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SARS-CoV-2 Evades Immune Detection in Alveolar Macrophages

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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 Revision - Editorial Decision 7th Aug 2020

Dear Dr. Hartmann,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, I will not detail them here.

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

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

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

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

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

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

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content.

Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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

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

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

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

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

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

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary

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

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

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our new reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Please add up to five keywords to the title page, and remove the summary (we only need the abstract).

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

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this manuscript the authors examine the induction of interferons and ISGs in respiratory virus infections of primary human alveolar macrophages. Similarly to what has been demonstrated in many cell types over several decades, they observe that Sendai virus is a strong inducer of Type I IFNs/ISGs and that influenza virus is a relatively weaker inducer of IFNs/ISGs. They newly test effects of SARS-CoV-2 treatment of the macrophages and observe that IFNs are not induced, though a limited subset of ISGs may be unregulated. However, whether the macrophages are actually infected by SARS-CoV-2 at a significant rate is not convincingly demonstrated, and further,

it is already published in multiple studies that SARS-CoV-2 is a poor inducer of type I IFNs. Overall, the manuscript appears rushed for publication and lacks new insights and essential controls.

Major concern:
Evidence for infection of macrophages by SARS-CoV-2 is poor. SARS-CoV-2 is an endocytosed virus, and a number of cellular restriction factors have already been shown to block SARS-CoV-2 membrane fusion (e.g., Ly6E, CH25H, IFITMs). Thus, detection of viral RNA within the cell does not indicate an actual infection in which genomic contents have been released into the cytosol for the genome amplification that is usually required for activation of a robust IFN response by RNA viruses in myeloid cells. Likewise, the lack of an active IFN suppression mechanism in the experiments with PolyI:C could be easily explained by a lack of infection.

What is the percent of cells that are truly infected when staining for viral protein 24 or 48 h post infection? How does this compare with the rates of infection that you are observing for the disparate doses of SeV and IAV that are being used? Further, how was the dose of SARS-CoV-2 for alveolar macrophages chosen without examining what MOI leads to actual infection of cells? Comparing RNA Ct values is not at all informative as to the percentage of infected cells within a culture.

Referee #2:

The study is straightforward and by and large the data support the conclusions (see specific comments). To my opinion the scope could be widened without asking for the impossible, given the obviously demanding experimental system.

Specific comments:

- One of the most interesting aspects is the inability of SARS CoV-2 to block an IFN response stimulated by pIC. However, there is a potential technical issue with the experiment: all cells will respond to pIC, but at MOI 1 not all cells in the culture are productively infected. Can the authors rule out that the pIC response stems from uninfected cells? IF studies might help to clarify this.

- The authors suggest an ability of SARS CoV-2 to avoid detection by the immune system, without however substantiating this conclusion. It should be fairly easy to analyze early signaling events indicating viral recognition such as pIRF3 or pTBK1 by western blot.

- In Fig.1 I don't understand why the ISG levels in the C controls are as high as those of the IFN-treated NT samples.

- To me the title doesn't sound right (not claiming infallibility): you can evade an immune response or inhibit/block induction, but you can't evade induction?

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Dalskov et al investigate the ability of alveolar macrophages (AMs) to generate an interferon (IFN)

response upon SARS-CoV-2 exposure. They report a lack of IFN or ISG induction upon viral exposure, and show that the cells generate an immune response thought TLR3 stimulation. Understanding the immune responses to SARS-CoV-2 pathogenesis is of interest.
Major concerns:
1. The access to Broncho Alveolar Lavages (BAL) is not easy and the number of AMs purified from the BAL can be low. This may explain the low number of independent samples tested (n=2 or 3 for most of the conditions). IFNA2 and IFNL1 were quantified only in samples from 2 donors, while IFNB1, ISG15, RSAD2 and IFIT1 were quantified in 3 donors. Using 2 donors is not sufficient to perform a statistical analysis. The authors should increase the number of samples analyzed.
2. The authors quantified IFN and ISG mRNA levels at one time point only (8 hours). This is not sufficient to conclude that AMs exposed to SARS-CoV-2 do not induce an immune response.
3. The susceptibility of tissue macrophages to SARS-CoV-2 and their capacity to produce de novo infectious viral particles remain controversial and should be discussed.
4. At 8 hours post-infection neither IFN nor IFN decrease the RNA levels of SEV, IAV and SarsCov2. These results suggest that no viral replication does not occur in AMs at this early time point. Infections should be performed at different MOI and viral RNA should be measured at different time points to assess whether AMs are productively infected by SARS-Cov-2 and whether SARS-CoV-2 is sensitive to IFNs in these cells.
5. A previous study demonstrated that in lung tissues, SARS-CoV-2 infection induces a weak IFN response compared to SARS-CoV-1, and that AMs contain SARS-CoV-2 RNA (Chu et al., CID, 2020). The present resulst are thus in large part confirmatory, since the authors do not investigate the potential mechanisms by which SARS-CoV-2 avoids sensing by PPRs.
6. In the quantification of cell-associated SARS-CoV-2 RNA, the limit of detection of the PCR should be added to the graph.
7. Sentences are too long. Many repetitions, unnecessary comments and grammatical errors make the reading difficult.

