now enlarged (Fig 3 and 6).

5. Fig. 4E,F: Can the authors consider including more immunofluorescence staining of NP at the lower MOI or earlier time point to show the spread of the virus?

A: In Fig 7C, we now show whole-well images of infected cells 72 hours post infection in cultures that were infected at a low MOI and treated with interferon lambda at 24 h post infection. The replication kinetics graph shows that this treatment halts viral replication, implying that the number of cells present here is indicative of the number of cells infected at 24 h post infection.

6. Fig. 5: Missing or mislabeled legend notes on 5F, no scale bar distance mentioned. Virions are difficult to see, can the authors consider using arrows to point out specific virions?

A: Thanks. This indeed was mislabeled as panel J, but should have been panel F. This is now corrected and this figure has been moved to the Expanded view (Fig EV4). Virions were already indicated by dotted lines. This is now mentioned in the legend.

Referee #2:

Summary
Lamers et al. (2020) developed an in vitro platform to generate airway and alveolar cells from human lung bud tip progenitor organoids. They used this model to study SARS-CoV-2 infection and identify target cell types and gene expression changes upon infection. Although a very interesting and important study, the present manuscript lacks extensive quantitative and statistical analysis to further validate suggested infection signatures and gene expression changes they observed in brochioalveolar cells upon SARS-CoV-2 infection. These analyses are critical to fully support their conclusions that susceptibility to SARS-CoV-2 infection differs between cell types and that there are regional differences in gene expression upon infection.

Major comments
- The authors did not assess variation between biological replicates in cell type complexity and composition of organoid-derived bronchioalveolar tissues, which confounds the interpretation of whether this model is robust enough to model in vivo lung complexity. The authors should provide quantification of proportion of bronchiolar and alveolar cell types in their 2D bronchioalveolar model using cell type specific markers. They should also assess variation in cell type proportion between biological replicates and donor-to-donor variation to further address this point.

A: Quantification of bronchiolar and alveolar cell types is now performed by single cell mRNA sequencing (Fig 4). Due to time restrictions, we were not able to perform this experiment for multiple donors.

- The gene expression differences the authors reported between airway-like and bronchioalveolar 2D organoids is based on 2 biological replicates. The authors should provide additional gene expression data to confirm these results with qPCR of relevant cell type markers.

A: The cell types present in these organoid cultures were confirmed using an independent single cell RNA sequencing experiment. We have also added additional immunofluorescent stainings to confirm cell types by protein marker expression (Fig 2; Fig EV1).

- The authors suggest that there are differences in the susceptibility to SARS-CoV-2 infection between cell types yet they do not provide quantification of infection levels for each cell type. For example, based on the data in Figure 1, they suggest goblet cells can get infected yet to a lower extent compared to ciliated cells and AT2 cells, but they do not provide quantitative evidence for this and estimation of variability between replicates. The authors should quantity and compare levels of SARS-CoV-2 infection in each cell type using cell type-specific markers to support this conclusion.

A: As others have already characterized the tropism of SARS-CoV-2 in airway cells, we decided to focus mainly on the novel 2D bronchioalveolar model to study SARS-CoV-2.

- It seems that in the bronchioalveolar 2D model, AT1 and AT2 cells are the main targets of SARS-CoV-2. Please, provide quantification of infection levels for these cell types and compare to airway cells present in 2D bronchioalveolar tissues they generate from bud tip progenitors. Are the levels of infection of ciliated cells in bronchioalveolar tissues similar to the levels observed in airway epithelial cell cultures? This is critical to determine whether this model recapitulates the gradient of SARS-CoV-2 infectivity observed in primary human lung tissues reported in Hou et al., Cell, 2020 (doi: 10.1016/j.cell.2020.05.042).

A: We have added an additional staining showing that most infected cells express SFTPC, indicating that these are alveolar cells with an AT2-like phenotype. HOPX+ cells are also infected but as HOPX can also be expressed in AT2 cells in humans we have removed our claims that AT1-L cells are infected.

- The authors suggest that there are regional differences in virus-induced cytokine expression when they compare airway-like and bronchioalveolar cells. As their RNA-seq analysis is statistically underpowered with only 2 replicates, they should confirm these results with an orthogonal assay such as qPCR or ELISA to measure regional differences in gene expression upon SARS-CoV-2 infection.

