EMBO reports

Immune-Epithelial Cell Cross-Talk Enhances Antiviral Responsiveness to SARS-CoV-2 in Children

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In the manuscript entitled "Enhanced Airway Epithelial Response to SARS-CoV-2 Infection in Children is Critically Tuned by the Cross-Talk Between Immune and Epithelial Cells", the authors report a mechanism how airway epithelium of children is in a primed state to more efficiently defense SARS-CoV-2 infection. They interestingly found that the expression level of MDA5 and RIG-I are essential for this primed stage. In details, they discovered that enhanced immune-epithelial cell interactions through cytokines play a major role in the upregulation of these two RNA sensors, where IRF1 may be the most important regulator. They fully used their single cell sequencing data and established a relative convincing in vitro model to validate their hypothesis. This story is quite attractive to me since they explained the behind mechanisms for a clinical phenomenon. However, there are still some issues need to be addressed.

Major comments:

1. A549 is a cancer cell line and cannot support SARS-CoV-2 replication. The authors reply on it too much. Some important experiments need to be performed in primary airway epithelial cells.
2. MDA5 and RIG-I protein levels need to be detected in some important experiments such as Figure 4D, Figure 5A, Figure 5B, Figure 6B.

Minor comments:

1. In figure 1B, the authors claimed that "As expected, pre-treatment alone did not trigger notable IFN-β transcription". But in my mind, pretreatment elevated IFN- β mRNA at least 5 folds compared to mock group (from 10-2.5 to 10-1.8). The authors need to make the statement more accurate.
2. In figure 2A, the authors used IFNARKO cells. This should be mentioned in the manuscript.
3. The statement of Figure 2C should be more accurate, "This was also confirmed at the downstream level of STAT2 activation, at which phosphorylation was still observed upon infection of RIG-I or MDA5 single KOs with SARS-CoV- 2, but was fully diminished only in the double KO cells (Fig 2C)." IRF3 phosphorylation is not downstream of STAT2. More downstream event can be shown as

STAT1/STAT2/IRF9 nuclear translocation or ISGs transcription. Also, total STAT2 levels should be shown here.
4. It is quite surprising that none of IRNAR, RIG-I, MDA5 affected SARS-CoV-2 replication in untreated cells.
5. If we compare the mRNA induction of MDA5 and RIG-I between Figure 4D and Figure 6B, they are not in the same amplitude. There are 10 folds induction in Figure 4D while 100-300 folds in Figure 6B.

2. Significance:

Significance (Required)

This is an interesting study with clear data. They fully used their single cell sequencing data and established a relative convincing in vitro model to validate their hypothesis. They uncovered an important mechanism why airway epithelium of children is in a primed state and more resistant to SARS-CoV-2 infection.

The limitation here is that they do not have in vivo model to support their conclusions.

Nevertheless, this manuscript is still able to answer a clinical question. Why children are much more resistant to SARS-CoV-2 infections compared to adults? It has good clinical significance.
I believe that a broad audience will be interested in this story.

I study virus and cancer induced metabolic reprogramming, virus and host interaction and innate immune regulation, and mass spectrometry (metabolomics and proteomics).

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at Web of Science

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Web of Science Reviewer Recognition

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In the manuscript entitled "Enhanced airway epithelial response to SARS-CoV-2 infection in children is critically tuned by the crosstalk between immune and epithelial cells" Gonçalves Magalhães et al address the molecular interactions that result in greater antiviral responses to coronavirus infections in children versus adults. The authors have re-analyzed previously reported scRNA data from the nasal passages of healthy donors and found heightened pro-inflammatory responses in the nasal passages of children relative to the signatures in adults. Thus, the authors posit that this could result in priming of epithelial cells subsequently boosting antiviral responses to SARS-CoV-2 infection in a RIG-I and MDA5 dependent manner. Beyond the expected antiviral protection conferred by type I IFNs, the authors demonstrate type II IFN and TNF can also promote antiviral priming. Although epithelial and immune cell crosstalk has been previously demonstrated, this study proposes an age-dependent differences in the magnitude of this crosstalk. Indeed, bacterial stimulation of PBMCs derived from children resulted in greater cytokine [production and A549 priming. The article was well written, the data presented is compelling, and appropriately controlled. Importantly, the authors appropriately acknowledge the study limitations. The mechanisms that govern the increased inflammatory responsiveness of tissues derived from children remain to be addressed.