Referee #1:

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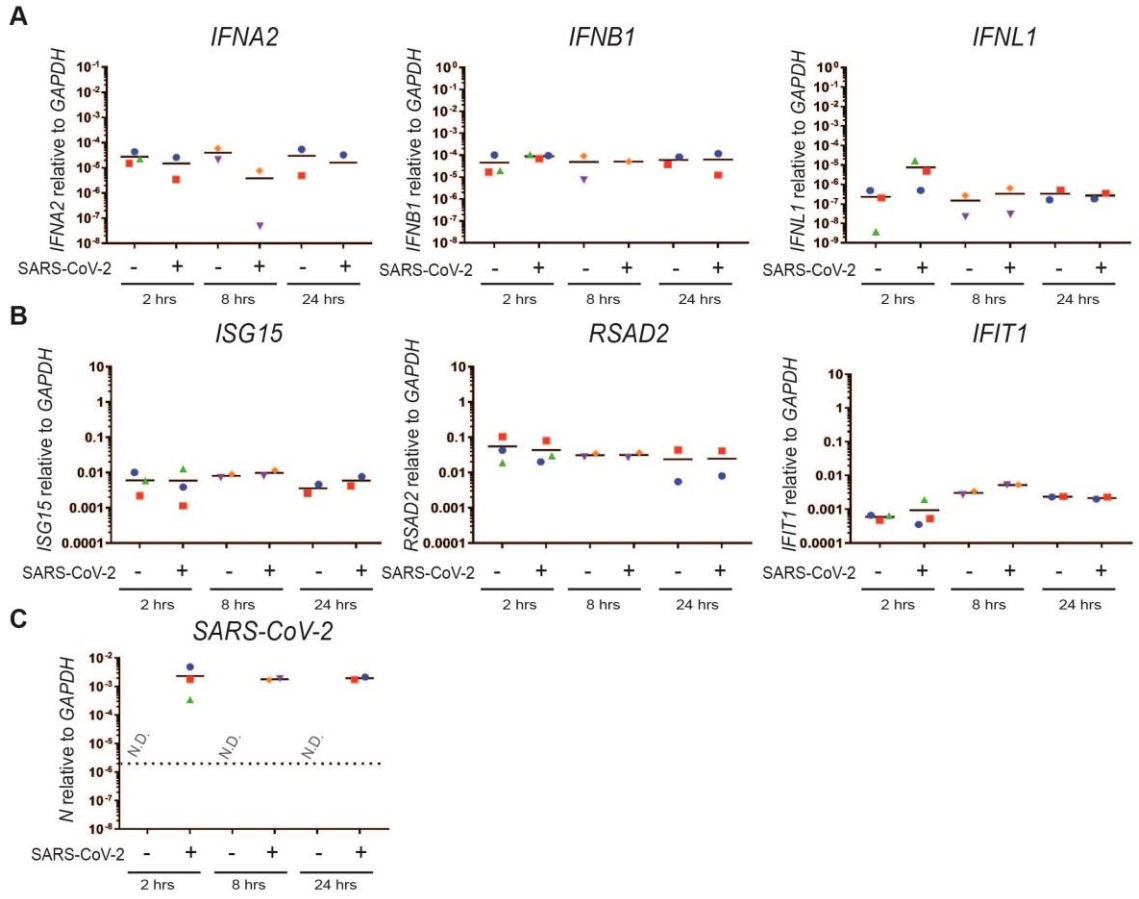
The reviewer voices concern that the SARS-CoV-2 RNA we detect is within the endosomes and has not been released to the cytosol. While this may indeed be true, it does not affect the conclusions drawn in our paper. Immune cells such as macrophages and dendritic cells express TLR receptors (TLR3, 7, 8 and 9), which recognize nucleic acids, and are localized within endosomes (1). The placement of TLR receptors within endosomes enables immune cells (like AMs) to survey the environment within which it lives for signs of infection and react to this without being infected themselves. As described below, we use Poly(I:C) as a control. Importantly, Poly(I:C) is simply added to the culture media in our experiments, something that we did not state clearly enough in the initial version of our manuscript, but which is now clearly described. Thus, the Poly(I:C) controls show that AMs will react towards nucleic acids within their immediate environment and that the nucleic acid does not need to be actively released into the cytosol by the virus.
The virus we use is highly infective in human airway epithelial cells (the MOI stated in the paper is derived from experiments on those cells). We did qPCR to detect SARS-CoV-2 RNA and compared its level to that of two other viruses known to elicit a clear and robust immune response (as the reviewer also points out) and those data are found in figure 1C and 2C. Danish regulations forbid that we work with other viruses while working with SARS-CoV-2, which is why the IAV and SeV experiments were carried out separately. This is also the reason why we use Poly(I:C) as a positive control instead of virus infection in figures 2 and 3.
Our data show slightly more IAV RNA and slightly less SeV RNA compared to the level of SARS-CoV-2 RNA found within the AMs. Thus, we are arguing that the amount of SARS-CoV-2 RNA found within the AMs should be sufficient to elicit an immune response as it was sufficient for the two other viruses tested. However, as the reviewer points out, we did not show a productive infection. It was not our attempt to do so, largely because we did not expect a productive infection in immune cells like AMs. However, immune cells are thought to be the main contributors to the cytokine storm that drives the pathogenic inflammation seen in SARS-CoV-2 infections as well as