A: This is an interesting subject, but we feel that elaboration of this issue feels beyond the scope of the current study. Due to the limited data we have moved the data to the extended view and limited the conclusions to speculations.

- In the discussion, the authors suggest their model is the only organoid alveolar-like model available to study SARS-CoV-2 infection. This is not completely accurate as lung organoids derived from human pluripotent stem cells were recently employed to study SARS-CoV-2 infection in alveolar cells (Han et al., 2020: https://eur01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1101%2F2020.05.05.079095&data=02%7C01%7Cb.haagmans%40erasmusmc.nl%7C36ce405a380e4f2ae2ee08d83aa2768f%7C526638ba6af34b0fa532a1a511f4ac80%7C0%7C0%7C637323816743999822&sdata=3R0tApocsYywRXXJjpOX6zA592ycbxBTcNphVpYTRHM%3D&reserved=0<https://eur01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1101%2F2020.05.05.079095&data=02%7C01%7Cb.haagmans%40erasmusmc.nl%7C36ce405a380e4f2ae2ee08d83aa2768f%7C526638ba6af34b0fa532a1a511f4ac80%7C0%7C0%7C637323816743999822&sdata=3R0tApocsYywRXXJjpOX6zA592ycbxBTcNphVpYTRHM%3D&reserved=0>). These models recapitulate similar aspects of in vivo lung physiology and can be used to follow SARS-CoV-2 infection in alveolar cells. The authors should discuss why this model offers advantages over these other established organoid models that have been used to study productive SARS-CoV-2 infection.

A: The iPSC model described by Han and colleagues (2020) has not yet been published but is available online as a preprint. In contrast to our study, Han et al do not shown SARS-CoV-2 replication using classical virology assays to determine viral titers over time. Another major difference between our model and the model described by Han and colleagues is that in our model, the alveolar cells are exposed to the air on the apical side of the cell, mimicking human physiology. Other advantages of our model are the accessibility of both the apical and basal side of the cells for high-throughput drug screening, and the rapid differentiation of our cultures, which can take up to several months for iPSC models.

Minor comments
- In Figure 1B, why is the TCID50 value at time 0 hrs post infection above background levels? In all other virus titer quantification panels such as in Figure 1 and Figure 4, this value is at background levels at 0 hpi, as expected. This confounds the comparison of virus titer quantification at different MOI.

A: Small airway cells produce sticky mucus-like secretions which makes it more difficult to wash these cells properly. This likely resulted in higher levels of input virus remaining on the cells after washing. This does not confound the data as we do not directly compare the viral titers from Fig 1 and Fig 4 (now Fig 5).

- The authors used mass spectrometry to identify various surfactant proteins but they only used one marker, HTII-280, to identify ATII cells by immunostaining. They should also provide immunostaining data for surfactant proteins to further validate these results.

A: Well taken. Immunostaining of SFTPC is now included in Fig 2 and Fig EV1.

- The gene labels in the heatmaps in Figure 3 and Figure 6 are too small. Please, provide clear legible labels for these figures.

A: Larger labels are now provided.

Referee #3:

The manuscript by Lamer and van der Vaart et. al. describes the establishment of a novel primary bronchioloalveolar cell culture model that is comprised out of a mixture of bronchial cell types and Type I and II-like alveolar epithelial cells which can be utilized to investigate virus - host interactions during respiratory virus infections. This model facilitates the potential investigation of mechanisms that drive virus-induced acute respiratory distress (ARDS) in an authentic environment. The authors performed a detailed characterization of the bronchioloalveolar cell culture model and supplemented this with SARS-CoV-2 infection studies to demonstrate that the Type I and II-like epithelial cell culture model are susceptible to virus infection. The model is interesting and would be useful for a broad audience working lung development and infectious diseases, although the authors should complement the current manuscript with some additional experiments, to clearly demonstrate the full potential of the novel cell culture model in the context of a respiratory virus infection.

