THE EMBO JOURNAL

SARS-CoV-2 sensing by RIG-I and MDA5 links epithelial infection to macrophage inflammation

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

1st Editorial Decision 23rd Feb 2021

Dear Greg,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by the three referees and their comments are provided below.

As you can see from the comments below the response is a bit mixed. Referee #3 is not convinced that we get enough new insight for consideration here while referees #1 and 2 are more supportive. I do find that the study makes an important contribution and I would like to invite a revision.

Can we discuss how to best address the referees' comments as some of the comments are very well taken, but properly also beyond the scope of a revised version. We can do so via video or email whatever works best for you.

This is also a very competitive field with PMID 33440148 being published shortly before your manuscript was submitted to us and PMID 33514628 published during the review process. Please make sure to discuss both papers

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further with you

best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).

- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Please see out instructions to authors
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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 24th May 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

Thorne and colleagues analyzed sensing of SARS-CoV-2 infection by the interferon system and its consequences. They show that the virus replicates efficiently in Calu-3 cells and induces expression of IFNs, proinflammatory cytokines and ISGs. However, expression of IFN/ISGs is delayed as compared to viral replication and, in keeping with this finding, addition of type I IFN prior but to but not at the same time as virus or later reduces viral replication. Further, it is show that efficient induction of gene expression and cell killing but not viral replication depends on the activity of the viral RdRp and on RIG-I/MDA5. In addition, induction of ISG expression is shown to depend on an intact JAK/STAT pathway while IL-6 expression is blocked by inhibitors targeting NFkB. Finally, it is demonstrated that supernatants from infected Calu-3 cells promote macrophage activation and that this process is enhanced upon pre-activation of macrophages. The manuscript is well written and the results are of interest to the field, although some of them are not novel (PMID 33440148, 33514628). The following points should be addressed.

Major

While Calu-3 cells are a good model for infection of human lung epithelial cells, key findings - efficient IFN/ISG induction but no viral inhibition and proinflammatory phenotype of macrophages - should be confirmed with primary cells/organoids.

It is unclear whether the induction of the proinflammatory phenotype in macrophages was solely due to soluble mediators in the culture supernatants or whether abortive infection played a role. This should be investigated and PMID: 33277988 should be discussed.

Related to the point above, it would be important to know which factors in the Calu-3 cell supernatants were responsible for promoting the proinflammatory phenotype in macrophages. Can initial insights be obtained using antibodies directed against IFNs?

Minor

Can the authors exclude that IFN/ISGs have been induced in Caco-2 cells upon SARS-CoV-2 infection but with faster kinetics as compared to Calu-3? Maybe at 72 h post infection expression had already decreased to background levels.

Referee #2:

Thorne et al have explored the mechanism of innate sensing of SARS-CoV2 in epithelial cells, and also the potential link to pathological inflammation driven by immune cells in the infected lungs. The authors propose that the virus is sensed by RIG-like receptors in epithelial, to promote an innate immune response, which does not contribute to antiviral defense, but rather amplifies inflammation in macrophages. The results presented a based on well designed, performed, and presented data, and the conclusions are generally supported by the data. However, the physiological relevance of the data is difficult, because a relatively simple setup with epithelial cell lines and transfer of supernatants to macrophages is used. This part should be strengthened.

1. The proposed model that epithelial-cell-derived innate responses amplify pathological macrophage activities in the COVID19 lung should be tested. Systems that could be used include primary co-culture models, organoids, K18-hACE2 mice, existing deposited scRNA data.

2. The inhibitor data in Figure 4 should be complemented with test of the a TBK1 inhibitor.

3. The data in Fig 6 is somewhat disconnected from the rest of the paper, and doe not "dig a later deeper" after the interesting data in Fig 5. The authors should do a serious effort to identify some of the factors in the virus-containing conditioned media that trigger macrophage activation.

4. Could also be interested to have the macrophage characterized with respect to whether the virus-containing conditioned media drive them in a M1, M2 or non-conventional activity direction.

5. In Fig. 2M, the autors show that IL6 responses are dependent on RIG-I and MAVS but not MDA5. To test whether the NF-kB response is dependent on RIG-I exclusively, the authors should test for other NFkB-induced genes, and also test for NF-kB nuclear translocation (as in Fig 2A-B) in cells treated with the different RNAi.