other respiratory infections, such as influenza, and these immune cells do not need to be productively infected in order to be activated (2,3), as discussed above. Furthermore, AMs are the most abundant immune cell in the lung and they react strongly towards a dsRNA mimic, Poly(I:C), if added to the culture media. Nevertheless, AMs do not react towards challenge with SARS-CoV-2 virus. Thus, we believe our data to be important as they suggest that AMs are not productively infected by SARS-CoV-2 and do not react to challenge by SARS-CoV-2 with production of IFNs. Therefore, we believe our data suggest that AMs are not the major contributors of inflammatory cytokines in SARS-CoV-2-infected patients, at least not early during the infection.



However, prompted by the reviewer comments, we did test if SARS-CoV-2 has the potential to replicate within AMs. We challenged AMs with SARS-CoV-2 and then measured viral RNA levels at 2, 8 and 24 hours post infection, we decided not to include those data in the manuscript, but they are shown to the reviewer here (see the figure above). We also performed a similar experiment where we harvested protein instead of RNA to investigate potential synthesis of SARS-CoV-2-related proteins post challenge. Those experiments did reinforce the concerns voiced by the reviewers, as SARS-CoV-2 RNA levels declined over time, suggesting that no replication took place. Using a targeted MS analysis approach, we tested for 11 SARS-CoV-2 related proteins, in the AMs post challenge, we could detect the Nucleocapsid protein in a reproducible manner at all time-points (new figure 4). Here we observed a similar pattern to the one observed for SARS-CoV-2 RNA with the level of the Nucleocapsid protein declining slightly over time. We suspect that the detected protein originated from the virions used for challenge and our data suggest that there is no translation of SARS-CoV-2 RNA in AMs.

We have therefore changed the word “infection” with “challenge” throughout the manuscript to emphasize that the cells were challenged with SARS-CoV-2 virus to establish if this leads to IFN production, and to make it clear to the reader that there is no productive infection of the AMs.

What is the percent of cells that are truly infected when staining for viral protein 24 or 48 h post infection?

Please refer to our extended response above

How does this compare with the rates of infection that you are observing for the disparate doses of SeV and IAV that are being used?

Unfortunately, we did not determine infection rates for those viruses but the amount of viral RNA within the AMs is comparable (refer to Supplementary Figure 1).

Further, how was the dose of SARS-CoV-2 for alveolar macrophages chosen without examining what MOI leads to actual infection of cells?

The dose of SARS-Cov-2 was determined based on our own and collaborators previous experiences with the virus. MOI of 0.05 is sufficient for infection in human airway epithelial cells. As we had limited availability of AM cells, we decided to challenge with a higher dose to ensure proper challenge.