Major comments:

- Although the authors acknowledge this limitation, do primary epithelial cells respond to priming? Is there an age difference in viral detection and/or the response to

priming?
- Optional: Does the deletion of IRF3 phenocopy MAVS deficiency in the context of type I IFN priming and blockade of IFN replication (Fig 2D)? Does priming induced increases in IRF1 and IRF7 and is this sufficient to overcome the loss of IRF3 (PMID: 25520509)?

Minor comments:

- Are the differences observed in MDA5 and RIG-I expression after PBMC stimulation across A549 IFNR KO cells significant?
- Figure 2C could be strengthened by the addition of total IRF3 and STAT2 immunoblot.
- Figure 3C, include tSTAT2 control.
- There is one typo on line 610. Change OSA2 to OAS2.
- Please expand the discussion to include relevant work on IFN and TNF-mediated antiviral priming (PMID: 16537619) and epithelial-immune cell crosstalk (PMID: 36563691).

2. Significance:

Significance (Required)

Advance: This study expands upon previous work by the authors that described that children have higher expression of RLRs and in epithelial and immune cells relative to adults. The current work, provides an incremental but important advance to the previous study by demonstrating that PBMC-mediated priming of epithelial cells (A549). However, it remains to be addressed whether epithelial cells from children have increased capacity to detect SARS-CoV-2 infection or respond to priming.

Audience: This work is of interest to pediatric clinicians, virologists, immunologists, cell biologists, bioinformaticians.

Reviewer expertise: viral innate immunity; IFN regulation

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at Web of Science Reviewer Recognition Service (formerly Publons); note that the content of your review will not be visible on Web of Science.

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Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary: This study by Magalhaes et al sheds light on the molecular underpinnings of the relative resistance of children to severe COVID-19. The authors found that priming of epithelial cells by resident immune cells to express tonic levels PRR receptors MDA-5 and RIG-I predisposes the epithelial cells for a faster and more robust onset of IFN-beta production upon SARS-CoV-2 infection. The study uses a combination of in vitro and ex vivo models, as well as mining of scRNA-Seq datasets from clinical specimens.

Major comments: The claims and conclusions are supported by the data and therefore no new experiments are needed.

Optional

1. The use of primary cells (i.e. human airway epithelial cultures cross talking to immune cells) would make this study more compelling, although I assume that the major findings would be recapitulated in such models.
2. It is not clear how the use of Yersinia enterocolitica to trigger activation of PBMC

is relevant to this story. Using different (commensal) pathogens to achieve PBMC activation may yield different and more physiologically relevant results.
3. The manuscripts would greatly benefit from improved structure and focus, particularly in the Abstract, Introduction and Results sections. The text is very dense, and makes it difficult for the reader to follow the flow and to distinguish important from less important information.
Particularly, the introduction starts very broadly introducing COVID-19, which I think we are by now all familiar with. Directly starting with the burning question why kids get less sick with SARS-CoV-2 would capture the readers' attention better. Figure 1 a is beautiful for a review but much too dense to help the reader as a graphical abstract. In the results section, for each experiment, leading with clearly stating the rationale of the specific question, the gap in knowledge and why the gap is there, then followed by the results, then summarizing the impact of said results, would make this a much more enjoyable read and help the reader evaluate the novelty and impact better, particularly for Figures 1, 2, and 3 (but also all others). The interaction wheel graphs (Figure 4. are amazing, but are not properly explained in the text (do I read this right that in adults, all the crosstalk is basically performed by proliferating T-cells?). In all, these scientific writing issues sell an otherwise beautiful story short.

Referees cross-commenting

I agree with reviewers 1 and 2 that the use of primary cells would significantly elevate the story. However, I think this should be "optional", as I do not think it would change the findings.

2. Significance:

Significance (Required)

General assessment:

The main strength of the study are its topic and clearly relevant question: why do kids rarely get severe COVID-19? The main novelty is the answer to this question, that immune cell-epithelial crosstalk in children elevates the tonic expression of MDA5 and RIG-I via the IRF1 axis, leading to faster onset of IFN production and signaling upon SARS-CoV-2 challenge, which ultimately mounts an antiviral response detrimental to robust SARS-CoV-2 replication. The study uses an innovative combination of in vivo and ex vivo experiments and analysis of clinical specimens.

The significant advance of this study to the field is clear to this reviewer, although it could be much better stated in the manuscript, as described at length above.