6. For some of the presented data n=2. This should be improved to reach at least n=3 for all data presented.

Referee #3:

In this manuscript, the authors show replication of SARS-CoV-2 in human lung epithelial cells is

largely unaffected by the innate immune response. Through a careful timecourse analysis they provide evidence that the antiviral immune response is delayed until significant viral replication has already occurred, suggesting that there may be a threshold of replication that must occur for this virus to be robustly detected. They further confirm these findings with elegant single cell imaging analysis of NF-kB, IRF3, and inflammatory mRNAs in infected and bystander cells. Additionally, they pinpoint the induction of type I IFN and inflammation to the RIG-I/MDA5 and MAVs signaling pathway. They then show that JAK/STAT and NF-kB signaling inhibitors diminish inflammatory responses without major effects of SARS-CoV-2 replication. Lastly, they show that macrophages are not susceptible to infection by SARS-CoV-2, but can be activated by media from infected epithelial cells.

While the experiments are extensive, well controlled, and use relevant cells, the findings and concepts are not novel. It is unfortunate that so much high-quality effort has gone into rediscovering things that are already well established (e.g., epithelial cell infection by CoV-2 and resulting inflammation, RLR detection of CoV-2, involvement of NF-kB and JAK/STATs in inflammatory and IFN responses to CoV-2, lack of macrophage infection by CoV-2, macrophage activation by cytokines). Given the lack of new insights provided by the work, I cannot recommend publication in a high impact journal.

Specific concerns/questions:

1. Knockdown efficiencies should be shown in the main text figures.
2. Figure 6, viral gene expression graphs are confusion given that it is stated that macrophages don't become infected.
3. The title of the paper/major conclusion is based on in vitro experiments with conditioned media or cytokines added to cultured macrophages. The same results with the limited panel of readouts would have been obtained with conditioned media from virtually any virus infection. SARS-CoV-2-specific insights or in vivo-relevant insights cannot be effectively gleaned from this work.

Referee #1:

Thorne and colleagues analyzed sensing of SARS-CoV-2 infection by the interferon system and its consequences. They show that the virus replicates efficiently in Calu-3 cells and induces expression of IFNs, proinflammatory cytokines and ISGs. However, expression of IFN/ISGs is delayed as compared to viral replication and, in keeping with this finding, addition of type I IFN prior but to but not at the same time as virus or later reduces viral replication. Further, it is show that efficient induction of gene expression and cell killing but not viral replication depends on the activity of the viral RdRp and on RIG-I/MDA5. In addition, induction of ISG expression is shown to depend on an intact JAK/STAT pathway while IL-6 expression is blocked by inhibitors targeting NFkB. Finally, it is demonstrated that supernatants from infected Calu-3 cells promote macrophage activation and that this process is enhanced upon pre-activation of macrophages. The manuscript is well written and the results are of interest to the field, although some of them are not novel (PMID 33440148, 33514628). The following points should be addressed.

Major

1. While Calu-3 cells are a good model for infection of human lung epithelial cells, key findings - efficient IFN/ISG induction but no viral inhibition and proinflammatory phenotype of macrophages - should be confirmed with primary cells/organoids.

This is a good suggestion for future work. Our key finding is that inflammation is directly downstream of RNA sensing, shown by suppressing pretty much all inflammatory responses measured by depleting RNA sensing machinery. We can't do this in primary airway cells because RNAi doesn't really work well. However, we already know that primary airway cells make inflammatory markers on CoV2 infection and we now cite this work in the following text in the discussion.

"Our data show that RNA sensing in infected Calu-3 cells creates a pro-inflammatory milieu capable of activating primary macrophages. Crucially the combined profile of pro-inflammatory mediators in this system mirrors that observed in COVID-19 *in vivo* (Bost et al., 2020;Laing et al., 2020;Szabo et al., 2020) and primary airway epithelial cells (Fiege et al., 2021)".

2. It is unclear whether the induction of the proinflammatory phenotype in macrophages was solely due to soluble mediators in the culture supernatants or whether abortive infection played a role. This should be investigated and PMID: 33277988 should be discussed.

This is an excellent point and we have included the following text in the discussion and cited the paper,

"A recent study suggested that sensing of abortive SARS-CoV-2 infection of macrophages may contribute to their activation (Zheng et al., 2021). Our data do not rule out a role for detection of abortive replication. But they suggest that inflammatory mediators produced from infected cells, perhaps with responses particularly skewed towards pro-inflammatory pathways after viral manipulation, are key to macrophage activation. Notably, exposure of macrophages to infected Caco2 supernatant, which contains virus but not significant levels

of cytokine or IFN, did not strongly activate the macrophages. Indeed, our results show that it is important to distinguish between the effects of virus and the effects of cytokines in the viral prep. Here, we have achieved this by using Caco2 cells to produce virus without significant inflammatory cytokines and interferons and Calu3 to produce virus with a corresponding inflammatory secretome."