Comparing RNA Ct values is not at all informative as to the percentage of infected cells within a culture.

While the reviewer is right, the Ct values do not reflect the percentage of infected cells, we still think this the appropriate thing to measure here. This is based upon the assumption that the amount of viral PAMP is important for detection. The Ct values are a good measure to detect the viral RNA found within the cells, and since it is RNA, which is being detected by pattern recognition receptors (PRR), we do believe measuring the amount of RNA is informative.

Referee #2:

The study is straightforward and by and large the data support the conclusions (see specific comments). To my opinion the scope could be widened without asking for the impossible, given the obviously demanding experimental system.

Specific comments:

- One of the most interesting aspects is the inability of SARS CoV-2 to block an IFN response stimulated by pIC. However, there is a potential technical issue with the experiment: all cells will respond to pIC, but at MOI 1 not all cells in the culture are productively infected. Can the authors rule out that the pIC response stems from uninfected cells? IF studies might help to clarify this.

We appreciate the praise by the reviewer. We simply included the Poly(I:C) as a control to demonstrate that the AMs are capable of detecting nucleic acid and initiating IFN production. However, our wording was clearly imprecise and we have rewritten the text to clarify this point. Nevertheless, the fact that SARS-CoV-2 RNA is found within the AMs at levels comparable to SeV and IAV supports our hypothesis that the SARS-CoV-2 RNA is modified and in a that way rendering it hard to detect by the PRR system.

- The authors suggest an ability of SARS CoV-2 to avoid detection by the immune system, without however substantiating this conclusion. It should be fairly easy to analyze early signaling events indicating viral recognition such as pIRF3 or pTBK1 by western blot.

In relation to other projects, we have tried extensively to produce phosphowestern blots or mass spectrometry detection of pIRF3 and pTBK1. However, we were never successful using this method on primary cells. In fact, most reported experiments with those antibodies use cells overexpressing the target protein.

- In Fig.1 I don't understand why the ISG levels in the C controls are as high as those of the IFN-treated NT samples.

This control is a control for background expression of ISGs in the donors and a test for signs of inflammation and/or infection in those donors. Donors with very high ISG expression (indicative of an ongoing infection) were excluded from the study. Thus, for the control measurement in figure 1, cells were collected immediately after receiving the BAL fluid from the hospital. The ISG expression seen here reflects endogenous levels of ISGs in living individuals, where there is a certain degree of tonic IFN signaling going on. For the non-treated samples, cells were isolated by attachment and then washed and grown in clean medium for 6 hours. Thus, the drop in ISG expression reflects the absence of tonic IFN signaling once the cells are in isolated cultures.

- To me the title doesn't sound right (not claiming infallibility): you can evade an immune response or inhibit/block induction, but you can't evade induction?

We agree with the reviewer and have altered the title to “SARS-CoV-2 Evades Immune Detection in Alveolar Macrophages”

Referee #3:

Dalskov et al investigate the ability of alveolar macrophages (AMs) to generate an interferon (IFN) response upon SARS-CoV-2 exposure. They report a lack of IFN or ISG induction upon viral exposure, and show that the cells generate an immune response thought TLR3 stimulation. Understanding the immune responses to SARS-CoV-2 pathogenesis is of interest.
Major concerns:
1. The access to Broncho Alveolar Lavages (BAL) is not easy and the number of AMs purified from the BAL can be low. This may explain the low number of independent samples tested (n=2 or 3 for most of the conditions). IFNA2 and IFNL1 were quantified only in samples from 2 donors, while IFNB1, ISG15, RSAD2 and IFIT1 were quantified in 3 donors. Using 2 donors is not

sufficient to perform a statistical analysis. The authors should increase the number of samples analyzed.

We apologies for this mistake. We set 50 cycles in the qPCR as our detection limit meaning that if a gene in a given donor failed to reach this criterion, the measurement was excluded. This is now clearly indicated in the figure legend. This occurred for two donors in Fig. 2A for both *IFN2A* and *IFNL1*. However, please note that we have a total of 5 donors that was treated with IFN-λ, as well as 5 donors in the untreated group. As the reviewer points out, we cannot do statistical analysis on the low IFN-λ concentration, due to lack of replicates. We have removed the statistical analysis from this data point (this was included by mistake) and are happy to remove this data point entirely, if the reviewer prefers this. However, we believe that the low and high doses of IFN-λ reinforce each other and therefore we will retain the low concentration data point unless instructed to do otherwise. At this point, it is not feasible to increase the number of samples as this would require selection of an entire new set of donors.
In general, we have taken care to include as many replicates as possible given the number of donors available. Some donors yield relative few AMs, and those donors could therefore not be included in all conditions. We have prioritized to have the most replicates when we compared SARS-CoV-2-challenged with non-challenged cells (at least 5 replicates). While this is imperfect, we believe this is the best compromise given the limited number of donors available.