The study is of great interest to the field of immunology and virology, and also has clinical and translational impact with respect to risk assessment for severe COVID-19 per age group, as well as epidemiological considerations for infection control.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

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Web of Science Reviewer Recognition

Yes

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In the manuscript entitled "Enhanced Airway Epithelial Response to SARS-CoV-2 Infection in Children is Critically Tuned by the Cross-Talk Between Immune and Epithelial Cells", the authors report a mechanism how airway epithelium of children is in a primed state to more efficiently defense SARS-CoV-2 infection. They interestingly found that the expression level of MDA5 and RIG-I are essential for this primed stage. In details, they discovered that enhanced immune-epithelial cell interactions through cytokines play a major role in the upregulation of these two RNA sensors, where IRF1 may be the most important regulator. They fully used their single cell sequencing data and established a relative convincing in vitro model to validate their hypothesis. This story is quite attractive to me since they explained the behind mechanisms for a clinical phenomenon. However, there are still some issues need to be addressed.

We are glad to read the reviewer finds our study interesting, particularly for its merit to address the molecular mechanisms of a widely appreciated clinical phenomenon.

Please find our point-by-point reply below. Note that we refer to the line numbering in the revised manuscript with tracked changes– unfortunately, Microsoft Word uses a different line numbering in the “track changes” mode.

Major comments:
1. A549 is a cancer cell line and cannot support SARS-CoV-2 replication. The authors reply on it too much. Some important experiments need to be performed in primary airway epithelial cells.

We agree with the reviewer that A549 is not the most physiological model system for respiratory epithelium. Nonetheless, it is a well-established and widely used model for respiratory virus infections incl. SARS-CoV-2 (upon transduction with hACE2, characterized e.g. in Xie et al., doi: 10.1038/s41467-020-19055-7). We, too, used them for their easy genetic amenability with a large panel of KOs already available in the lab, and a huge body of data and experience with regards to their innate antiviral system, also in comparison to other cell lines (please see e.g. our manuscript Burkart et al. on bioRxiv, doi: 10.1101/2022.08.05.502818, including a comparison of A549 to HepG2; additional data from primary human foreskin fibroblasts are included in the current revision of that manuscript and available for this reviewer upon request).

Important to our presented study, A549 cells exhibit rather low basal expression levels of MDA5 and also RIG-I, rendering them similar to “adult” nasal epithelial cells in this regard (compare scRNA-Seq in Fig. 3B). Therefore, they are a good model to mimic the “adult state” of epithelial cells, that can be transformed to the “adolescent state” (elevated RLR expression, robust IFN-β response upon SARS-CoV-2 infection) purely by defined cytokine treatment. Other model cell lines / systems may have different basal RLR expression and, hence, may less clearly mimic this adult *vs* adolescent transition. In Supplement Fig. 1B we demonstrate the concept of priming in an independent lung epithelial cell line, Calu-3. These cells indeed have substantial basal expression particularly of MDA5 (see also e.g. Rebendenne et al., doi: 10.1128/JVI.02415-20), and, hence, exhibit a smaller effect window upon priming (compare Suppl. Fig. 1B to Fig. 1C). Please also see our response to reviewer #2’s first major comment.

We fully align with the reviewer’s assessment that further investigating the concept of priming by immune cells / PBMCs in a more complex (and more authentic) culture model such as primary human airway epithelium in air-liquid-interface cultures would be very exciting. This should then involve primary cells of adolescent and adult donors, in order to rule out an age-effect on side of the epithelial cells themselves. However, given the complexity of such a set-up, we consider this beyond the scope of the present study and very strongly prefer to do this as part of a follow-up study. Please also see reviewer #2’s first major comment and our response, as well as the statement of reviewer #3, who considers validation in primary cells optional as it is unlikely to reveal fundamentally different effects.

2. MDA5 and RIG-I protein levels need to be detected in some important experiments such as Figure 4D, Figure 5A, Figure 5B, Figure 6B.

The reviewer is correct; this is useful additional data that should have been included in the initial submission already. We now supply western blots for Figures 4D (in Suppl. Fig. S3D), 5A and 5B (in Suppl. Fig. S4A and S4B); due to limited material, we regrettably could not perform protein analyses for the experiments in Fig. 6B. However, the overall concordance of mRNA and protein data in Figs. 4 and 5, clearly suggests a high agreement of mRNA and protein level effects upon priming.

Minor comments:
1. In figure 1B, the authors claimed that "As expected, pre-treatment alone did not trigger notable IFN-β transcription". But in my mind, pretreatment elevated IFN- β mRNA at least 5 folds compared to mock group (from 10-2.5 to 10-1.8). The authors need to make the statement more accurate.

