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SARS-CoV-2 targets neurons of 3D human brain organoids

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Transaction Report:

(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 7th Aug 2020

Dear Jay,

Thank you again for the submission of your manuscript (EMBOJ-2020-106230) to The EMBO Journal, and in addition providing further input in your preliminary revision outline. As mentioned earlier your manuscript was sent to three reviewers for evaluation, who cover neurobiology - tau (referee#1), 3D cerebral models (referee#2) and virology (referee#3) as their core expertise. We have now received reports from all of them, which I enclose below.

The referees state potential interest and novelty of your findings, at the same time they raise a number of issues with the current results presented and their discussion that will have to be conclusively addressed before they can be supportive of publication of your manuscript in 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.

Importantly, the following issues are required in our view to be addressed in order to achieve the level of robustness and comprehensive analysis we have to request:

>> complementary cytoskeleton-tau stainings/biochemistry (ref#1)
>> longer time points virus exposure (ref#2, pt.2)
>> additional TUNEL analyses (ref#2, pt.4)
>> assessment of ACE2 expression in organoids (ref#3, pt.3)
>> assessment of viral replication-productive infection (ref#3, pt.6)
>> documentation MOI infections/ titration curve (ref#3, pt.7)
>> controls on antibody validity (ref3, pt.1)

Related to the last point, referee#3 raises the matter of accessibility of the SARS-CoV-2-N specific antibodies generated during your study. As to our journal policies we kindly ask you to confirm that these antibodies will be made publically available post publication of the study.

Further, the following referee concerns are per se reasonable, but in our view beyond the scope of the current study.

>> lack of sufficient mechanistic depth on SARS-CoV-2 entry and proof of causalities for tau-phosphorylation and localization (ref#1; ref#2 standfirst).
>> integration data on aged organoids (ref#2, pt.1).
>> viral penetrance and diffusion into the 3D model (ref#2, pt.3; ref#3, pt.4).

They should nevertheless be acknowledged and carefully discussed, and the claims toned down where appropriate.

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

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.

Please also 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:
http://bit.ly/EMBOPressFigurePreparationGuideline

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
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

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.

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 5th Nov 2020.

Link Not Available

Referee #1:

The data included in the study is well-done and supports the conclusions. Notably the data seem to confirm a recent report that SARS-Cov2 infects neurons in this case more "cortical" like neurons in the orgnaoid. The new data claims evidence of tau mislocalization following IPSc derived neuronal organoid infection. The data is intriguing but without additional markers for other cytoskeletal elements hard to interpret. Further, there is no biochemistry. Blots of tau and other cytoskeletal elements could be informative. Solubility profiles woudl help as well.
Cause and effect here are not interpretable, though the authors try suggest that there may be a role of tau in SARS-COV2 neuronal death. Such insights are not possible without some manipulation of tau.

Referee #2:

The manuscript from Ramani et al. investigates the effects of SARS-CoV-2 on the central nervous system via a brain organoid model system. The authors expose day 15 and 60 organoids to SARS-CoV-2 and study viral targeting, Tau pathology, and neuronal cell death within the organoids. The hypothesis is interesting and the authors demonstrate some evidence that the SARS-CoV-2 disrupts Tau localization and preferentially targets neurons. The manuscript raises a number of new questions that remain unanswered-perhaps in light of the urgency and speed to which the experiments were conducted. For example, while potentially outside the scope of this work, it would be interesting to investigate the mechanism in which the SARS-CoV-2 enters neurons since they express less ACE-2.

Considering rigor and scientific approach, there are several concerns and additional experiments that are necessary to support the current claims. Additionally, many justification and limitations of the current study should be further discussed. As noted below, the organoid system is a reductionist model that contains no vasculature (thus no BBB), immune component (microglia), and at the ages investigated here, no glia. There are heterogeneity issues and concerns about viral penetration throughout the organoid. Please see concerns outlined below:

Major:
1. The authors show that SARS-CoV-2 preferentially targets neurons, dysregulates Tau, and can induce neuronal death. They begin to mention limitations in that the organoids tested were young and aged organoids would be necessary to conduct future studies. I agree that using older organoids that contain more mature neurons and other glial cells is crucial. If the authors now have

older organoids with glia present, it would be extremely helpful to include that data too. Given the time constraint, excluding older timepoints is acceptable but would positively add to the work. If this is the case, I believe it is imperative to discuss other limitations that could play a role in studying SARS-CoV-2 in this organoid model system. For example, these organoids do not contain a blood-brain barrier or vasculature and are lacking an immune system (microglia). These components would influence how SARS-CoV-2 replicates and interacts in the environment. Ideally, microglia could be engrafted into the organoid system prior to SARS-CoV-2 administration. Again, given the time constraint this is not likely possible to achieve, but should be mentioned in the discussion.

2. Most of the analysis were conducted 2- or 4-days post exposure to the virus and showed differences compared to the controls. However, I believe it would be important to contain longer timepoints (10 days, 21 days). It has been shown that patients who recover from COVID still experience deficits weeks later (e.g. taste and smell) and it would be valuable investigate the pathology of the virus at longer time periods in the organoids.

3. Given that the experiments are all focused on using immature organoids to distinguish whether NPCs or neurons are more susceptible, the necessity of the organoid systems is a bit confusing. Why not perform all studies in a 2D system? My concern for the 3D system is about viral diffusion into the deeper components of the organoid. In response to this there was one controlled comparison to 2D rosettes, but I still have concerns about viral diffusion. Could the authors use another viral control (ZIKV) to confirm that they are capable of observing viral infection within the VZ of the organoids and that absence of SARS-CoV-2 replication in those cells is definitively a tropism issue and nothing technical? This has been a similar observation and potential issue with other viral studies in organoid (see Sun et al 2020 re: CMV infection) where viral particles only are detected on the outermost layers but pathology appears to be widespread.

4. The TUNEL comparison in Figure 3B is not a fair assessment. In the Mock (i), a segment of the organoid with a rosette is shown, whereas (ii) contains a part of the organoid without a visable rosette. These are two clearly heterogeneous parts of the representative organoids. TUNEL staining is surely likely to be less near healthy rosettes than it is at locations physically farther from the "VZ-like" zones.
Along the lines of point 4, it would be helpful to include a few zoomed out (or stitched-together) images of whole organoids to see AB4 at the macro-level. At the moment, we are only able to see a few zoomed in panels, which can be tricky to interpret given the heterogeneity of organoid cultures in general.

Minor:
1. In Figure S1b, the text at the top of the table is cut off. It reads "ISA against the spike protein of SARS-CoV-2" instead of "ELISA against the spike protein of SARS-CoV-2."

2. I did not see a legend for Figure 3D.

Overall, this study feels quite rushed. I recognize that is in large part due to the extraordinary circumstances surround SARS-CoV-2. I do feel, however, that it is imperative to include all relevant controls and to be very direct about limitations. The current text reads like this is definitive proof that human neurons are susceptible to SARS-CoV-2. After reading carefully, however, I have a number of questions. Given the low infectivity rate, would the BBB and microglial defenses normally provide a serious obstacle to neuronal infection at all? Alternatively, since ACE2 is highest expressed in brain vasculature, does that serve as a nidus for further spread into cells in the brain? These are issues that should be more clearly included in the discussion. What is most important to

me is that the authors confirm no technical issues in viral penetration to deeper regions of the organoid, test later timepoints after viral administration, address the organoid heterogeneity issues with more zoomed out images, and greatly tone down the language that insinuates this is evidence for what might be happening in the human brain. I applaud the authors for this ambitious endeavor and just want to be sure that rigor is appropriate given the current climate surrounding SARS-CoV-2.