2. The authors quantified IFN and ISG mRNA levels at one time point only (8 hours). This is not sufficient to conclude that AMs exposed to SARS-CoV-2 do not induce an immune response.

This is not true. In figure 2, the SARS-CoV-2 challenge is 8 hours while the IFN treatment is 20 hours (see figure 2D), and in figure 3, it is reverse (see figure 3A). We have clarified this in the text. However, we did an additional experiment where we measured SARS-CoV-2 RNA at 2, 8 and 24 hours, but as the infection is not productive. What we observe is a slow decline in SARS-CoV-2 RNA over time as shown below. However, we decided not to include this data in the manuscript and left it to the discretion of the reviewer.

3. The susceptibility of tissue macrophages to SARS-CoV-2 and their capacity to produce de novo infectious viral particles remain controversial and should be discussed.

Please refer to our response to R1.

4. At 8 hours post-infection neither IFNλ nor IFNα decrease the RNA levels of SEV, IAV and SarsCov2. These results suggest that no viral replication does not occur in AMs at this early time point. Infections should be performed at different MOI and viral RNA should be measured at different time points to assess whether AMs are productively infected by SARS-Cov-2 and whether SARS-CoV-2 is sensitive to IFNs in these cells.

Our purpose was not to establish a productive infection of the AMs but rather to test their ability to detect viruses in the environment. Please refer to our response to R1 for further details.

5. A previous study demonstrated that in lung tissues, SARS-CoV-2 infection induces a weak IFN response compared to SARS-CoV-1, and that AMs contain SARS-CoV-2 RNA (Chu et al., CID,

2020). The present resulst are thus in large part confirmatory, since the authors do not investigate the potential mechanisms by which SARS-CoV-2 avoids sensing by PPRs.

We do not agree that our results are simply confirmatory in relation to the Chu *et al*. paper. In that paper, the authors used lung biopsies where AMs are a minority population. Thus, any signal from AMs would be quite diluted, and therefore no conclusion was drawn about the role of AMs in the Chu *et al*. paper. As for the second part of the comment, she/he is quite right. We did not investigate the mechanism whereby SARS-CoV-2 evades immune detection by AMs, and unfortunately this is beyond the scope of this study. However, we speculate that the SARS-CoV-2 RNA is modified in a way that renders it difficult to detect. This is now discussed more extensively in a revised version of our discussion.

6. In the quantification of cell-associated SARS-CoV-2 RNA, the limit of detection of the PCR should be added to the graph.

The detection limit for SARS-CoV-2 RNA is $2x10^{-6}$ relative to *GAPDH*. This has been added to the figures as requested by the reviewer. The detection limits for SeV and IAV RNA have furthermore been added to figure 1 as well.

7. Sentences are too long. Many repetitions, unnecessary comments and grammatical errors make the reading difficult.

We apologies. We have carefully revised the manuscript with the help of a professional proofreader available through our university.

1. Lee, B.L. and Barton, G.M. (2014) Trafficking of endosomal Toll-like receptors. *Trends Cell Biol*, **24**, 360-369.
2. Davidson, S., McCabe, T.M., Crotta, S., Gad, H.H., Hessel, E.M., Beinke, S., Hartmann, R. and Wack, A. (2016) IFNlambda is a potent anti-influenza therapeutic without the inflammatory side effects of IFNalpha treatment. *EMBO molecular medicine*, **8**, 1099-1112.
3. Kopf, M., Schneider, C. and Nobs, S.P. (2015) The development and function of lung-resident macrophages and dendritic cells. *Nat Immunol*, **16**, 36-44.

2nd Revision - Editorial Decision 24th Sep 2020

Dear Dr. Hartmann,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, all referees have remaining concerns or suggestions to improve the study, we ask you to address in a final revised manuscript. I also added referee cross-comments below, with suggestions how to address remaining concerns. Please provide a detailed point-by-point response addressing these remaining points and the cross-comments.

Moreover, I have these editorial requests:

- Please add up to five keywords to the title page.