3. Related to the point above, it would be important to know which factors in the Calu-3 cell supernatants were responsible for promoting the proinflammatory phenotype in macrophages. Can initial insights be obtained using antibodies directed against IFNs?

We agree that this is key. We have addressed this experimentally by treated the MDM with ruxolitinib and anti-IFNAR antibody to inhibit IFN signalling during exposure to the Calu-3 conditioned media. We have measured the same genes and macrophage activation markers, and the data suggest that IFNb produced by the Calu-3 cells contributes to MDM activation and the gene expression we have measured. We have included this data in Figure 5, given its importance in providing additional mechanistic insight, with controls in SupFig6, and modified the methods, results (pg 8-9) and discussion (pg12) text to reference it. This experiment has also addressed a similar point raised by reviewer 2 below.

Minor

4. Can the authors exclude that IFN/ISGs have been induced in Caco-2 cells upon SARS-CoV-2 infection but with faster kinetics as compared to Calu-3? Maybe at 72 h post infection expression had already decreased to background levels.

This is a really important point. In fact, we did this experiment right at the beginning of the study to make sure this wasn't a problem, but didn't include the data. We have now included these data showing no induction of gene expression at 24h post infection in Caco2s even at MOIs 500x higher than we use to prepare the viral stocks. These data are now included in supplementary figure 1 to clarify this point.

Referee #2:

Thorne et al have explored the mechanism of innate sensing of SARS-CoV2 in epithelial cells, and also the potential link to pathological inflammation driven by immune cells in the infected lungs. The authors propose that the virus is sensed by RIG-like receptors in epithelial, to promote an innate immune response, which does not contribute to antiviral defense, but rather amplifies inflammation in macrophages. The results presented a based on well designed, performed, and presented data, and the conclusions are generally supported by the data. However, the physiological relevance of the data is difficult, because a relatively simple setup with epithelial cell lines and transfer of supernatants to macrophages is used. This part should be strengthened.

1. The proposed model that epithelial-cell-derived innate responses amplify pathological

macrophage activities in the COVID19 lung should be tested. Systems that could be used include primary co-culture models, organoids, K18-hACE2 mice, existing deposited scRNA data.

Thank you for this suggestion, as explained above in answer to reviewer 1 we agree that in the long term these alternative systems will provide valuable insight to support our data. However, given primary airway experiments have been done by others, we are now citing these data in our discussion as follows

"Our data show that RNA sensing in infected Calu-3 cells creates a pro-inflammatory milieu capable of activating primary macrophages. Crucially the combined profile of pro-inflammatory mediators in this system mirrors that observed in COVID-19 *in vivo* (Bost et al., 2020;Laing et al., 2020;Szabo et al., 2020) and primary airway epithelial cells and primary airway epithelial cells (Fiege et al., 2021)".

2. The inhibitor data in Figure 4 should be complemented with test of the a TBK1 inhibitor.
Thank you for this suggestion, we agree that there many inhibitors that we could have included such as a TBK1 inhibitor. As we have mapped the RNA sensors involved (Figure 3) and confirmed that inhibiting NFkB activation directly downstream of their activation as well as the second wave of IFN signalling dampens the innate and inflammatory response (Figure 4), we do not feel that including a TBK1 inhibitor will add significant mechanistic insight.

3. The data in Fig 6 is somewhat disconnected from the rest of the paper, and doe not "dig a later deeper" after the interesting data in Fig 5. The authors should do a serious effort to identify some of the factors in the virus-containing conditioned media that trigger macrophage activation.

This point is similar to that of reviewer one (point 3), and we have now addressed this point by demonstrating that IFN is a key driver in MDM activation. We inhibit IFN signalling in MDM with Ruxolitinib, or anti-IFNAR antibody, during exposure to infected Calu3 supernatant and show that activation markers and inflammatory gene expression are suppressed. We interpret these new data, presented in Fig 5k-O, as showing that IFN is a key feature of the proinflammatory response of infected cells. Failure to suppress all gene expression suggest further cytokines/chemokines also have a role.