Major:
The manuscript, is largely framed around the clinical picture that is been observed been among patients diagnoses with COVID-19, namely Acute Respiratory Distress Syndrome (ARDS) and the need of a model that allows the investigation of mechanistic aspects driving virus-induced ARDS, which currently cannot be addressed by other cell culture models, as they do not recapitulate the natural environment. The authors suggest that the novel bronchioloalveolar cell culture model could be used to address this, however only demonstrate that it is susceptible to SARS-CoV-2. Therefore, and regrettably, the authors do not demonstrate the full potential of the model by including additional experimental data on some of the hallmarks of ARDS, to confirm that the new model is indeed suitable to address these mechanistic aspects. Therefore, the reviewer would strongly encourage the authors to

include some additional experimental data, such as the measurement of Trans Epithelial Electrical Resistance (TEER) and/or accumulation of fluids in the apical compartment during SARS-CoV-2 infection.

A: Instead of assaying TEER, we have opted to provide medical relevance of our model in an experiment in which IFN lambda was used both prophylactically and as a treatment to block viral replication and dissemination. Viral replication was dramatically reduced by ~5 logs implying that IFN lambda treatment could be an option to block replication and ARDS. In addition, this data shows that the model can be used for drug screens. This data is now included in Fig 7.

Minor:
Line 96 - 99; "ciliated cells were the main target" Please provide some quantitative data to support this.

A: As our model primarily models alveolar cells, we have tuned down this statement.

Line 130 - 136; based on the limit number of donors used the authors should be careful with their interpretations, especially with terms as "significantly different".

A: The word 'significant' has been removed in this sentence.

Line 150 - 151; the authors mention that the donor-to-donor variation is limited, while there is approximately 1-log variation visible between donors at a MOI 0.01 (Figure 4 and EV5), nonetheless with the limit number of donors used such statement should be supported by providing data from additional donors.

A: The statement that donor-to-donor variation is limited has been removed as this is indeed rather subjective.

Line 152; The results demonstrated that SARS-CoV-2 infects both ATI-like and ATII-like cells, but is unclear whether this corresponds with the expression of ACE-2 and TMPRSS2, or that the virus uses an alternative receptor. It would therefore be interesting to assesses whether viral entry overlaps with the corresponding receptor expression in these cells. Furthermore, it would be interestingly to assess whether this is similar or distinct from SARS-CoV?

A: We have now included expression analyses for the entry receptors ACE2, TMPRSS2 and other family members. The expression of the main receptor ACE2 is relatively low and uniformly distributed in the different cell clusters (Figure 4N).

Line 198 - 212; the authors performed mRNA sequencing analysis, that provides interesting data, however based on the limit number of donors used the authors should be careful with their interpretations of their RNA-seq data and statements, as differential gene expression analysis should be performed on at least 3 independent biological replicates. Moreover, there is a lot of variation between donors, and therefore, inclusion of additional donors would be recommended.

A: Including additional donors was not possible within the time granted for these revisions.

Line 92 (Figure 1 / EV1D); From the figure legend it is unclear whether the data is from independent biological replicates/preparations from multiple donors, or technical replicates (e.g. 3 - 4 inserts from on donor/preparation infected at the same time)? Furthermore, it is unclear whether images are representative for multiple donors. This comment also applies to other figures.

A: These are technical replicates from a single donor. Images are representative images from at least three experiments from the same donor. This is now clarified in the materials and methods section "One small airway donor was used in this study."

Line 129: "data not shown", please include data.
A: The identity of these TP63+ cells was confirmed using single cell sequencing. The TP63+ population co-expresses Krt5 (Fig 4).

Line 463: AdDF++ abbreviation should be included earlier in the M&M.

A: Corrected.

Please change "live" virus titer to "infectious" virus titer, as it is questionable if a virus is alive.

A: Agreed and corrected.

How was the MOI calculated, as there is no information given regarding the number of cells in each culture?

A: The number of cells in each culture was calculated by trypsinizing and counting the cells from single wells. This has been added to the methods.

Line 642, please clarify why the mapping data on SARS-CoV-2 omitted from the manuscript?

A: Since the mRNA reads are normalized during processing, reads that map to the genome of SARS-CoV-2 will interfere with normalization and comparison with uninfected cultures. Therefore, SARS-CoV-2 reads were not included in the normalization to allow for correct comparison to uninfected controls.

Certain aspects of the M&M can be reduced, as they are already published elsewhere (Lamers et.al 2020).

A: Rather than referring to our previous papers and mentioning the differences in the methods, we have chosen to describe the methods 'in whole' as this is in line with the EMBO press guidelines.