This is a attentive observation by the reviewer– in fact, we should have be more careful in our statement. Please note, however, that basal IFN-β expression is virtually zero (literally zero in RNA-Seq of A549, not shown), so a factor of 5-fold is not meaningful and reliable in qRT-PCR (ct around 35). Still, this modest influence was visible in both repeats, therefore we rephrased our statement (lines 465ff).

2. In figure 2A, the authors used IFNARKO cells. This should be mentioned in the manuscript.

We now explicitly state that we used IFNAR-KO cells as a negative control incapable of priming through IFN-β (line 528f).

3. The statement of Figure 2C should be more accurate, "This was also confirmed at the downstream level of STAT2 activation, at which phosphorylation was still observed upon infection of RIG-I or MDA5 single KOs with SARS-CoV- 2, but was fully diminished only in the double KO cells (Fig 2C)." IRF3 phosphorylation is not downstream of STAT2. More downstream event can be shown as STAT1/STAT2/IRF9 nuclear translocation or ISGs transcription. Also, total STAT2 levels should be shown here.

We apologize for the misunderstanding, which we now try to avoid by a more careful phrasing (lines 540ff). Indeed, we intended to refer to STAT2-phosphorylation being downstream of IRF3 activation and of IFN production (it occurs downstream of IFNAR engagement). We do not think assessing ISGF3 formation or nuclear translocation would be more informative than STAT2 phosphorylation in order to demonstrate absence or presence of IFN signaling. Following the advice of the reviewer, we have updated Fig. 2C (as well as Fig. 3A) to display total STAT2 levels. Note of the increase in overall STAT2 levels as result of IFN-β priming (STAT2 is an ISG).

4. It is quite surprising that none of IRNAR, RIG-I, MDA5 affected SARS-CoV-2 replication in untreated cells.

Yes, we agree this is a surprising feature for an RNA virus. However, it has been described early in the pandemic, that SARS-CoV-2 is special in its very low induction of type I and III IFNs (Blanco-Melo et al., 2020, doi: 10.1016/j.cell.2020.04.026), explaining its apparent indifference towards the RLR/IFN system. Others, too, observed this independence on RLRs and IFN signaling, e.g. compare Figure 9 of Rebendenne et al., doi: 10.1128/jvi.02415-20.

5. If we compare the mRNA induction of MDA5 and RIG-I between Figure 4D and Figure 6B, they are not in the same amplitude. There are 10 folds induction in Figure 4D while 100-300 folds in Figure 6B.

Again, a very attentive observation by the reviewer! Please note, that the PBMCs in Figure 6 were obtained from a well-controlled cohort study into COVID-19 using very stringent procedures, whereas the PBMCs in Figure 4 were bulk processed, aliquoted and frozen down for general purpose use following a slightly different protocol. Comparing quantitatively the cytokine responses of PBMCs from different sources or at different sites is generally challenging and not advisable.

Reviewer #1 (Significance (Required)):

This is an interesting study with clear data. They fully used their single cell sequencing data and established a relative convincing in vitro model to validate their hypothesis. They uncovered an important mechanism why airway epithelium of children is in a primed state and more resistant to SARS-CoV-2 infection. The limitation here is that they do not have in vivo model to support their conclusions.
Nevertheless, this manuscript is still able to answer a clinical question. Why children are much more resistant to SARS-CoV-2 infections compared to adults? It has good clinical significance.
I believe that a broad audience will be interested in this story.

We gratefully acknowledge the reviewer's highly favorable overall evaluation of our study and appreciation for its addressing a clinical matter of broad relevance. We fully agree that an *in vivo* model, e.g. a Syrian hamster or a suitable murine model, would be fascinating (this limitation is also explicitly stated and discussed in lines 1123ff). However, we figure this issue is beyond the scope of the current manuscript and intend to address it in a follow-up study. Nonetheless, we believe the striking agreement of our cell culture findings with our human single cell data (arguable the finest "*in vivo model*") is a pretty solid (albeit circumstantial) corroboration of our proposed model of epithelial cell priming.

I study virus and cancer induced metabolic reprogramming, virus and host interaction and innate immune regulation, and mass spectrometry (metabolomics and proteomics).