4. Could also be interested to have the macrophage characterized with respect to whether the virus-containing conditioned media drive them in a M1, M2 or non-conventional activity direction.

This is a reasonable suggestion but we feel it is beyond the scope of the current manuscript.

5. In Fig. 2M, the autors show that IL6 responses are dependent on RIG-I and MAVS but not MDA5. To test whether the NF-kB response is dependent on RIG-I exclusively, the authors should test for other NFkB-induced genes, and also test for NF-kB nuclear translocation (as in Fig 2A-B) in cells treated with the different RNAi.

Thank you for this suggestion, we agree that this is interesting and we have measured TNF expression as another NFkB-dependent gene. We found the same result as for IL-6, that is was not affected by MDA5 depletion. These new data are included in figure 3 with the following text

“Concordantly, depletion of cytoplasmic RNA sensors RIG-I or MDA-5 also reduced inflammatory gene expression after infection (Figures 3J-N). This suggested sensing of multiple RNA-species given the different specificities of RIG-I and MDA5 (Hornung et al., 2006;Kato et al., 2006;Rehwinkel et al., 2010;Wu et al., 2013). Intriguingly, unlike RIG-I, MDA5 was not required for induction of NF-□B-sensitive genes IL-6 or TNF, consistent with differences in downstream consequences of RIG-I and MDA5 activation (Figure 3N and O) (Brisse and Ly, 2019).”

6. For some of the presented data n=2. This should be improved to reach at least n=3 for all data presented.

This comment refers to the growth curves presented in figures 1 and 2. We have now performed this experiment again, clarifying in the figure legends that the data represent three independent experiments. We show two of the independent repeats between the main figure and supplementary figures. Please note that each growth curve derives from replicate wells and 3 or 4 MOIs to confirm our observations of replication timing versus innate response. This approach is more thorough than typical, as single MOI growth curve experiments are often presented and we are very confident that our findings are robust and representative. In the single cell analyses, we present data on thousands of cells for each repeat and condition, making it a very reliable dataset. Furthermore, the replication and innate response data is also repeated in experiments throughout the paper, the timing for which were based on the growth curve data, which are all consistent and confirm the reliability of the phenotypes described.

Referee #3:

In this manuscript, the authors show replication of SARS-CoV-2 in human lung epithelial cells is largely unaffected by the innate immune response. Through a careful timecourse analysis they provide evidence that the antiviral immune response is delayed until significant viral replication has already occurred, suggesting that there may be a threshold of replication that must occur for this virus to be robustly detected. They further confirm these findings with elegant single cell imaging analysis of NF-kB, IRF3, and inflammatory mRNAs in infected and bystander cells. Additionally, they pinpoint the induction of type I IFN and inflammation to the RIG-I/MDA5 and MAVs signaling pathway. They then show that JAK/STAT and NF-kB signaling inhibitors diminish inflammatory responses without major effects of SARS-CoV-2 replication. Lastly, they show that macrophages are not susceptible to infection by SARS-CoV-2, but can be activated by media from infected epithelial cells.

While the experiments are extensive, well controlled, and use relevant cells, the findings and

concepts are not novel. It is unfortunate that so much high-quality effort has gone into rediscovering things that are already well established (e.g., epithelial cell infection by CoV-2 and resulting inflammation, RLR detection of CoV-2, involvement of NF-kB and JAK/STATs in inflammatory and IFN responses to CoV-2, lack of macrophage infection by CoV-2, macrophage activation by cytokines). Given the lack of new insights provided by the work, I cannot recommend publication in a high impact journal.

Specific concerns/questions:

1. Knockdown efficiencies should be shown in the main text figures.

The knockdown efficiencies are already shown in the main figure 3.

2. Figure 6, viral gene expression graphs are confusion given that it is stated that macrophages don't become infected.

We are sorry if this was not clear. Viral gene PCR is performed to show that we see the same amount of viral genome, ie dose of virus, when we're comparing host gene expression in LPS treated and untreated target cells. This is important, because it allows us to be sure the difference in response is due to a difference in the behaviour of the target cells rather than a different dose of virus being used, or induction of viral replication by LPS. We have clarified this point with the following text,

"Importantly, LPS treatment of MDM, before or after virus challenge, did not alter SARS-CoV-2 permissivity of MDM, evidenced by no change in the level of detectable viral E gene in MDM supernatants (Figure 6B and J). Thus, the changes in MDM gene induction by virus after LPS treatment are due to differences in the MDM response to virus and not due to a difference in the amount of viral genome added or induction of viral gene expression."