- Please remove the section called 'Summary' from the manuscript. This could be used as synopsis blurb (if shortened). See below.

- Please deposit all primary datasets produced in this study (i.e. the mass spec data) in an appropriate public database and list the accession numbers and database in a formal "Data Availability" section (placed after Materials & Methods).
See also: http://embor.embopress.org/authorguide#datadeposition

- In the author contributions it seems that Soren Skaarup is listed as SHK. This should be SK. Please check.

- We have recently changed our reference format. Please make sure the final manuscript file is formatted accordingly:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Please include the diagram shown in the Supplementary Figure into one of the main figures. I think there is enough space, and the data are important.

- Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 40 words).
- two to three bullet points highlighting the key findings of your study (2 lines).
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this manuscript the authors conclude that alveolar macrophages are unable to sense SARS-CoV-2 infection and thus do not mount an IFN response. I remain unconvinced of the core conclusions of the manuscript for the following reasons:

1. It is still not clear that macrophages in this manuscript were infected by SARS-CoV-2. Is ACE2 present on these cells? A search for ACE2 within the manuscript shows a primer set is listed, but data were not included with these primers.

2. The authors suggest in their reviewer response that virus is simply endocytosed by macrophages and likely degraded. They contend that a small amount of endocytosed virus should be expected to activate TLR signaling. However, this is not supported by the decades of experimentation on virus detection by myeloid cells. Non-replicative RNA virus (e.g., mild UV-inactivation) has been used as a control throughout the literature to demonstrate that RNA virus replication is generally necessary for induction of IFN responses in myeloid cells. This is true for both influenza virus and SeV, i.e., that replication-defective virus does not activate a significant IFN response in myeloid cells. Thus, it should not be expected that an RNA virus that is not able to infect myeloid cells would induce any type of IFN response even if endocytosed.

3. The SARS-CoV-2 stock used was not shown to be infectious in any context nor was any information provided as to its propagation or titering. It is becoming widely known that propagation of SARS-CoV-2 in Vero cells selectively promotes evolution of Spike protein furin site mutants that overtake virus stocks and drastically limit infection of most human cell types. Was the virus tested by the authors to ensure it is replicative in other cell types, such as human epithelial cells? Were the virus stocks sequenced to ensure WT virus lacking furin site mutations was used?

Referee #2:

My comments did not have much impact on the revision as in one case I had apparently misunderstood the intention (and interpretation) of the experiment and in the other the authors argue that the suggested experiment is technically not feasible.

Thus the major change with regard to the original manuscript is an answer to referee 1, the demonstration that infection of alveolar macrophages is not productive. To my opinion this is a significant new aspect. I am somewhat confused now by the interpretation of the finding that the IFN system is not engaged: On the one hand the authors argue this may result from modification, i.e. lack of recognition of the RNA, on the other hand they write:
'The lack of a functional interaction between SARS-CoV-2 and the IFN system suggests that AMs are not productively infected, and that the SARS-CoV-2 RNA detected within the AMs after challenge with the virus, is the result of an abortive infection'

To my opinion lack of recognition and lack of productive infection are two separate aspects that may not have anything to do with each other mechanistically.

In conclusion this paper is in some aspects preliminary, but its relevance is strongly increased by the

SARS-CoV2 pandemic.

Referee #3:

Dalskov et al. report that alveolar macrophages (BAL) fail to detect SARS-CoV-2 and to generate an IFN-type I response in vitro. The mechanisms involved are not investigated, and results are somewhat negative. The number of independent BAL samples tested remains quite low. The manuscript has been improved and the authors have addressed some of my comments.

Cross comments:

Referee #1:
At minimum, I would ask that they provide details on the growth and characterization of their CoV-2 stock, including whether or not the stock was sequenced. I also agree with reviewer 2 that lack of virus recognition and lack of productive infection are two separate aspects that may not have anything to do with each other mechanistically. They should be more careful with their language and describe more precisely what their data suggest is happening in their cells. If one considers virus entry into the cytosol to be required to consider a cell infected, then I would not consider virus phagocytosis of macrophages and degradation to be an abortive infection or non-productive "infection."

-
Referee #2:
Referee #1's objection that the lack of an IFN response might be directly linked to its inability to replicate is a possibility worth considering, but the fact that AM are no niche for virus replication or stimulation of an IFN response remains, and thus the main conclusion derived from the data. The critique that the virus or its RNA might never enter the cells is another matter. The detection of viral RNA would then reflect extracellular virus? If the authors can convincingly rebut this point, the third criticism (viral genetic drift) would also be taken care of.