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In the manuscript entitled "Enhanced airway epithelial response to SARS-CoV-2 infection in children is critically tuned by the crosstalk between immune and epithelial cells" Gonçalves Magalhães et al address the molecular interactions that result in greater antiviral responses to coronavirus infections in children versus adults. The authors have re-analyzed previously reported scRNA data from the nasal passages of healthy donors and found heightened pro-inflammatory responses in the nasal passages of children relative to the signatures in adults. Thus, the authors posit that this could result in priming of epithelial cells subsequently boosting antiviral responses to SARS-CoV-2 infection in a RIG-I and MDA5 dependent manner. Beyond the expected antiviral protection conferred by type I IFNs, the authors demonstrate type II IFN and TNF can also promote antiviral priming. Although epithelial and immune cell crosstalk has been previously demonstrated, this study proposes an age-dependent differences in the magnitude of this crosstalk. Indeed, bacterial stimulation of PBMCs derived from children resulted in greater cytokine [production and A549 priming. The article was well written, the data presented is compelling, and appropriately controlled. Importantly, the authors appropriately acknowledge the study limitations. The mechanisms that govern the increased inflammatory responsiveness of tissues derived from children remain to be addressed.

We very much thank the reviewer for their so positive assessment of our work and manuscript.

Major comments:

- Although the authors acknowledge this limitation, do primary epithelial cells respond to priming? Is there an age difference in viral detection and/or the response to priming?

The reviewer raises an important question: what is the relative contribution of immune cells *vs* epithelial cells in the observed “priming” phenotype and, particularly, how does it vary with age? In our study, we focus on the effects of immune cells and their cytokines on epithelial cells, as we found a clear basis for this in our primary human samples (scRNASeq data). To characterize these effects *in vitro,* we rely on one single, well-controlled and -characterized model of epithelial cells, namely A549 (note the validation experiment in Calu-3 cells in Supplement Figure 1B).

Nonetheless, the reviewer is absolutely right in that the epithelial cells themselves might be intrinsically distinct between adults and children/adolescents. However, this cannot simply be addressed *in vivo* (e.g. by scRNASeq of nasal swabs), as the respiratory mucosa always contains immune cells and cytokines. Hence, one would have to prepare *ex vivo* primary airway epithelium cultures from a substantial number of adolescent and adult donors in order to compare cellular states and responses in the absence of any immune-epithelial cross-talk. We deem this to be well beyond the scope of the present study and would be happy to be able tackling this challenge in a dedicated follow-up study.

- Optional: Does the deletion of IRF3 phenocopy MAVS deficiency in the context of type I IFN priming and blockade of IFN replication (Fig 2D)? Does priming induced increases in IRF1 and IRF7 and is this sufficient to overcome the loss of IRF3 (PMID: 25520509)?

Indeed, we had performed the experiments using IRF3-KO cells, but excluded them from the manuscript to focus more on the different sensor pathways– Fig. 2A are all adaptors, 2B are the sensors; note, however, the western blot in Fig. 2C, in which IRF3-KO cells are included and show no STAT2 phosphorylation upon SARS-CoV-2 infection even when primed. This argues against sufficient IRF7 (IRF1 is not stimulated by RLR signaling) upon priming, at least in our system. This has also been confirmed at the IFNB1 mRNA level, which the reviewer can appreciate below. Unlike the non-targeting control cells, infection of IRF3KO cells with SARS-CoV-2 after IFN-b priming does not trigger an immune response (compare the IFNB1 mRNA levels in the blue bars). If requested, we could include this information also in the supplements of our manuscript, however, we are concerned that it might appear slightly out of place.

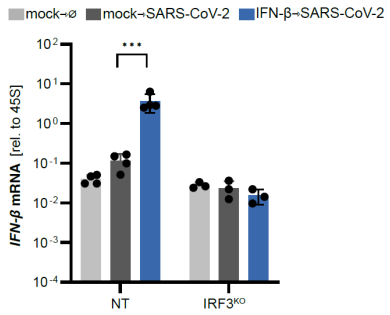

Figure 1: A549$^{ACE2/TMPRSS2}$ cells harboring the CRISPR/Cas9-mediated functional KO of IRF3 were mock-treated or primed with IFN-β followed by infection with SARS-CoV-2 (MOI of 0.1). At 24 hours post infection, IFN-β (**IFNB1**) gene expression was determined by qRT-PCR. Values were normalized to 45S rRNA levels. Bar represent mean ±SD of three indepedent experiments (individual experiments shown as dots). NT: non-targeting CRIPSPR gRNA used as wildtype control.