Figure 4A-D; please combine MOI data from different donors.

A: This data has now been combined into Fig 5A and B.

Figure 1 and 4; why is the titer at 0 h h.i for a MOI 0.1 so markedly different?

A: Small airway cells produce sticky mucus-like secretions which makes it more difficult to wash these cells properly. This likely resulted in higher levels of input virus remaining on the cells after washing.

1st Revision - Editorial Decision 27th Nov 2020

Dear Bart,

Thank you again for submitting your revised manuscript for consideration by the EMBO Journal, and in addition providing us with a preliminary rebuttal response detailing additional analyses. As mentioned, your work has been sent back for re-evaluation to the three referees, and we have at this point received feedback from all of them, which I enclose below.

As you will see, the referees acknowledge your amended work and that the study has been strengthened by the revisions. At the same time, they also point to important remaining concerns.

We have carefully considered the remaining points raised in light of your complemented manuscript and response, and concluded that we can consider your study for publication at the EMBO Journal pending a final revision and amendments to address the remaining referee concerns as follows:

>> Contextualisation with primary healthy lung tissue and adult alveolar cell identity (ref#1, pt.1): please add the complementary expression plots and tSNE meta-analyses integrating single-cell data of healthy adult AT2 cells to Figure 4.

>> Evidence for 'ATI-like clusters' (ref#1, pt.2): adjust title, main text and Figure 4 focussing on AT2-cells and AT2-like identity.

>> Validation infection response datasets (ref#1, pt.3, ref#3): While we understand the point raised by the referees regarding number of replicates for the sequencing analysis we concur with your argument that the induced IFN signature is sufficiently consolidated by orthogonal p-STAT1 staining and IFN-lambda response assay. We agree with the reviewer #3 though that the claims made on differential individual cytokine gene expression upon infection are not sufficiently supported by data from two donors considering potential donor-to-donor variability; we thus ask you to remove these data from the manuscript and add them as suggestive evidence to the rebuttal letter.

>> Complement methods annotation for single-cell RNA-seq and AB stainings (ref#1 minor pts.1,2).

>> Remove reference to single-cell RNA-seq experiments on the infected culture from the manuscript but add as suggestive evidence to the rebuttal letter.

>> Avoid phrasing as 'only available alveolar-like model' in your discussion (ref#1, minor pt.3) and cite related recent published articles (PMIDs: 33128895 33142113 32979316) and preprint (https://doi.org/10.1101/2020.07.27.212076).

Note that while per se well taken, we do not consider the remaining concern of referee #3 regarding additional TEER measurements as overriding in light of the complementary functional IFN treatment data.

Please let me know any time in case you have additional questions or need more input on the referees' points.

In addition, we need you to consider some issues related to formatting and data representation as listed below, which need to be adjusted at re-submission.

Further, I will share additional changes and comments from our production team during the next days to be considered.

As this is an article related to COVID-19, it will be published as a pre-typeset version within a couple of days post acceptance. Thus, please re-check the text carefully as we cannot make any other changes to typos etc until typesetting is completed about ten days later.

Please submit a revised version of the manuscript using the link enclosed below, addressing the advisor's comments.

Once we have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your final revised version of the manuscript.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Editor
The EMBO Journal.

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Formatting changes required for the revised version of the manuscript:

>> Please specify author contributions for C.D.K. and M.P.G.K. .

>> Rename the current 'Declaration of Interests' section to 'Conflict of interest'.

>> Adjust the reference format to journal style with 10 author names before et al. .

>> Please re-check publication status for the preprint bioRxiv entries in your reference list and update with journal information in case.

>> Introduce ORCID IDs for all corresponding authors (B.H.) via our online manuscript system. Please see below for additional information.

>>Please add a separate data accessibility section to the Material and Methods part and update the GEO entry for the single-cell RNA-seq data.

>>Update the Author Checklist with the single-cell data access information.

>> There are currently 6 Appendix supplementary figures. These need to be compiled into one appendix file, in PDF format, with a ToC, with their legends added. Please remove the legends from the main text.

>> Please remove legends from main and EV figure files.

>> Dataset EV legends: 5 EV datasets are uploaded as Table EV1-5, all need their titles/legends added to the files.