-
Referee #3:
I agree with the other reviewers, there is not a big enthusiasm regarding this work, but the manuscript has improved. In the context of the epidemics it may be interesting to publish it rapidly. Regarding reviewer #1, I would suggest modifying the text to take into account points 1 and 2. It is not necessary to sequence the virus, but the authors should indicate whether their viral stock is infectious in other cell types and indicate the titers if not done yet.

Referee #1:

In this manuscript the authors conclude that alveolar macrophages are unable to sense SARS-CoV-2 infection and thus do not mount an IFN response. I remain unconvinced of the core conclusions of the manuscript for the following reasons:

1. It is still not clear that macrophages in this manuscript were infected by SARS-CoV-2. Is ACE2 present on these cells? A search for ACE2 within the manuscript shows a primer set is listed, but data were not included with these primers.

We did qPCR to measure ACE2 expression in the AMs. However, we were unable to detect expression of ACE2 mRNA within the AMs. In contrast, we detected a robust expression of ACE2 in primary lung epithelial cells. We have now described this in the manuscript and the data are shown in Figure 5A.

2. The authors suggest in their reviewer response that virus is simply endocytosed by macrophages and likely degraded. They contend that a small amount of endocytosed virus should be expected to activate TLR signaling. However, this is not supported by the decades of experimentation on virus detection by myeloid cells. Non-replicative RNA virus (e.g., mild UV-inactivation) has been used as a control throughout the literature to demonstrate that RNA virus replication is generally necessary for induction of IFN responses in myeloid cells. This is true for both influenza virus and SeV, i.e., that replication-defective virus does not activate a significant IFN response in myeloid cells. Thus, it should not be expected that an RNA virus that is not able to infect myeloid cells would induce any type of IFN response even if endocytosed.

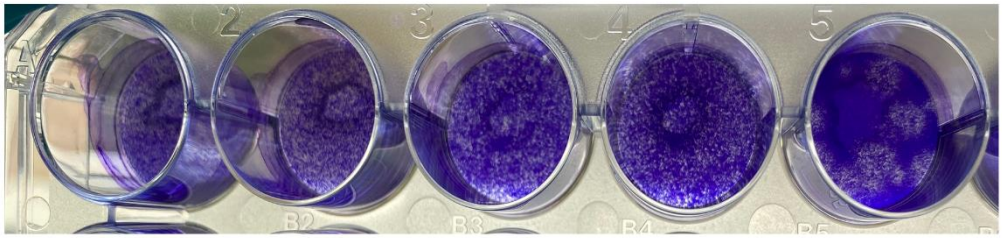
Agree that this is currently actively debated within the scientific literature and we hope that we have made it clear that we do not see our study as a final, but part of an ongoing debate. In our favor speaks that the Poly(I:C) control clearly demonstrates the ability of the AMs to recognize dsRNA in the absence of replication. Furthermore, we detect very low levels of SeV RNA, yet this virus results in a robust IFN response.

Replication deficient viruses should have been used in this study to prove our point, but a combination of lack of time, regulation which prohibit working with other viruses while working with SARS-CoV-2 and a limited supply of AMs, resulted in our choice of using Poly (I:C) as our positive control.

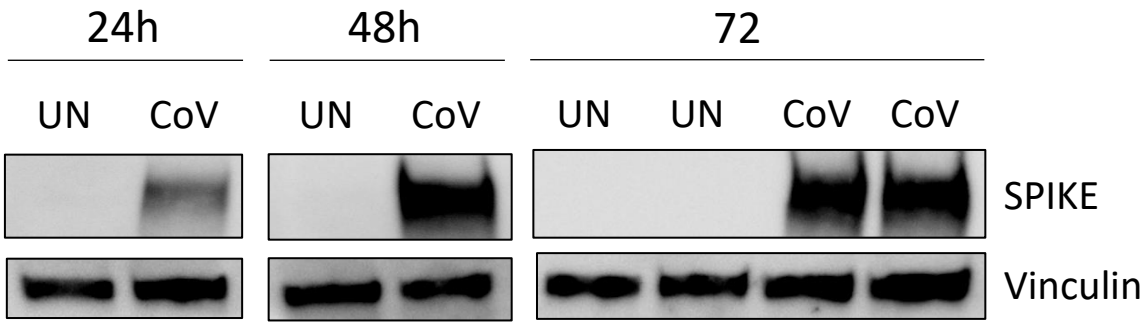
3. The SARS-CoV-2 stock used was not shown to be infectious in any context nor was any information provided as to its propagation or titering. It is becoming widely known that propagation of SARS-CoV-2 in Vero cells selectively promotes evolution of Spike protein furin site mutants that overtake virus stocks and drastically limit infection of most human cell types. Was the virus tested by the authors to ensure it is replicative in other cell types, such as human epithelial cells? Were the virus stocks sequenced to ensure WT virus lacking furin site mutations was used?