Minor comments:

- Are the differences observed in MDA5 and RIG-I expression after PBMC stimulation across A549 IFNR KO cells significant?

We assume the reviewer refers to Figure 4D. As indicated by the asterisks, the differences in RLR expression upon PBMC supernatant treatment are indeed statistically significant even in IFNR-TKO (unpaired, one-tailed Student's t-test), albeit, we agree, small. Nonetheless, they were reproducible across all biologically independent repetitions. Moreover, please note that other sources / preparations of PBMCs can lead to significantly stronger overall RLR induction, as seen in Figure 6B (see also our response to reviewer #1's minor comment 5), in which case also the contribution of non-IFN cytokines could become clearer. Unfortunately, we had to limit our experiments in Fig. 6 and have not included KO A549 cells.

- Figure 2C could be strengthened by the addition of total IRF3 and STAT2 immunoblot.

We thank the reviewer for this suggestion and have now included total IRF3 and STAT2. As expected, STAT2 but not IRF3 levels increase with IFN pre-treatment, as STAT2 is an ISG. See also our comment to reviewer #1's minor comment 3.

- Figure 3C, include tSTAT2 control.

We now include total STAT2 in Fig. 3C.

- There is one typo on line 610. Change OSA2 to OAS2.

We corrected the typo, thank you for spotting!

- Please expand the discussion to include relevant work on IFN and TNF-mediated antiviral priming (PMID: 16537619) and epithelial-immune cell crosstalk (PMID: 36563691).

These are two great suggestions for literature to discuss. We now cite the antiviral priming of RIG-I expression in line 1077f and discuss the epithelial-immune cross-talk with regards to inflammasome priming by myeloid cells in lines 1117ff.

Reviewer #2 (Significance (Required)):

Advance: This study expands upon previous work by the authors that described that children have higher expression of RLRs and in epithelial and immune cells relative to adults. The current work, provides an incremental but important advance to the previous study by demonstrating that PBMC-mediated priming of epithelial cells (A549). However, it remains to be addressed whether epithelial cells from children have increased capacity to detect SARS-CoV-2 infection or respond to priming.

We greatly appreciate the reviewer's assessment that our manuscript meaningfully expands our previous study and represents an important advance. We want to underscore that we not only confirm and corroborate those previous findings, but that we are able to delineate a molecular mechanism *in vitro* that is compatible with and can explain the *in vivo* findings.

Audience: This work is of interest to pediatric clinicians, virologists, immunologists, cell biologists, bioinformaticians.

Reviewer expertise: viral innate immunity; IFN regulation

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary: This study by Magalhaes et al sheds light on the molecular underpinnings of the relative resistance of children to severe COVID-19. The authors found that priming of epithelial cells by resident immune cells to express tonic levels PRR receptors MDA-5 and RIG-I predisposes the epithelial cells for a faster and more robust onset of IFN-beta production upon SARS-CoV-2 infection. The study uses a combination of in vitro and ex vivo models, as well as mining of scRNA-Seq datasets from clinical specimens.

Major comments: The claims and conclusions are supported by the data and therefore no new experiments are needed.

We very much appreciate the reviewer's summary and assessment of our manuscript and are delighted that they fully agree with our experimental setup and interpretation of data.

OPTIONAL:

(1) The use of primary cells (i.e. human airway epithelial cultures cross talking to immune cells) would make this study more compelling, although I assume that the major findings would be recapitulated in such models.

We fully agree with the reviewer that a more sophisticated cellular model system would be desirable to further corroborate our model. The principal effect of epithelial cell priming through IFN / cytokines is highly plausible to be independent of the exact cell line or system– please also note our validation experiment in Calu-3 in Supplement Figure 1B. More interesting would be two questions: 1) are epithelial cells from adolescent donors different from those of adult donors with regard to PRR expression already in the absence of cytokine priming, and/or are they differently susceptible to priming? See also major comment 1 of reviewer #2 above. And 2) do immune cells of children / adolescents really interact with primary airway epithelial cells (of adolescents vs adults?) through homeostatic production of the cytokines identified in our study, type I IFN, type II IFN, and TNF? We are truly intrigued by both questions, however, both require an elaborate experimental setup involving tedious establishing of techniques and conditions (e.g. to isolate and culture primary airway epithelial cells of adolescent and adult donors), and even then these would only be better but still limited models (e.g. peripheral blood immune cells are likely inadequate surrogates of tissue (mucosa) resident immune cells). We, hence, consider such experiments well beyond the scope of the present study, but a fantastic subject for a follow-up study by us or others.