3*. The title of the paper/major conclusion is based on in vitro experiments with conditioned media or cytokines added to cultured macrophages. The same results with the limited panel of readouts would have been obtained with conditioned media from virtually any virus infection. SARS-CoV-2-specific insights or in vivo-relevant insights cannot be effectively gleaned from this work.*

We do not think that any viral infection would lead to the same cocktail of soluble mediators produced in epithelial cells. This is certainly not true for the other viruses we work on, eg HIV and HCV. We make the case in the discussion that this response is a result of the unique combination of evasion and antagonism strategies employed by SARS-CoV-2 to counter the innate response and permit human to human transmission. A particular manipulation of IRF3, leaving NF-kB pathways intact to drive inflammation, is reflected in our transcription factor translocation data in Fig 2. We observed significantly higher activation of NFkB than IRF3, suggesting SARS-CoV-2 more effectively inhibits IRF3 activation, consistent with the current literature. To clarify these points we have added the following text to the results and discussion sections.

Results

“IRF3 activation was also virus dose dependent but did not maximise until 72 hpi, later than NF-κB (Figures 2C and D, Figure S3), and we observed a more modest shift in IRF3 nuclear intensity compared to NF-κB throughout infection. These data are consistent with the requirement of a threshold of viral RNA replication to induce transcription factor translocation and innate immune activation, and suggest that SARS-CoV-2 may antagonise IRF3 activation to a greater extent than NF-κB.”

Discussion

“Consistent with the literature, our data indicate that SARS-CoV-2 more effectively antagonises IRF3 activation and nuclear translocation than NF-κB. Indeed, it is possible that the innate immune response and the secreted signals produced by infected cells are dysregulated by viral manipulation, and that this imbalanced response contributes to disease particularly in the context of underlying inflammatory pathology (Blanco-Melo et al., 2020;Giamarellos-Bourboulis et al., 2020;Lucas et al., 2020).”

1st Revision - Editorial Decision 18th May 2021

Dear Greg,

Thanks for submitting your manuscript to The EMBO Journal.

Your study has now been seen by referee #1 and as you can see from the comments below, the referee appreciates the introduced changes and support publication here.

I am therefore very happy to let you know that we will accept the manuscript for publication here. There are just a few remaining editorial issues to resolve before I can send you the formal accept letter

- we are missing 3-5 keywords

- we need a Data Availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements

- We need a conflict of interest statement

- please double check the reference format. Author list should be capped after 10 authors

- we are missing an ORCID ID for Lucy - please see guide to authors

- I think that the grant numbers are missing from the manuscript text

- Callouts to Fig 2G and 3R are missing. There is a callout to supplemental table 1, but no table

- "Methods" needs correcting to Materials and Methods

- Fig EV3 only has one panel so does not need the 'A' label.

- "Extended view Figure Legends" should be corrected to Expanded view Figure Legends.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data edited manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

That should be all - let me know if you have any further questions

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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

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

IMPORTANT: When you send the revision we will require
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- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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.

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 16th Aug 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have adequately addressed the points raised by this reviewer. The demonstration that antibodies targeting components of the IFN system blocked expression of macrophage activation markers provided additional mechanistic insights and strengthened the study.

Please find below a point-by-point response addressing all of the changes required for publication.

- we are missing 3-5 keywords
Keywords have been added to the start of the manuscript:
' SARS-CoV-2, inflammation, RNA sensing, epithelial, macrophage'

- **we need a Data Availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements**
This section and sentence has been added to the manuscript.

- We need a conflict of interest statement
The following text has been added to the manuscript after the materials and methods section:

'Conflict of Interest Statement

The authors have no conflicts of interest to declare.'

- **please double check the reference format. Author list should be capped after 10 authors**
The format has been updated to EMBO J and author lists are now capped at 10 authors.

- **we are missing an ORCID ID for Lucy - please see guide to authors**
I have added this, apologies I thought I had already!