We apologies for not being clearer on this point. We amplified the SARS-CoV-2 virus on primary human airway epithelial (HAE) cells, not Vero and then we titrated the SARS-CoV-2 virus stock used in this study on Vero cells. The MOI derived from this titration is what was used in the study. Please see the figure below showing the plaques formed on Vero cells. This is now also mentioned in the Materials and Methods section

10-fold serial dilution of supernatants from SARS-CoV2 infected HAE cells transferred to Vero cells. Gel overlay placed 2hours post infection.



Infection with this virus also led to a clear and time dependent production of spike protein in HAE cell cultures (see below). However, we did not sequence the virus.



Referee #2

My comments did not have much impact on the revision as in one case I had apparently misunderstood the intention (and interpretation) of the experiment and in the other the authors argue that the suggested experiment is technically not feasible.

Thus the major change with regard to the original manuscript is an answer to referee 1, the demonstration that infection of alveolar macrophages is not productive. To my opinion this is a significant new aspect. I am somewhat confused now by the interpretation of the finding that the IFN system is not engaged: On the one hand the authors argue this may result from modification, i.e. lack of recognition of the RNA, on the

other hand they write:
'The lack of a functional interaction between SARS-CoV-2 and the IFN system suggests that AMs are not productively infected, and that the SARS-CoV-2 RNA detected within the AMs after challenge with the virus, is the result of an abortive infection'

To my opinion lack of recognition and lack of productive infection are two separate aspects that may not have anything to do with each other mechanistically.

We agree with the reviewer and have modified the manuscript by deleting the latter part of the sentence quoted above.

In conclusion this paper is in some aspects preliminary, but its relevance is strongly increased by the SARS-CoV2 pandemic.

Referee #3

Dalskov et al. report that alveolar macrophages (BAL) fail to detect SARS-CoV-2 and to generate an IFN-type I response in vitro. The mechanisms involved are not investigated, and results are somewhat negative. The number of independent BAL samples tested remains quite low.

We regret not being able to perform more duplicates, but limited availability of AMs (they are sourced directly from patients) and limited time, in particular limited time available in the SARS2-CoV-19 certified laboratory has limited the volume of experiments.

The manuscript has been improved and the authors have addressed some of my comments.

3rd Revision - Editorial Decision 30th Sep 2020

Dr. Rune Hartmann
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Department of Molecular Biology and Genetics
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Aarhus 8000
Denmark

Dear Dr. Hartmann,

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

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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 iustified
- ➔ 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?	Based on the small derivation between different donors (see figure 1, control conditions) a sample size of 5-9 donors were chosen for the different experimental set ups.
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?	It was ensured that samples were collected from non-infectious donors. No samples were excluded from the analysis based on this criteria.
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 from each donor were treated under as many different conditions as the sample size allowed us to, ensuring allocation of different donors under the same test conditions and preventing donor bias.
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.	NA
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?	Yes, we have performed an one-way-ANOVA test to perform our statistical analysis
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, the data meets the assumptions of the test. The necessary requirements were evaluated before any tests were performed.
Is there an estimate of variation within each group of data?	No

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Is the variance similar between the groups that are being statistically compared?	NA

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).	anti-Spike (GeneTex, #GTX632604), anti-IFIT1 (Cell Signalling, #14769), anti-RSAD2 (Cell Signalling, #13996), anti-Vinculin (Cell Signalling, #18799), peroxidase-conjugated F(ab)2 donkey anti-mouse IgG (Jackson, #715-036-150), and peroxidase-conjugated F(ab)2 donkey anti-rabbit IgG (Jackson, #711-036-152)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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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 project was performed in accordance with local and national Danish laws and regulations. The project has been approved by the Health Research Ethics Committee of Region Midtjylland, Denmark (Journal nr. 1-10-72-103-20).
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.	Consent as obtained from all participating donors.
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	NA
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

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