(2) It is not clear how the use of Yersinia enterocolitica to trigger activation of PBMC is relevant to this story. Using different (commensal) pathogens to achieve PBMC activation may yield different and more physiologically relevant results.

The reviewer is of course absolutely right in that Yersinia is not at all common in the respiratory tract; we sincerely hope our manuscript doesn't suggest any different. In fact, we only used *Y.e.* as a model gram-negative bacterium to activate immune cells (explicitly stated as such e.g. in line 712f), which was readily available to and is routinely used by our collaborator Stella Autenrieth. We initially contemplated treatment with purified LPS, but then preferred the more physiological mode using live bacteria instead.

The reviewer again raises a really intriguing point– does the possibly more frequent microbial encounter of mucosal immune cells in children / adolescents contribute to the higher cytokine production observed in our scRNASeq data (briefly mentioned in our discussion, line 1065ff and 1088ff), and if so, are certain genera of bacteria more efficient in stimulating this effect. Again, we are afraid this goes well beyond the scope of the present manuscript and would require much deeper microbiological expertise.

(3) The manuscripts would greatly benefit from improved structure and focus, particularly in the Abstract, Introduction and Results sections. The text is very dense, and makes it difficult for the reader to follow the flow and to distinguish important from less important information.
Particularly, the introduction starts very broadly introducing COVID-19, which I think we are by now all familiar with. Directly starting with the burning question why kids get less sick with SARS-CoV-2 would capture the readers' attention better. Figure 1 a is beautiful for a review but much too dense to help the reader as a graphical abstract.

In the results section, for each experiment, leading with clearly stating the rationale of the specific question, the gap in knowledge and why the gap is there, then followed by the results, then summarizing the impact of said results, would make this a much more enjoyable read and help the reader evaluate the novelty and impact better, particularly for Figures 1, 2, and 3 (but also all others). The interaction wheel graphs (Figure 4) are amazing, but are not properly explained in the text (do I read this right that in adults, all the crosstalk is basically performed by proliferating T-cells?). In all, these scientific writing issues sell an otherwise beautiful story short.

After perusing and re-reading this comment by the reviewer, we acknowledge they have a valid point ;-) We have made every effort to accommodate the reviewer's suggestions by rewriting and revising the abstract and introduction, but also the results section. We specifically changed the abstract such that it immediately states the central question of why children suffer so much less from COVID-19 and make clear this is the overarching topic of our study. We have further restructured the introduction to make it more coherent and significantly more explicitly centered on this theme. We removed large parts of the "boring" introduction into COVID-19 and open the introduction, similar to the abstract, with a much clearer focus on the evident clinical phenomenon of substantially less morbidity and mortality among SARS2 infected children and adolescents. In the results part, we have paid particular attention to the opening and closing of individual paragraphs, and now more clearly state questions /hypothesis in the beginning and concisely sum up the experimental outcome as the reviewer has suggested (e.g. line 445f and 470f; 520ff and 544ff; etc.). We furthermore introduced a new paragraph heading on the immune-epithelial interaction analyses, describing the wheel graphs in Figure 4 much more in detail now (lines 646ff "Nasal mucosa of children…"). We sincerely hope our revision is now easier and more pleasant to read and follow– we feel it has improved significantly by these changes and thank the reviewer for directing us!

As for the scheme in Figure 1A, we decided to keep it in the manuscript. We agree it does not serve well as a graphical abstract, but we got the explicit request by several colleagues in the past, primarily from the clinics, to include such a scheme to serve as a go-to reference throughout the manuscript for readers not fully acquainted with the molecular topology of the central pathway(s).

Referees cross-commenting

I agree with reviewers 1 and 2 that the use of primary cells would significantly elevate the story. However, I think this should be "optional", as I do not think it would change the findings.

We thank the reviewer for making this point. As we stated in our individual responses to the three reviewers above, we feel that primary cell experiments that would significantly advance the overall message of your study would require a degree of complexity that is beyond the feasible scope of the current study.