- **I think that the grant numbers are missing from the manuscript text**

The grant numbers have been added to the manuscript:

- Callouts to Fig 2G and 3R are missing. There is a callout to supplemental table 1, but no table

We have modified the text to reference Fig2G: *'IFIT1 transcripts (a prototypic ISG) measured by FISH also demonstrated rapid induction in N-positive cells with increased signal from at 6 hpi (Figure 2G and H).'*

We have modified the text to reference Fig3R and correct the figure 3 call outs: *'Depletion of RNA sensing adaptor MAVS abolished SARS-CoV-2-induced IL-6, CXCL10, IFNβ and IFIT2 gene expression (Figures 3 J-O), consistent with RNA sensing being a key driver of SARS-CoV-2-induced innate immune activation. Concordantly, depletion of cytoplasmic RNA sensors RIG-I or MDA-5 also reduced inflammatory gene expression after infection (Figures 3J-O). This suggested sensing of multiple RNA-species given the different specificities of RIG-I and MDA5 (Hornung et al, 2006; Kato et al, 2006; Rehwinkel et al, 2010; Wu et al, 2013). Intriguingly, unlike RIG-I, MDA5 was not required for induction of NF-κB-sensitive genes IL-6 or TNF, consistent with differences in downstream consequences of RIG-I and MDA5 activation (Figure 3N and O) (Brisse & Ly, 2019). Abrogating SARS-CoV-2 sensing via MDA5 and MAVS depletion also reduced cell death, suggesting cell death is mediated by the host*

response rather than direct virus-induced damage (Figure 3P). Notably, sensor depletion did not strongly increase viral RNA levels (Figure 3Q), or the amount of released infectious virus (Figure 3R), confirming that innate immune activation via RNA sensing did not potently inhibit viral replication.'

We have now provided file for Supplementary Tables 1A-G.

- **"Methods" needs correcting to Materials and Methods**
This has been changed in the manuscript to 'Materials and Methods'.

- Fig EV3 only has one panel so does not need the 'A' label.
The 'A' label has been removed and a modified figure provided.

- "Extended view Figure Legends" should be corrected to Expanded view Figure Legends.
This has been changed in the manuscript to Expanded view Figure Legends.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data edited manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

We have checked and corrected each of the comments in the data edited manuscript file which all related to the figure legends. We have modified these in the updated, newly uploaded manuscript.

- **We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.**

We have included the summary at the start of the manuscript:

'SARS-CoV-2 induces a robust, delayed innate immune response in airway epithelial cells, driven by activation of RNA sensors, which propagates inflammation through macrophage activation.'

The following bullet points have been added to the start of the manuscript and are provided below:

'Key points:

- *SARS-CoV-2 activates RNA sensors and consequent inflammatory responses in lung epithelial cells.*
- *Epithelial RNA sensing responses drive pro-inflammatory macrophage activation.*
- *Exogenous inflammatory stimuli exacerbate responses to SARS-CoV-2 in both eplithelial cells and macrophages.*
- *Immunomodulators inhibit RNA sensing responses and consequent macrophage inflammation.'*

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

We would like to use Figure7 as the summary figure for the synopsis if this is possible?

the grey bars in Fig 5E and H look identical - did you use the same sample or is it a mistake. If you used the same samples please juts make sure that this is clearly indicated in the figure legend.

Yes we performed the inhibitors in FigE (Rux) and Fig5H (TPCA1) side-by-side so the mocks are the same, we have made this clear in the figure legend:

'The inhibitors in (E) and (H) were tested side-by-side with the same mock condition.'

The Mock lane in Fig EV3 is the same within panels for NF-Kbeta and within the IRF3 panels. I presume that is how the experiment was done, but please state this clearly in the figure legend.

We have modified the figure legend to state that the conditions were all performed side-by-side with the same mock:

'All MOIs and mock were performed side-by-side and the mock is the same within panels for NF-Kbeta and within the IRF3 panels.'

2nd Revision - Editorial Decision 28th May 2021

Dear Greg,

Thank you for submitting your manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Greg J Towers
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2021-107826

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In macrophage experiments, a minimum sample size of six independent experiments using cells derived from separate donors was used to give 90% power in order for a two sided test to detect >two-fold differences between two groups with an estimated standard deviation of 0.5.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes

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

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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-tun

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

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

http://biomodels.net/

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

Is there an estimate of variation within each group of data?	yes see point one above
Is the variance similar between the groups that are being statistically compared?	yes, see point 1 above

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 following text is included. "All antibody sources are cited with sample identifiers and all antibodies were validated for their specific use by manufacturers or by previously published work as cited."
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells sources are described in text as is negative mycoplasma status

* 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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	UCL/UCLH NHS Trust Human Research Ethics Committee
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.	This text is included
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No human data included
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
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).	N/A
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).	N/A
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.	N/A

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.	No