>>Please state redisplay of TMPRSS2 single cell data (Fig 4O) in the legend of Figure S5D.

>> Thank you for providing the synopsis image. We would suggest a minor adjustment, i.e. including a human silhouette and indicating the lungs, so the reader will at first glance catch what the main subject of the study is. Please remove the bullet point highlight from the main text and provide them as separate doc file.

>>Structured Methods: the Reagent Table uploaded can be removed from manuscript text.

>>Methods & Protocols should be moved up in the manuscript after Discussion.

>>We kindly ask you to add the Medical Ethical Committee / Medical Ethical Review Board approval statements to your study.

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In order to link your ORCID iD to your account in our manuscript tracking system, please do the following:

1. Click the 'Modify Profile' link at the bottom of your homepage in our system.
2. On the next page you will see a box half-way down the page titled ORCID*. Below this box is red text reading 'To Register/Link to ORCID, click here'. Please follow that link: you will be taken to ORCID where you can log in to your account (or create an account if you don't have one)
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Thank you very much in advance.

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Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the

revision online before 25th Feb 2021.

Link Not Available

Referee #1:

The authors have addressed some of my concerns with the addition of a single-cell RNA-sequencing dataset and have added further validation of the model and its usefulness in studying SARS-CoV-2 infections. The addition of IFN-lambda treatment data is also interesting and strengthens the applicability of this model for drug screening. However, still unaddressed and of remaining major concern are the following:
1) the lack of a primary control (uncultured lung tissue);
2) the remaining claims of ATI-like cells.
3) the validation of host responses from the authors' bulk-sequencing dataset, as the new single-cell data and immunofluorescence staining only address cellular composition and not viral response.
Additional minor concerns are listed below. Furthermore, as research on COVID-19 has been advancing quickly, some additional literature in the field should be included in the discussion.
Major concerns:
1. Fresh primary control: As previously mentioned, the data presented is difficult to interpret without the presence of a freshly HTII-280-sorted, not-cultured control. To understand what the levels of expression mean (RT-qPCR for example) inclusion of a positive control is crucial, otherwise expression levels are uninterpretable.
2. "ATI-like" cluster: The authors have appropriately toned down the claims on the production of ATI cells, but I suggest that the title should retain the specific mention of type II-like cells. In addition, Figure 4 still maintains the language of an "ATI-like" cluster despite the lack of specificity of AGER in this cluster. The new scRNA seq data establishes that ATI cells have not been generated. Can the authors please remove this ATI-like phrasing?
3. Validation of transcriptomics dataset: Although the manuscript has been significantly strengthened in this revised version, the authors have not validated host responses in terms of immunofluorescence staining experiments, ELISAs, etc. of IFN signaling or inflammatory response.

Minor concerns:
1. Single-cell RNA sequencing characterization and methods:
a. The authors should clarify if the cells have been sorted prior to methanol fixation, or if cells were fixed immediately after dissociation.
b. In Figure 4 and Line 147, the authors show the subclusters of ATI-like and ATII-like cells, but there remains a population of "alveolar cells" that are not subclustered. Can the authors please address this in the text?
2. New ACE2 and TMPRSS2 antibodies not in reagents table nor Methods.
3. Line 282: due to the advance in the field of COVID research in lung organoids in the past few months, several studies have since been published that show SARS-CoV-2 infection of human iPSC-derived and primary alveolar-like cells (Katsura et al., Cell Stem Cell, 2020) (Huang et al., Cell Stem Cell, 2020) (Youk et al., Cell Stem Cell, 2020). The authors should reference these studies and remove phrasing of "only available alveolar-like model".

Referee #2:

Summary

Lamers et al. (2020) developed an in vitro platform to generate airway and alveolar cells from human lung bud tip progenitor organoids. They used this model to study SARS-CoV-2 infection and identify target cell types and gene expression changes upon infection. Although a very interesting and important study, the present manuscript lacks extensive quantitative and statistical analysis to further validate suggested infection signatures and gene expression changes they observed in brochioalveolar cells upon SARS-CoV-2 infection. These analyses are critical to fully support their conclusions that susceptibility to SARS-CoV-2 infection differs between cell types and that there are regional differences in gene expression upon infection.

I've reviewed the materials of this new revision, and the authors have addressed all my concerns.