Reviewer #3 (Significance (Required)):

General assessment:
The main strength of the study are its topic and clearly relevant question: why do kids rarely get severe COVID-19? The main novelty is the answer to this question, that immune cell-epithelial crosstalk in children elevates the tonic expression of MDA5 and RIG-I via the IRF1 axis, leading to faster onset of IFN production and signaling upon SARS-CoV-2 challenge, which ultimately mounts an antiviral response detrimental to robust SARS-CoV-2 replication. The study uses an innovative combination of in vivo and ex vivo experiments and analysis of clinical specimens.
The significant advance of this study to the field is clear to this reviewer, although it could be much better stated in the manuscript, as described at length above.
The study is of great interest to the field of immunology and virology, and also has clinical and translational impact with respect to risk assessment for severe COVID-19 per age group, as well as epidemiological considerations for infection control.

We are flattered by the so clear and positive assessment of our work, its novelty and merit by the reviewer. As stated above, we now tried to make the core message much clearer in the text, and to streamline the manuscript along this central message.

Dear Dr. Binder,

Thank you for the transfer of your revised manuscript to our editorial offices. I 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, the referees fully support publication of your study in EMBO reports.

The manuscript now needs formatting according to our journal style. Please 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 final revised manuscript, we will require:

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures 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. In this case, I think it will be possible to combine some of the Supplementary Figures to have in the end 5 EV figures. It is not necessary to have one EV figure related to one main figure. Just make sure that the panels are called out correctly.

For more details, please refer to our guide to authors:
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Please consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) 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

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

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

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

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

Data availability

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

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

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

Moreover, I have these editorial requests:

5) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator (in cc) will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

6) 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

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

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

8) Please also note our reference format:
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9) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

10) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions and remove the author contributions section from the manuscript text file. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

11) Please add up to 5 keywords to the manuscript and order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

12) Please provide a title with not more than 100 characters (including spaces).

13) Please move the funding information into the acknowledgements section. Please enter all the funding information also into our submission system and make sure this is complete and similar to the one mentioned in the manuscript text file.

14) Please provide an abstract with not more than 175 words.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- three to four short (!) one sentence bullet points highlighting the key findings of your study.
- a schematic summary figure (synopsis image) 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 a revised version of your manuscript when it is ready. Please let me know if you have questions or

comments regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The author's have addressed the major comments of raised by this reviewer and included important controls.

Referee #2:

The authors addressed all my concerns. I do not have further questions.

Referee #3:

My comments and concerns have been addressed adequately.

***
Rev_Com_number: RC-2023-02008
New_manu_number: EMBOR-2023-57912-T
Corr_author: Binder
Title: Enhanced Airway Epithelial Response to SARS-CoV-2 Infection in Children is Critically Tuned by the Cross-Talk Between Immune and Epithelial Cells

The authors have addressed all minor editorial requests.

Dear Dr. Binder,

Thank you for the submission of your revised manuscript to our editorial offices. Before I can proceed with formal acceptance, I have these few editorial requests I ask you to address in a final revised manuscript:

- Please provide the abstract written in present tense throughout.

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

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

Presently, information related to n is missing in the legends of figures 3b, 4f and EV3c.

In Fig. 1B, EV3D and EV3E n=2 (although in E three points are shown). Please show the data as separate datapoints without error bars and statistics.

In figures 4d-e; 5b, e-f there is a mismatch between the annotated p values in the figure legends and the annotated p values in the figure. Please check/correct.

- I would suggest combining all information about statistics in a Data information section for each figure legend, at least for figures 2, 4, 5, 6 and EV3 (the attached word file what I mean).

- Presently the figure legends are rather wordy and long and partly the text is redundant with the Results section and/or the Materials and Methods section. Please try to shorten and re-write these presenting the data actually shown in the panels. See also our guide to authors (section Figure Legends):

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- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Presently, the Helmholtz International Graduate School is only mentioned in the acknowledgements.

- There are callouts for Suppl. Fig. S4D and 6B in the manuscript. Please check and provide the correct callouts.

- Thanks for providing the source data. However, please also fill in and upload the source data checklist (attached again) and upload the source data ZIPped together in one folder per figure (not everything in one folder).

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 final revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

The authors have addressed all minor editorial requests.

Dr. Marco Binder
German Cancer Research Center (DKFZ)
Heidelberg D-69120
Germany

Dear Dr. Binder,

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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Achim Breiling
Senior Editor
EMBO Reports

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Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

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

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods section

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods section

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Material and Methods section
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Material and Methods section
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Mycoplasma testing is conducted as a routine procedure, details provided in Material and Methods

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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Material and Methods section

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	**Yes**	Sample sizes are given in legends, individual samples also represented in plots where feasible
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Material and Methods section and in the figure legends

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	In the figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	In the figure legends

Ethics

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

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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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