Referee #3:

The manuscript "SARS-CoV-2 targets neurons of 3D human brain organoids" by Ramani et al investigates SARS-CoV-2 infection of human brain organoids. The authors are prompted to investigate these questions by several reports describing neuro complications in COVID-19 patients. The authors inoculate human brain organoids with SARS-CoV-2 virus and identify neurons with the virus. They go on to show that there is altered Tau localization in SARS-CoV-2 stained cells and see evidence of neuronal death. The authors use a self-identified human anti-SARS-CoV-2 antibody. In addition the authors show no evidence of viral replication or evidence of productive infection in human brain organoids. Overall the authors need to more clearly state that at best the virus enters neurons but does not actively replicate. It is not clear if the findings of neuronal Tau localization change is due to SARS-CoV-2 or any RNA or viral entry event in the cell. Given these concerns additional supporting experiments are needed and would require major revisions for consideration.
Comments for the author:
1. The authors recognize that SARS-CoV-2 infection is somewhat controversial in neuronal platforms. They use a antibody against SARS-CoV-2 that they identified and self-purified. Confirmation of all staining with a commercially available antibody to demonstrate that this isn't an artifact of an antibody that will not be available in the community is necessary.
2. The brain organoid imaging and stainings are beautiful and well annotated and the authors should be commended.
3. hACE2 staining of the organoids would be helpful for elucidating which cells in the organoids have the potential to even support viral entry. A SARS-CoV-2 pseudoparticle would also help support these studies if available.
4. The authors demonstrate that the the brain organoids have localization of the AB4+ cells on the periphery. Is that because the interior cells are not seeing the virus, have cells not permissive to the virus, or have cells with resistance to the virus?
5. Do the authors have any explanation for why the SARS-CoV-2 staining (which ostensibly is staining for SARS-N) is only labeling the somas and not the axons as it is not clear why the protein would be localized in the neuronal cells.
6. Given that there is no expansion in number of SARS-CoV-2 labeled cells 2 and 4 dpi strongly suggests that there is no evidence of viral replication. Further confirmation with TCID50 studies and qRT-PCR for subgenomic RNA and replicating strand would also validate these findings.
7. The MOI of infections is not reported. A dose titration curve for SARS-CoV-2 would help support the authors findings e.g. 0.001-1.0.
8. The authors should add additional discussion on prior reports of viral infections and its impact os Tau localization and neuronal impacts.
9. Given the authors's argument that viral replication does not seem to be required for neuronal entry and injury is one of the viral proteins responsible? Have the authors examined any of the viral proteins for similar effects?

We thank the referees and editor for expediting the process at these extraordinary times. The comments are constructive, and they help to improve the manuscript. To this end, we have addressed most of the editors' concerns with valid experiments. Besides, we have also provided additional data and interpretations for referee's concerns. Below are our point-by-point comments in blue.

Referee #1:

The data included in the study is well-done and supports the conclusions. Notably the data seem to confirm a recent report that SARS-Cov2 infects neurons in this case more "cortical" like neurons in the organoid. The new data claims evidence of tau mislocalization following IPSc derived neuronal organoid infection. The data is intriguing but without additional markers for other cytoskeletal elements hard to interpret. Further, there is no biochemistry. Blots of tau and other cytoskeletal elements could be informative. Solubility profiles would help as well.
Cause and effect here are not interpretable, though the authors try suggest that there may be a role of tau in SARS-COV2 neuronal death. Such insights are not possible without some manipulation of tau.

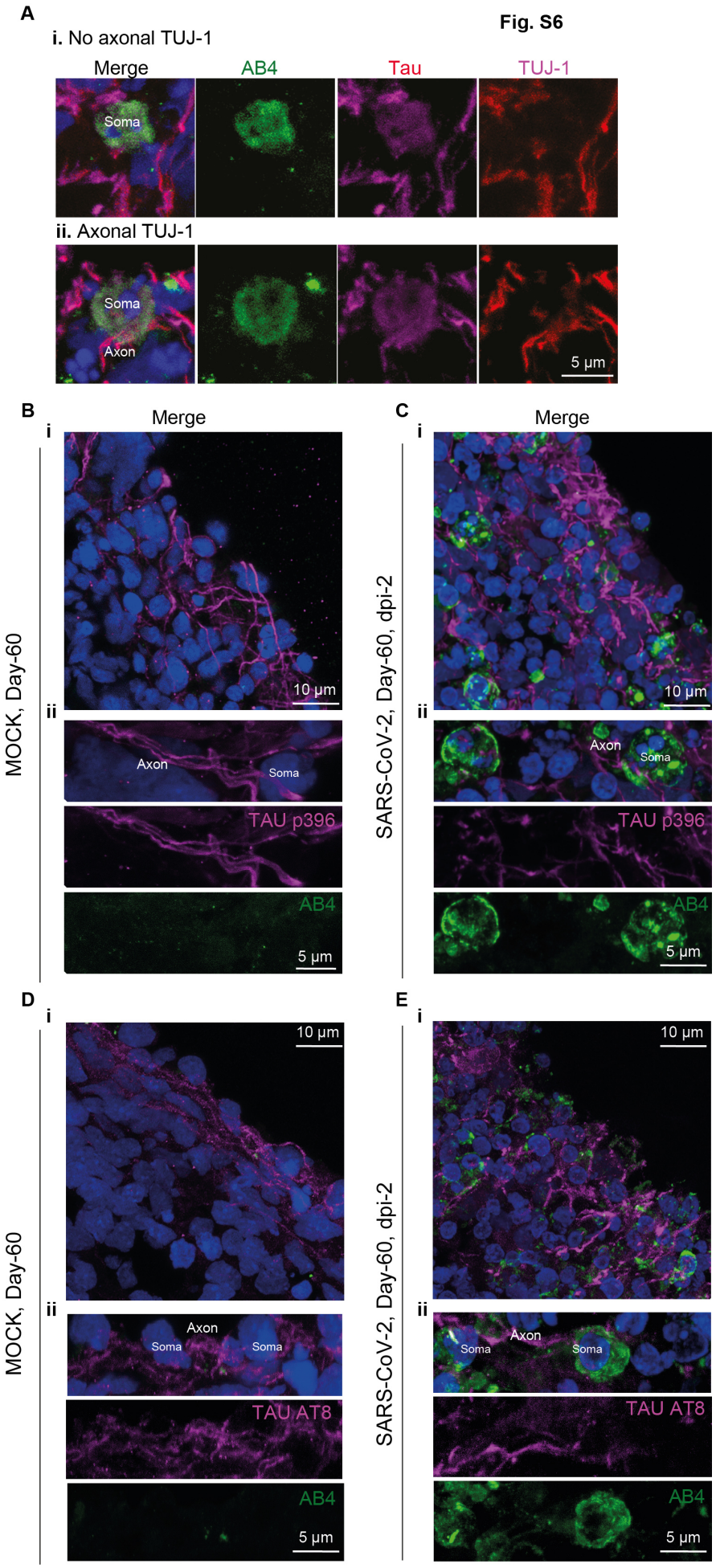
Editor: >> complementary cytoskeleton-tau stainings/biochemistry (ref#1)

We thank this reviewer for her/his encouraging comments and asking us to provide very useful data. We fully agree with this referee's concern that further experiments are required to get more insights into the cause and effect of Tau abnormalities in SARS-CoV2-positive neurons. Due to the time constraint of the current situation, we consider the current work is descriptive, and we will consider doing more mechanistic studies in the future.

As for the other cytoskeleton markers: We indeed anticipated this concern, which is truly a valid point. We are afraid that this referee perhaps missed seeing Figure S4B where we show both Tau and MAP2 label SARS-CoV2-positive neurons.

Agreeing with this referee, we analyzed several sections to get complementary neuronal staining with TUJ-1 (A pan neuronal maker). We must admit that we could not find several examples. This is because most of the SARS-CoV2-positive neurons lacked axonal Tau, and merely about 10% of the SARS-CoV2-positive neurons contained intact axons as determined by Tau-immunoreactivity **(Figure 2B and quantification graph 2Bv).** This could indirectly mean that those neurons are not healthy and intact. Thus, we had a difficult time to label the axons with an additional cytoskeleton marker.

However, we hope that the provided images convince this referee. We provided a representative figure for no axonal TUJ-1 **(i)** and axonal TUJ-1 **(ii)** as **Figure S6** shows the staining of SARS-CoV2-positive neurons by two other Tau antibodies specific to phosphorylated Tau. Accordingly, we changed the legends at the quantification graph Figure 2Bv. For your quick reference, below is the figure.


A
Fig. S6
i. No axonal TUJ-1
Merge
AB4
Tau
TUJ-1
Soma
ii. Axonal TUJ-1
Soma
Axon
5 µm
B
C
Merge
MOCK, Day-60
SARS-CoV-2, Day-60, dpi-2
10 µm
Axon
Soma
TAU p396
AB4
5 µm
D
E
TAU AT8

Biochemical experiments: We do believe performing biochemical experiments are beneficial. However, the current situations unfortunately, make this task impossible. There are at least two reasons as below:

-We are required to infect a large number of organoids with an infective strain (not a pseudovirus). At this point, this is a time-consuming process, and it may even take more than three months.
-Most importantly, testing the soluble form of Tau and sarkosyl-stable form of Tau must be processed under native conditions at the BSL3 lab. This is an impossible task at the moment. As we will deal with infective strain, this will put people under the risk of passive infection.

For clarity, we are capable of doing this kind of experiment. Indeed we have done it using organoids derived Tauopathies patients (happy to share the figures). But with SARS-CoV-2-exposed organoids, it is a hazardous task with limited access to the BSL3 lab. We believe the reviewer can waive this experiment considering the current situations. However, we have discussed this issue in the revised manuscript below **(Between page 9 to 10 before the conclusion paragraph).**

"Future biochemical experiments dissecting the ratio of soluble and sarkosyl-stable Tau extracted from SARS-CoV-2-positive neurons are required to obtain insights into the cause and effect of potential Tau pathology and neuronal death".

Referee #2:

The manuscript from Ramani et al. investigates the effects of SARS-CoV-2 on the central nervous system via a brain organoid model system. The authors expose day 15 and 60 organoids to SARS-CoV-2 and study viral targeting, Tau pathology, and neuronal cell death within the organoids. The hypothesis is interesting and the authors demonstrate some evidence that the SARS-CoV-2 disrupts Tau localization and preferentially targets neurons. The manuscript raises a number of new questions that remain unanswered-perhaps in light of the urgency and speed to which the experiments were conducted. For example, while potentially outside the scope of this work, it would be interesting to investigate the mechanism in which the SARS-CoV-2 enters neurons since they express less ACE-2.

We are thankful for this insightful comment. We believe that in neurons, there are alternative viral entry mechanisms besides ACE2. One possibility is Integrin-mediated signaling mechanisms, which are abundant in neurons. Thus it is worth testing integrin-dependent mechanisms in viral entry. Nevertheless, even a low level of ACE2 could still support the viral entry into neurons. To test this further, we are now generating iPSCs overexpressing ACE2. We will check if brain organoids/neurons differentiated from these transgenic iPSCs facilitate the viral entry and eventually allow them to replicate actively.

Considering rigor and scientific approach, there are several concerns and additional experiments that are necessary to support the current claims. Additionally, many justification and limitations of the current study should be further discussed. As noted below, the organoid system is a reductionist model that contains no vasculature (thus no BBB), immune component (microglia), and at the ages investigated here, no glia. There are heterogeneity issues and concerns about viral penetration throughout the organoid. Please see concerns outlined below:

Major:
1. The authors show that SARS-CoV-2 preferentially targets neurons, dysregulates Tau, and can induce neuronal death. They begin to mention limitations in that the organoids tested were young and aged organoids would be necessary to conduct future studies. I agree that using older organoids that contain more mature neurons and other glial cells is crucial. If the authors now have older organoids with glia present, it would be extremely helpful to include that data too. Given the time constraint, excluding older timepoints is acceptable but would positively add to the work. If this is the case, I believe it is imperative to discuss other limitations that could play a role in studying SARS-CoV-2 in this organoid model system. For example, these organoids do not contain a blood-brain barrier or vasculature and are lacking an immune system (microglia). These components would influence how SARS-CoV-2 replicates and interacts in the environment. Ideally, microglia could be engrafted into the organoid system prior to SARS-CoV-2 administration. Again, given the time constraint this is not likely possible to achieve, but should be mentioned in the discussion.

We are very much encouraged by this referee and fully agree that organoids are reductionist approaches and have some limitations. We have discussed it in the discussion section **(Page 9, the first paragraph)**

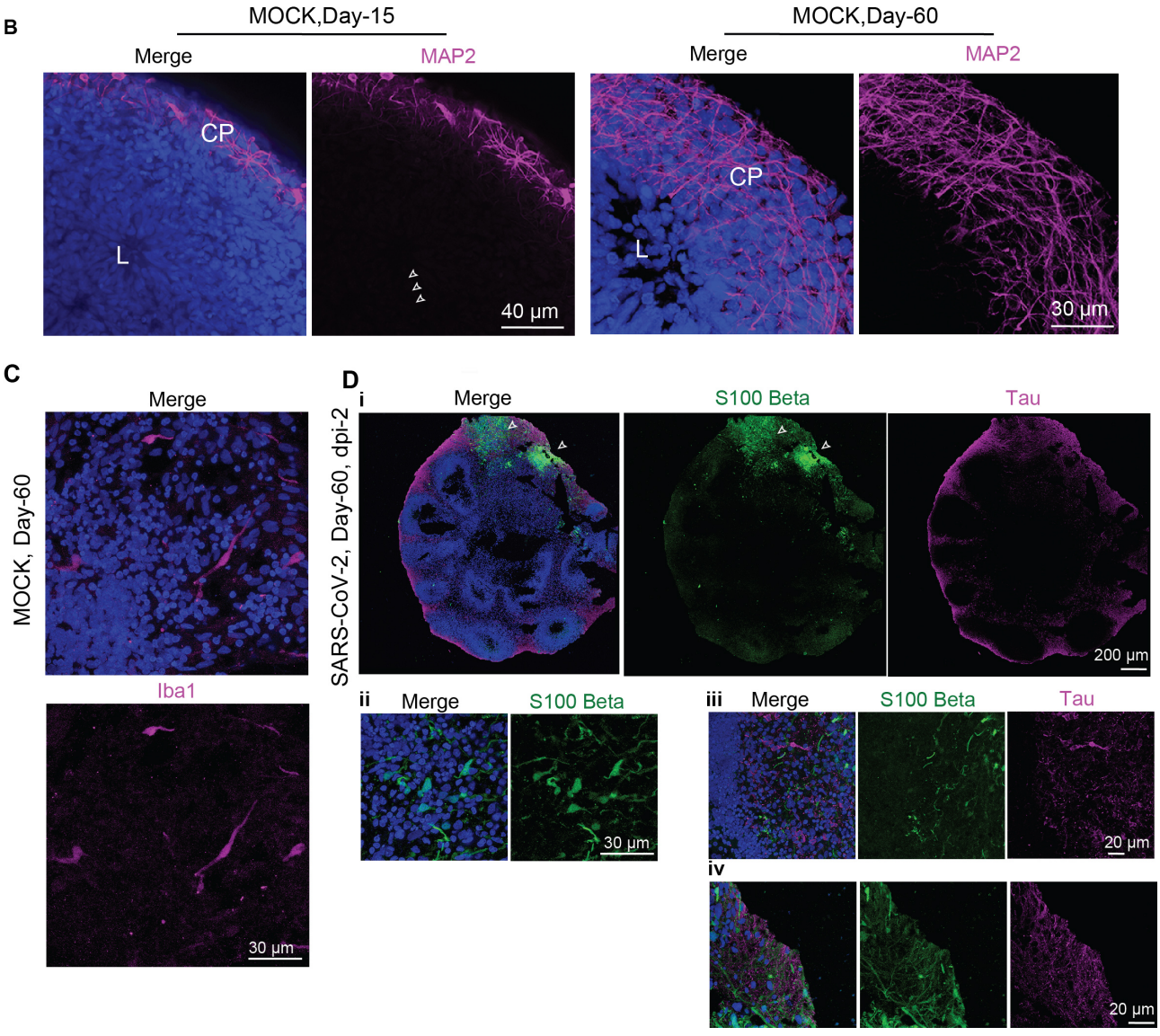
We value the referee's comments, and thus we have included the following experiments in the revised version.

-Day 60 organoids do express astrocytes as labeled by S100β that is known to mark a subtype of mature astrocytes. We noticed that S100β-positive cells specifically restricted to a region within the organoid. However, we could not detect GFAP-positive cortical astrocytes, which could appear in a much older stage. As per this reviewer's instruction, we have provided this data as an overview image (low mag) and a zoomed region.

-Iba1 that labels reactive microglial cells in organoids: Recently, via an orthogonal experiment, we realized that our protocol for culturing organoids allows the differentiation of microglial-like cells. This was surprising to us as we directed the differentiation of iPS cells towards neuroectoderm by simultaneously blocking mesoderm lineages at initial stages of differentiation.

Staining for Iba1, we notice that day 60 organoids do exhibit microglial cells. We, however, did not see many Iba1-positive cells exhibiting their typical ramification morphology. Thus, microglial cells could be one of the factors why our organoids better respond to infection studies (both ZIKV and SARS-CoV2) and can model the diseases. We have now provided these data as **Figure S3B-D**. For your quick reference, below is the figure.

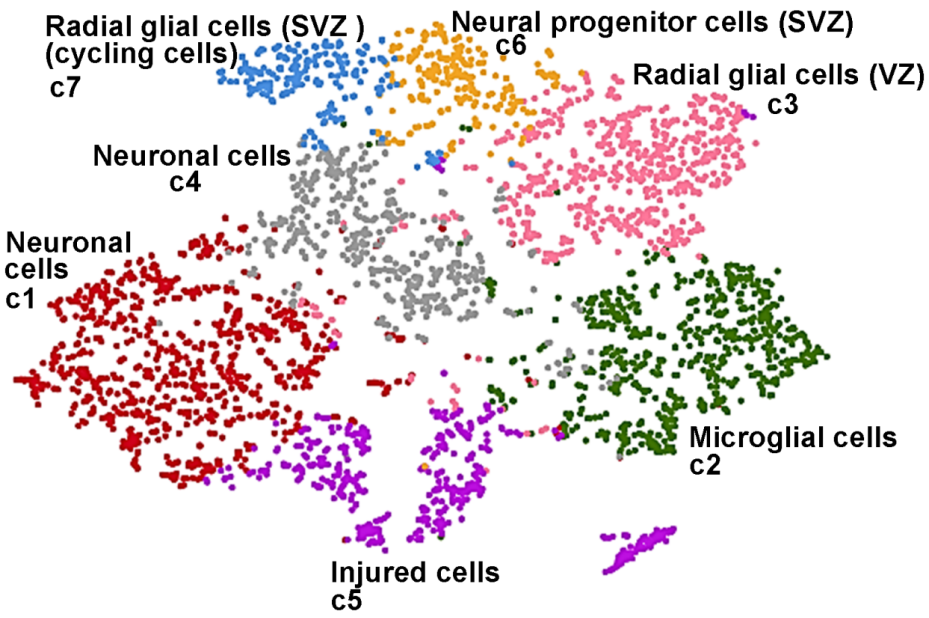
Fig. S3



-To further convince this reviewer, we would like to share our preliminary single-cell mRNA sequencing data, which suggests the presence of astrocytes and microglial cells (These data are still under analysis).

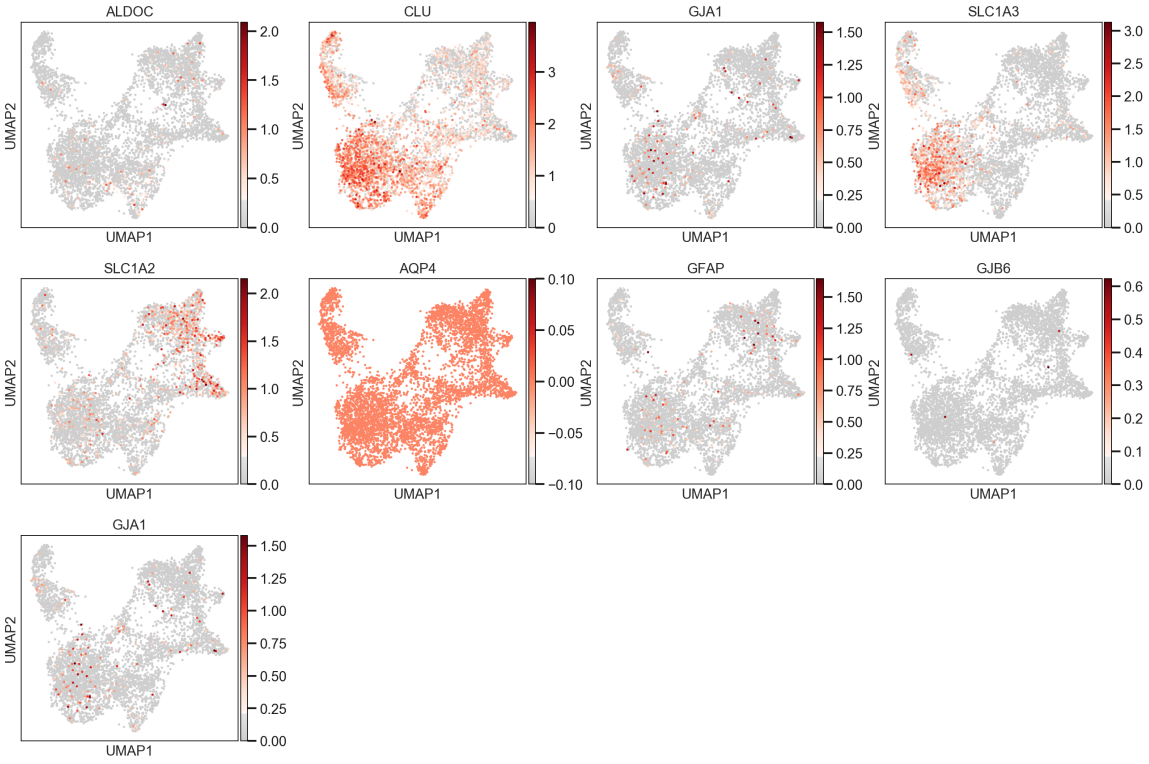
Microglial cell clusters:

Below is t-SNE plot for 60-day-old organoids. We found the C2 cluster of microglia in 60-day-old organoids. This assumption was based on following published microglial markers, AIF1, DNAJC3, RRBP1, HIST1H1C, TMEM50B, and DNAJB9. Interestingly, we observed DNAJC3 with a maximum fold change of 2,6 (p value 8,00E-19). Similarly, we found the highest expression (4,19 fold change, p value 2,00E-72) of AIF1.



Astrocyte clusters:

Based on the known astrocyte markers, we conducted similar analysis and identified few clusters suggesting the presence of astrocytes. Interestingly, these organoid groups still did not develop GFAP-positive astrocytes.



2. Most of the analysis were conducted 2- or 4-days post exposure to the virus and showed differences compared to the controls. However, I believe it would be important to contain longer timepoints (10 days, 21 days). It has been shown that patients who recover from COVID still experience deficits weeks later (e.g. taste and smell) and it would be valuable investigate the pathology of the virus at longer time periods in the organoids.

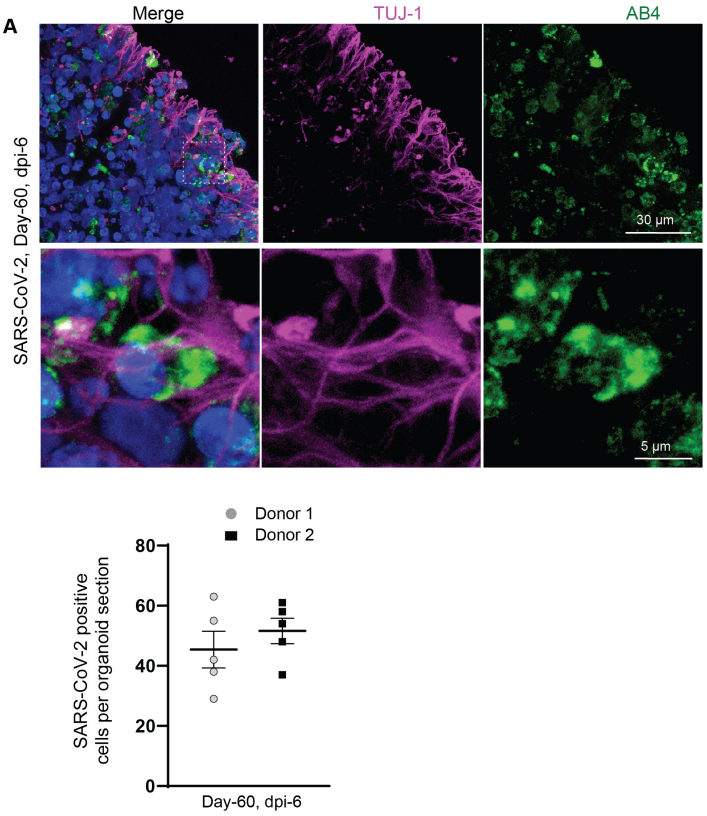
We indeed performed longer time-period experiment experiments up to dpi-6. We did not include the data due to the following reasons.

-We did not notice a significant increase in infectivity between dpi-4 and 6
-We noticed that the integrity of virus-exposed organoids was damaged after dpi-6. Thus, it was logically tricky for us to derive any specific conclusions. Even control organoids (which were kept under the stationary conditions identical to virus-exposed) have exhibited a slightly damaged integrity. Our organoids were cultured in spinner flasks and never been trained to grow in stationary cultures.

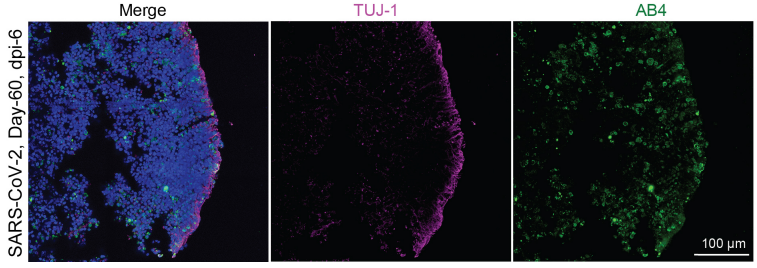
-Therefore, we limited our experiments with dpi-6. The revised version includes data for dpi-6. We provided a representative image along with a quantification graph as **Figure S4A**. For your quick reference, below is the figure.

For this reviewer's reference, we also provide a representative image showing AB-4-positive cells in a low magnification organoid **(dpi-6, Figure S4B).** Note that the organoid displays a slightly damaged architecture.

Fig. S4



B Compromised organoid architecture after longer viral exposure (dpi-6) - **For reviewers**



3. Given that the experiments are all focused on using immature organoids to distinguish whether NPCs or neurons are more susceptible, the necessity of the organoid systems is a bit confusing. Why not perform all studies in a 2D system? My concern for the 3D system is about viral diffusion into the deeper components of the organoid. In response to this there was one controlled comparison to 2D rosettes, but I still have concerns about viral diffusion. Could the authors use another viral control (ZIKV) to confirm that they are capable of observing viral infection within the VZ of the organoids and that absence of SARS-CoV-2 replication in those cells is definitively a tropism issue and nothing technical? This has been a similar observation and potential issue with other viral studies in organoid (see Sun et al 2020 re: CMV infection) where viral particles only are detected on the outermost layers but pathology appears to be widespread.

Referring to Sun et al., Cell Reports Medicine 2020; we noticed that HCMV is majorly at the outer surface of the organoids (their organoids were of high quality). As the reviewer points out, this raises an issue of penetrability of viral strains into organoids. As far as we know, Sun et al. did not directly expose neural stem cells, neurons, or organoid slices to HCMV.

We performed all of these procedures **(Figure S3A and S5)** to ensure technical issues do not mask the tropism of SARS-CoV-2.

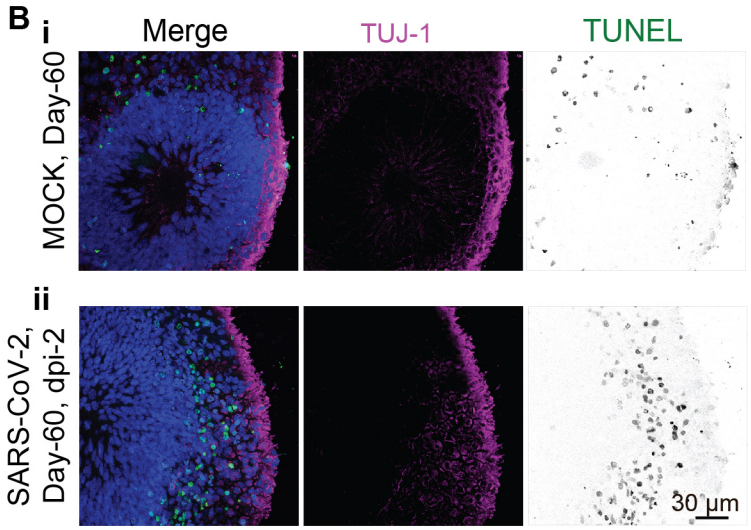
We thought of performing a parallel ZIKV experiment. However, we do have limitations with the BSL3 laboratory that we share. However, referring to our earlier work on ZIKV **(Gabriel et al. 2017, Cell Stem Cell),** the conditions for viral exposure are very same. In contrast to ZIKV, SARS-CoV-2 preferably targets the cortical region, which we have now mentioned in the revised version **(First paragraph on page 6).**

Finally, we think there is no technical issue that can block the diffusion of SARS-CoV-2 into organoids. Unlike HCMV (almost exclusively at the surface), SARS-CoV-2 does penetrate substantially into the organoid to the entire depth of the cortical region (Figure below). As a further example, at dpi-6 (see above image), we noticed that SARS-CoV-2 could penetrate to a great extent. Here we have to pay caution because the organoid's cytoarchitecture is not intact **(See above dpi-6 image above).** From these findings, we think, the behavior of SARS-CoV-2 in organoids is tropism dependent. Accordingly, we have carefully sated these interpretations in the manuscript **(First paragraph on page 6)**

4. The TUNEL comparison in Figure 3B is not a fair assessment. In the Mock (i), a segment of the organoid with a rosette is shown, whereas (ii) contains a part of the organoid without a visable rosette. These are two clearly heterogeneous parts of the representative organoids. TUNEL staining is surely likely to be less near healthy rosettes than it is at locations physically farther from the "VZ-like" zones.
Along the lines of point 4, it would be helpful to include a few zoomed out (or stitched-together) images of whole organoids to see AB4 at the macro-level. At the moment, we are only able to see a few zoomed in panels, which can be tricky to interpret given the heterogeneity of organoid cultures in general.

Our apologies. We have now provided comparable sections where healthy VZ is present in both groups in **Figure 3B** (see below for your quick reference).

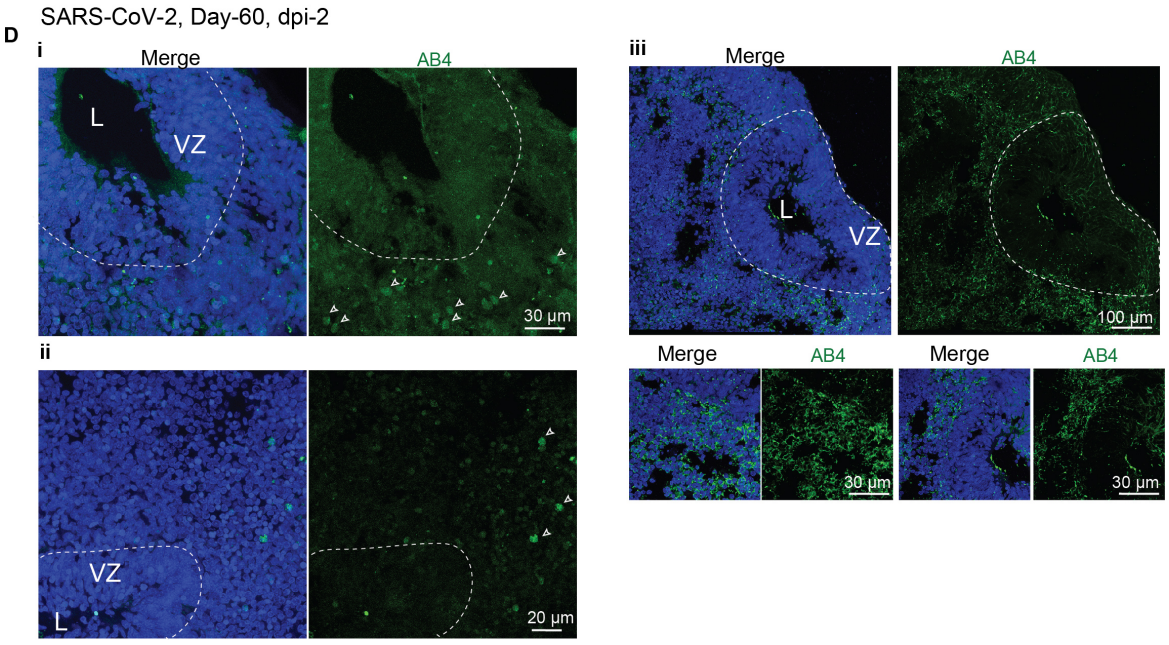
Fig. 3



Low magnification images: We consistently gave a zoomed out image covering VZ and cortical region as a panel. We then zoomed into a region (which is cortical region) where we could detect majority of the virus.

We wish we had several whole representative organoids intact. Referring to our ZIKV work, we did present complete organoid images **(Figure S5 of Gabriel et al Cell Stem Cell 2017).** In the current case, as mentioned above (See above dpi-6 image above), after SARS-CoV-2 exposure, we noticed compromised structural integrity. Nevertheless, we consistently detected a similar pattern of virus-positive cells in each organoid we tested. To convince this reviewer, we have managed to display a few zoomed out images where one can see AB4-positive cells consistently away from the VZ **(Figure S2D).**

Fig. S2



Minor:
1. In Figure S1b, the text at the top of the table is cut off. It reads "ISA against the spike protein of SARS-CoV-2" instead of "ELISA against the spike protein of SARS-CoV-2."

2. I did not see a legend for Figure 3D.

Our response: Our apologies. We have now fixed both of the minor points. Panel D (which was a control mock for AT180 staining) has now been changed into **Ciii.**

Overall, this study feels quite rushed. I recognize that is in large part due to the extraordinary circumstances surround SARS-CoV-2. I do feel, however, that it is imperative to include all relevant controls and to be very direct about limitations. The current text reads like this is definitive proof that human neurons are susceptible to SARS-CoV-2. After reading carefully, however, I have a number of questions. Given the low infectivity rate, would the BBB and microglial defenses normally provide a serious obstacle to neuronal infection at all? Alternatively, since ACE2 is highest expressed in brain vasculature, does that serve as a nidus for further spread into cells in the brain? These are issues that should be more clearly included in the discussion. What is most important to me is that the authors confirm no technical issues in viral penetration to deeper regions of the organoid, test later timepoints after viral administration, address the organoid heterogeneity issues with more zoomed out images, and greatly tone down the language that insinuates this is evidence for what might be happening in the human brain. I applaud the authors for this ambitious endeavor and just want to be sure that rigor is appropriate given the current climate surrounding SARS-CoV-2.

We highly value this referee' suggestions. We took them entirely and carefully addressed them at the discussion section tampered down our statements and clearly discussed the limitations of using brain organoids in COVID-19 research.

Editor: >> consider longer time points virus exposure (ref#2, pt.2)
Editor: >> additional TUNEL analyses (ref#2, pt.4)

Referee #3:

The manuscript "SARS-CoV-2 targets neurons of 3D human brain organoids" by Ramani et al investigates SARS-CoV-2 infection of human brain organoids. The authors are prompted to investigate these questions by several reports describing neuro complications in COVID-19 patients. The authors inoculate human brain organoids with SARS-CoV-2 virus and identify neurons with the virus. They go on to show that there is altered Tau localization in SARS-CoV-2 stained cells and see evidence of neuronal death. The authors use a self-identified human anti-SARS-CoV-2 antibody. In addition the authors show no evidence of viral replication or evidence of productive infection in human brain organoids. Overall the authors need to more clearly state that at best the virus enters neurons but does not actively replicate. It is not clear if the findings of neuronal Tau localization change is due to SARS-CoV-2 or any RNA or viral entry event in the cell. Given these concerns additional supporting experiments are needed and would require major revisions for consideration.

We agree with this reviewer's constructive concerns. We indeed made a statement that the virus can enter neurons and do not actively replicate. We made this point more evident in the revised version **(At the end of the introduction and results section before the Tau part).**

Comments for the author:
1. The authors recognize that SARS-CoV-2 infection is somewhat controversial in neuronal platforms. They use a antibody against SARS-CoV-2 that they identified and self-purified. Confirmation of all staining with a commercially available antibody to demonstrate that this isn't an artifact of an antibody that will not be available in the community is necessary.

As mentioned in the manuscript, as of April 1st 2020, we could not procure commercial antibodies that can specifically determine SARS-CoV-2 infection. Therefore, we isolated COVID-19 convalescent serum and tested if they can specifically recognize SARS-CoV-2 infections in our experiments. In later days, we have used two commercially available SARS-CoV-2 antibodies, which work well in Vero cells **(Figure S2A).** Only SARS-CoV-2-S (mouse monoclonal) could detect the virus in organoid sections co-localizing with AB4 (Figure S2B). Even then, commercial antibodies were not always the best ones in our routine experiments. This was the reason; we generated convalescent serum and

stringently validated them side-by-side with commercial antibodies. We dedicated two Figures **(Figures S1 and S2)** to prove the AB4 in multiple ways (ELISA, WB, and IF).

As further notes: (i) similar convalescent serum have also been used at the latest preprint by Guo ming Li (doi: https://doi.org/10.1101/2020.07.28.225151). (ii) Numerous studies have successfully demonstrated the convalescent serum's capacity in blocking viral entry (Bloch, Shoham et al., 2020, Casadevall & Pirofski, 2020)

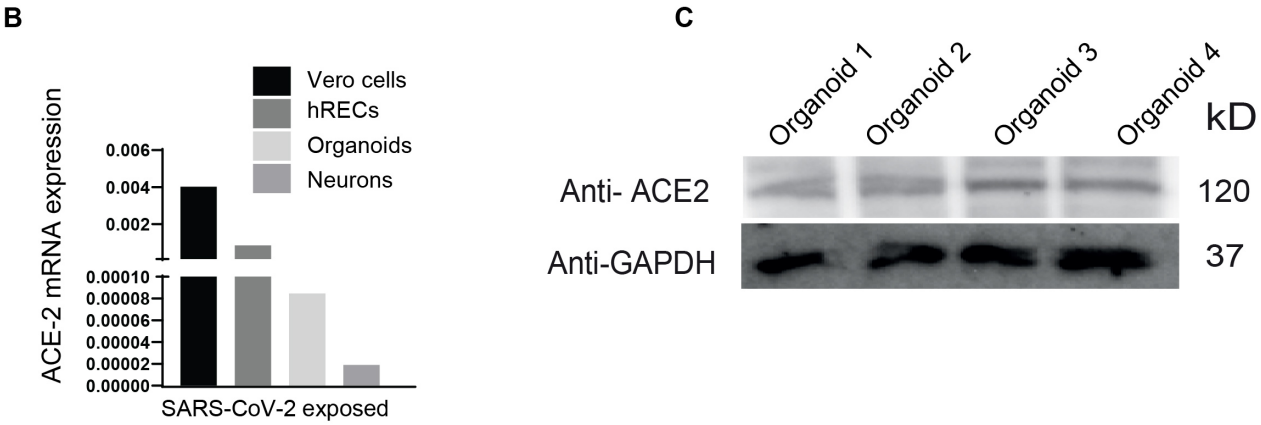
2. The brain organoid imaging and stainings are beautiful and well annotated and the authors should be commended.

We appreciate the reviewer for this comment. Addressing #1, #2 and #3, we have now provided more images.

3. hACE2 staining of the organoids would be helpful for elucidating which cells in the organoids have the potential to even support viral entry. A SARS-CoV-2 pseudoparticle would also help support these studies if available.

We determined a low level of ACE2 mRNA in our brain organoids compare to human respiratory epithelial cells **(Figure S4B)**. We then conducted a Western blot experiment to determine the ACE2 protein level. In accordance with mRNA, we could detect ACE2 only at more extended exposure conditions **(Figure S4C).**

Fig. S4



We then tried to image the subcellular localization of ACE2 in SARS-CoV-2-positive neurons. We could not identify any staining. We think one of the following is the potential reason.

-The commercial anti-ACE2 is not suitable for IF
-The antibody does not recognize ACE2 since it is expressed at a low level

We want to point here that a commercial anti-ACE2 from Santa Cruz has cross-reactivity to secondary antibodies (human, mouse, goat and donkey). After realizing it, we stopped using the antibody as it could mislead the interpretations.

4. The authors demonstrate that the the brain organoids have localization of the AB4+ cells on the periphery. Is that because the interior cells are not seeing the virus, have cells not permissive to the virus, or have cells with resistance to the virus?

We think this is due to the preferential tropism of the virus to neurons at the cortical region. To exclude that there is a technical problem of penetration to the interior of the organoid, we directly exposed 2D cultures of neural stem cells, cortical neurons or organoid slices to virus **(Figure S3A and S4B)**. These experiments further strengthen that the finding that the virus has a preferred tropism which are the neurons of the cortical region.

5. Do the authors have any explanation for why the SARS-CoV-2 staining (which ostensibly is staining for SARS-N) is only labeling the somas and not the axons as it is not clear why the protein would be localized in the neuronal cells.

Based on the observations, SARS-CoV-2 staining is very similar to what has been observed with Vero cells, mostly perinuclear (we have mentioned it in the manuscript). However, we cannot rule out how other antibodies (raised against the nucleoprotein, spike or any other domain) would determine a different localization pattern of the virus in neurons.

6. Given that there is no expansion in number of SARS-CoV-2 labeled cells 2 and 4 dpi strongly suggests that there is no evidence of viral replication. Further confirmation with TCID50 studies and qRT-PCR for subgenomic RNA and replicating strand would also validate these findings.

We are glad that this reviewer agrees with our interpretation that there is no active replication in brain organoids. We are afraid that this referee perhaps did miss seeing TCID50 studies, which we presented in our earlier version. We now present these assays as **Figure 1G** (for brain organoids) and **Figure S5C** for organoid outgrowths. Real-time qPCR analysis for quantification of SARS-CoV-2 RNA copies per mL is described in the method section.

Alternatively, one could also extract the virus present within the organoid and perform a similar experiment. We did not prefer doing it because; the virus loosely attached to the organoid's surface might lead to false values.

7. The MOI of infections is not reported. A dose titration curve for SARS-CoV-2 would help support the authors findings e.g. 0.001-1.0.

There is a technical problem why we could not specify MOI values because we are using organoid tissues and not cells. In the tissues, one cannot determine the precise cell numbers, and thus we did not intend to provide an arbitrary MOI value. To solve this problem, we disassociate few organoids and estimate the cell number.

Here is our detailed response in determining MOI:

To estimate MOI, we first calculated the viral titer as TCID50/mL of our generated SARS-CoV-2 by an end-point dilution assay. This assay has been described in detail in the methods section of our manuscript (Flint, Racaniello et al., 2015).

In brief, based on induced cytopathic effects, we calculated the TCID50/mL using a formula based on the Spearman-Karber method, which is also mentioned in detail in the methods section (Ramakrishnan, 2016). To further confirm this calculation with respect to the novelty of this formula, we also applied the commonly used Reed and Muench method (Lei, Yang et al., 2020). Both of these methods resulted in a TCID50/mL of 5000 that we then used to calculate the PFU/mL. Applying poisson distribution, we estimated that the amount of infectious viral particles per mL (PFU/mL) in our stock is 3500 PFU/mL.

In the context of our infection experiments, we provided 5 µL virus stock per organoid. According to our calculation, the 5 µL volume of SARS-CoV-2 stock contains approximately 17.5 PFUs. Having then estimated the number of viable cells after disintegrating organoids (an average of 100,000 for Day 15 and 200,000 for day 60), we could determine the multiplicity of infection (MOI). Considering 17.5 PFUs, our estimated MOI is 1.8×10-4 and 8.8×10-5 for day 15 and day 60, respectively. Importantly, we found that such a low viral load is sufficient for our studies.

Compared to other studies (appear as preprint in BioRxiv) the authors have used 100,000 to 750,000 PFUs per organoids (https://doi.org/10.1101/2020.05.30.125856, https://doi.org/10.1101/2020.07.28.225151). We think our approach seems more reasonable since in a natural system one would expect a lower amount of infectious particles per cell to reach specific target cell types. It is also possible that these authors have used different methods to estimate PFUs.

It is noteworthy that another coronavirus (MHV-1) is lethal to infected mice at a dose of only 50 infectious particles (PFUs) and with a calculated lethal dose 50 (LD50) of 240 PFUs (De Albuquerque, Baig et al., 2006). Moreover, in a different study, recombinant SARS-CoV, a close relative to SARS-CoV-2, was lethal to infected hACE-2 transgenic mice after infection with also only 240 PFUs (Dediego, Pewe et al., 2008).

8. The authors should add additional discussion on prior reports of viral infections and its impact os Tau localization and neuronal impacts.

We appreciate this point. We did mention the latest work from the David Kaplan lab showing Tau abnormalities with HSV virus. In the revised version highlights it further at the discussion section.

9. Given the authors's argument that viral replication does not seem to be required for neuronal entry and injury is one of the viral proteins responsible? Have the authors examined any of the viral proteins for similar effects?

Due to the time constraint, we did not explore this option. We are tempted to test individual viral proteins of SARS-CoV-2.

Editor>> ACE2 staining organoids (ref#3, pt.3)
Editor >> provide evidence on viral replication-productive infection (ref#3, pt.6)
Editor >> provide MOI infections/ titration curve (ref#3, pt.7)
Editor >> add controls on antibody validity (ref3, pt.1)

Bloch EM, Shoham S, Casadevall A, Sachais BS, Shaz B, Winters JL, van Buskirk C, Grossman BJ, Joyner M, Henderson JP, Pekosz A, Lau B, Wesolowski A, Katz L, Shan H, Auwaerter PG, Thomas D, Sullivan DJ, Paneth N, Gehrie E et al. (2020) Deployment of convalescent plasma for the prevention and treatment of COVID-19. *The Journal of clinical investigation* 130: 2757-2765
Casadevall A, Pirofski LA (2020) The convalescent sera option for containing COVID-19. *The Journal of clinical investigation* 130: 1545-1548
De Albuquerque N, Baig E, Ma X, Zhang J, He W, Rowe A, Habal M, Liu M, Shalev I, Downey GP, Gorczynski R, Butany J, Leibowitz J, Weiss SR, McGilvray ID, Phillips MJ, Fish EN, Levy GA (2006) Murine hepatitis virus strain 1 produces a clinically relevant model of severe acute respiratory syndrome in A/J mice. *Journal of virology* 80: 10382-94
Dediego ML, Pewe L, Alvarez E, Rejas MT, Perlman S, Enjuanes L (2008) Pathogenicity of severe acute respiratory coronavirus deletion mutants in hACE-2 transgenic mice. *Virology* 376: 379-89
Flint SJ, Racaniello VR, Rall GF, Skalka AM, Enquist LW (2015) *Principles of virology*. ASM Press, Washington, DC
Lei C, Yang J, Hu J, Sun X (2020) On the Calculation of TCID50 for Quantitation of Virus Infectivity. *Virologica Sinica*
Ramakrishnan MA (2016) Determination of 50% endpoint titer using a simple formula. *World J Virol* 5: 85-6

1st Revision - Editorial Decision 25th Aug 2020

Dear Jay,

Thank you for submitting your amended manuscript (EMBOJ-2020-106230R) to The EMBO Journal. As mentioned, your revised study was sent back to the three referees for re-evaluation, and we have received comments from two of them, which I enclose below. Please note that while referee #3 has imminently promised his-her report, we have in light of the other reviewers' re-evaluation and interest of time decided to proceed with our decision.

As you will see from their reports enclosed below, the referees #1 and #2 find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending the following remaining minor textual changes, introducing caveats as follows:

>> ensure it is clearly stated that the link to tau could be effect rather than causal.

>> viral penetrance into the center of the organoids can only be deferred indirectly at this time.

Please note, that if the third re-report arrives before the proofs, we expect any additional crucial issues to be addressed textually.

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.

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 contact me at any time if you need any help or have further questions.

As you know, every paper now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Again, we expect your revised manuscript version shortly and are prepared to swiftly proceed with acceptance and expedited production of your article.

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

Kind regards,

Daniel

Daniel Klimmeck PhD
Editor

The EMBO Journal.

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

>> Please provide a completed author checklist for your study.

>> Add a separate 'Conflict of interest" section to your manuscript.

>> Add a separate 'Statistical Analysis" section in the material and methods part.

>>Please move the materials and methods to the main manuscript after the discussion part.

>> Please rename the Supplementary Information to 'Appendix', and the Supplementary Figures 1...' to 'Appendix Figure S1...'; update the legends and callouts in the main text accordingly. Please add a ToC on the first page of the Appendix.

>> Please introduce a 'Data availability" section in the material and methods part stating 'no amenable data sets'.

>> Adjust reference format to 10 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.

>> Provide all figures as individual high resolution .tiff files. When submitting the figures as individual files, the legends need to be removed from the figure files and added as a Figure Legends section to the manuscript file.

>> Recheck callouts for Figure 1D and all subpanels of Figures 2 and 3 in the main text.

>> Please add the Jürgen Manchot Foundation as funding body in our online manuscript system.

>> IF confocal microscopy: Please consistently add zoom dashed boxes in overview microcraphs Figures 1A, 1B, S4A, S6A-E i versus ii. Specify DAPI counterstain and explain z axis differences between overview and zoomed in micrographs (eg in Figure 1A) in the figure legends.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

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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 23rd Nov 2020.

Link Not Available

Referee #1:

I think given the circumstances the authors have done a reasonable job of trying to respond to the reviewer's critiques. I still find the link to tau more likely to be effect rather than causal. Other points raised by the other reviewer's seem to be partially addressed.

Referee #2:

Overall, and in the context of current limitations and time pressures, I am satisfied with the authors' revisions. I feel that they have made a substantial attempt to address the concerns raised by the editors. In particular, I was happy to see some zoomed out images and recognize that tissue deterioration at later time points is a confounding issue. I also appreciate the toning down of some of the language and conclusions. I feel strongly that given the limited resources and time for this study, it's imperative that it read as a descriptive and preliminary study rather than definitive and causal proof.

I still have concerns about viral penetrance into the center of the organoids. This matters because the primary conclusion is that the virus is infecting cortical neurons on the outside exclusively. It's possible I missed this in the revised text, but I think it is an important discussion point just to emphasize that further work could be done in the future to test viral accessibility uniformly throughout the organoids.

Overall, the authors should be commended. Great work in such a short period of time.

2nd Authors' Response to Reviewers 28th Aug 2020

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 31st Aug 2020

Dear Jay,

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. I would thus like to ask for your consent on keeping the additional referee reply figures included in this file.

Also in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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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.

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Finally, we have noted that the submitted version of your article is also posted on the preprint platform bioRxiv. We thus appreciate if you could alert bioRxiv on the acceptance of this manuscript at The EMBO Journal in order to allow for an update of the entry status. Thank you in advance!

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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Corresponding Author Name: Jay Gopalakrishnan
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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

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The data shown in figures should satisfy the following conditions:

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- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
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- 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 $\chi 2$ 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.

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?	To have appropriate sample size and to avoid random variability, data were collected and analysed at least from triplicate independent experiments. For each experiment, the amount of cell numbers and appropriate statistical methods used are described under each figure legend in the manuscript. We have also provided this under the methods section on page # 19.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No animal studies have been performed for our manuscript.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animal studies have been performed for our manuscript.
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.	Subjective bias was minimized by randomizing our control and infected samples during immunofluorescence (IF) analysis.
For animal studies, include a statement about randomization even if no randomization was used.	No animal studies have been performed for our manuscript.
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.	Blinding of investigators was not relevent to our study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No animal studies have been performed for our manuscript.
5. For every figure, are statistical tests justified as appropriate?	The appropriate statistical analysis, including the sample size, replicates and appropriate cell and organoid numbers have beeen clearly described in the figure legends in our manuscript. This has also been provided under the methods section on page #19
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used Student's t-test and non-parametric one-way ANOVA followed by apprropriate Post-hoc tests for our analysis.
Is there an estimate of variation within each group of data?	Not applicable.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Not applicable.

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).	The antibodies used in this study have been described with the corresponding catalog numbers on page #17 and #18
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source for the cell lines have been described under the methods section on page # 16. All cell lines were tested 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.	No animals were used for this study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No animals were used for this study.
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.	Not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Serum samples AB1 and AB2 were obtained under a protocol approved by the ethical commitee, medical faculty, University Hospital Düsseldorf, Heinrich-Heine-University (study number 5350). Serum samples AB3 and AB4 were obtained under a protocol approved by the Institutional Review Board of the University of Cologne (protocol 16-054). Human respiratory epithelial cells (hREC) were obtained by nasal brush biopsy from healthy control individuals. The study was endorsed by the local ethical committee at the University of Münster, and each patient gave written informed consent (Study number, 2015-104-f-S, Flimmerepithel) and 2020-274-f-S (COVID-19). Trained physicians from the Department of General Pediatrics, University Hospital of Münster, performed biopsies.
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.	All patients participating in this study signed informed consent forms approved by the responsible authority.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Future distribution of resources to any third party will have to be authorised by niversity Hospital Düsseldorf, Heinrich-Heine-University and University of Cologne.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
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.	Not applicable.
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.	Not applicable.

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	No data have been generated in this study, to deposit in the public database.
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).	Not applicable.
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).	Not applicable.
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.	Not applicable.

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.	Not applicable.