Referee #3:

In the revised manuscript from Lamers et. al., the authors have provided minimal complementary data to demonstrate that their model system can be applied to evaluate potential therapeutic interventions against SARS-CoV-2. However, it remains unclear why the authors have not chosen to address the reviewer's previous suggestion to include complementary experimental data on some of the hallmarks of ARDS, such as the measurement of Trans Epithelial Electrical Resistance (TEER) during SARS-CoV-2 infection. These experiments could easily have been performed in parallel to the IFN pretreatment experiments, and would have provided important biological data on the physiological relevance of the bronchioalveolar-like model.

Minor points:
The revised manuscript still lacks an adequate number of biological replicates to employ differential gene expression (statistical) analysis on RNA-seq data.

Dear Daniel,

Please find our response below:

>> Contextualisation with primary healthy lung tissue and adult alveolar cell identity (ref#1, pt.1): please add the complementary expression plots and tSNE meta-analyses integrating single-cell data of healthy adult AT2 cells to Figure 4.

We have now added the tSNE plots to Figure 4L-M. The expression plots have been added to Figure EV3B-C, alongside a comparison of our bulk mRNA expression data with two bulk mRNA sequencing datasets of freshly purified AT2 cells.

>> Evidence for 'ATI-like clusters' (ref#1, pt.2): adjust title, main text and Figure 4 focussing on AT2- cells and AT2-like identity.

We replaced 'alveolar cells' in the title with 'alveolar type II-like cells'. In the main text we rephrased our claims on the AT1-like cells:

- *LINE 165: "...and alveolar cells with a ATI-like transcriptional profile" was replaced with "...and alveolar cells that showed an increased expression of several ATI markers..."*

- *LINE 188: "These data show that the bronchioalveolar model consists mainly of alveolar-like cells with both ATI- and ATII-like characteristics and potentially is susceptible to SARS-CoV-2 infection." was replaced with "These data show that the bronchioalveolar model consists mainly of ATII-like cells and potentially is susceptible to SARS-CoV-2 infection."*

>> Validation infection response datasets (ref#1, pt.3, ref#3): While we understand the point raised by the referees regarding number of replicates for the sequencing analysis we concur with your argument that the induced IFN signature is sufficiently consolidated by orthogonal p-STAT1 staining and IFN-lambda response assay. We agree with the reviewer #3 though that the claims made on differential individual cytokine gene expression upon infection are not sufficiently supported by data from two donors considering potential donor-to-donor variability; we thus ask you to remove these data from the manuscript and add them as suggestive evidence to the rebuttal letter.

The cytokine expression plots (former Figure EV5) have been remove from the manuscript. Any claims regarding the individual expression levels of cytokines have been removed from the main text.

>> Complement methods annotation for single-cell RNA-seq and AB stainings (ref#1 minor pts.1,2).

These have been added to the methods section and reagent and resource table.

>> Remove reference to single-cell RNA-seq experiments on the infected culture from the manuscript but add as suggestive evidence to the rebuttal letter.

The reference to the infected culture has been removed. Authors sequenced single cells of infected sample yet do not include this in the manuscript due to low infection percentages.

>> Avoid phrasing as 'only available alveolar-like model' in your discussion (ref#1, minor pt.3) and cite related recent published articles (PMIDs: 33128895 33142113 32979316) and preprint (https://doi.org/10.1101/2020.07.27.212076).

Lines 331-334: We now referred to the recently published alveolar models: The bronchioalveolar-like model presented here shows a robust increase in infectious virus titers over time of ~5 logs, whereas other recently developed adult-derived 3D alveolar organoid models show a more limited increase in viral titers (~1-2 logs) (Katsura et al., 2020, Salahudeen et al., 2020, Youk et al., 2020).

Line 338: Similar results may be obtained with the recently developed iPSC-derived 2D ATII-like cultures (Huang et al., 2020).

Formatting changes required for the revised version of the manuscript:

>> Please specify author contributions for C.D.K. and M.P.G.K. .

Done.

>> Rename the current 'Declaration of Interests' section to 'Conflict of interest'.

Done.

>> Adjust the reference format to journal style with 10 author names before et al. .

Done.

>> Please re-check publication status for the preprint bioRxiv entries in your reference list and update with journal information in case.

Done.

>> Introduce ORCID IDs for all corresponding authors (B.H.) via our online manuscript system. Please see below for additional information.

This will be done during in the resubmission.

>>Please add a separate data accessibility section to the Material and Methods part and update the GEO entry for the single-cell RNA-seq data.

Done.

>>Update the Author Checklist with the single-cell data access information.

Done.

>> There are currently 6 Appendix supplementary figures. These need to be compiled into one appendix file, in PDF format, with a ToC, with their legends added. Please remove the legends

from the main text.

Done.

>> Please remove legends from main and EV figure files.

Done.

>> Dataset EV legends: 5 EV datasets are uploaded as Table EV1-5, all need their titles/legends added to the files.

Done.

>>Please state redisplay of TMPRSS2 single cell data (Fig 4O) in the legend of Figure S5D.

Done. Another redisplay was found: Figure S5C is a redisplay of Fig 4M. This has also been added to the Fig S5 legend.

>> Thank you for providing the synopsis image. We would suggest a minor adjustment, i.e. including a human silhouette and indicating the lungs, so the reader will at first glance catch what the main subject of the study is. Please remove the bullet point highlight from the main text and provide them as separate doc file.

We have added an image of human lungs to the synopsis. We have not included a human silhouette as this may suggest that our model is adult-derived, while it is derived from fetal material.

The highlights are now in a separate file.

>>Structured Methods: the Reagent Table uploaded can be removed from manuscript text.

Done.

>>Methods & Protocols should be moved up in the manuscript after Discussion.

Done.

>>We kindly ask you to add the Medical Ethical Committee / Medical Ethical Review Board approval statements to your study.

This was already included in the manuscript: "Adult lung tissue was obtained from residual, tumor-free, material obtained at lung resection surgery for lung cancer. The Medical Ethical Committee of the Erasmus MC Rotterdam granted permission for this study (METC 2012-512). Human fetal lung tissue was obtained from legally terminated second trimester pregnancies (15–20 weeks) by the HIS-Mouse Facility of Academic Medical Center (AMC; Amsterdam, The Netherlands), after written informed consent of the mother for the tissue's

use in research and with approval of the Medical Ethical Review Board of the AMC (MEC: 03/038). Study procedures were performed according to the Declaration of Helsinki, and in compliance with relevant Dutch laws and institutional guidelines. The tissues obtained were anonymized and non-traceable to the donor. On request by the researchers, only gender and gestational age is provided."

2nd Revision - Editorial Decision 4th Dec 2020

Dear Bart,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work.
Please see the following link for a representative example:
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The videos are embedded in the respective article html page, see e.g.
https://www.embopress.org/doi/abs/10.15252/embj.2019103009

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Bart Haagmans
Journal Submitted to: EMBO
Manuscript Number: EMBOJ-2020-105912

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 χ2 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sampe size was chosen based of the availabilty of organoid or donor material. No tests were performed to ensure adequate power.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were subjected to treatments groups randomly.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	All statistical tests on all figures are justified based on sample size including bulk and single cell mRNA sequencing. For sequencing, published statistical analyses were used to determine significance, for example for differential expression the non-parametric wilcoxon rank sum test was used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Assumptions of the tests were always checked using similar methods as described in DESeq2 package (Love et al., 2014) for bulk mRNA sequencing and Seurat1 R toolkit (Butler et al., 2018) for single cell sequencing.

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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-report

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes, these variations were always analysed before statistical analysis
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This information is listed in the reagents and tools table in the structured methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	VeroE6 cells were obtained from ATCC (ATCC® CRL 1586TM). Cells tested negative for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The Medical Ethical Committee of the Erasmus MC Rotterdam (METC 2012-512) and the Medical Ethical Review Board of the AMC (MEC: 03/038) granted permission for this study.
12. 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.	Written or informed consent was obtained from all subjects (or in the case of fetal tissues from the mother). Study procedures were performed according to the Declaration of Helsinki, and in compliance with relevant Dutch laws and institutional guidelines. The tissues obtained were anonymized and non-traceable to the donor. On request by the researchers, only gender and gestational age is provided.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. 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.	NA
17. 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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Bulk mRNA sequencing was deposited at Gene Expression Omnibus GSE153218. Single cell mRNA sequencing was deposited at Gene Expression Omnibus GSE161934.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA