EMBO reports

Influenza A viruses limit NLRP3-NEK7-complex formation and pyroptosis in human macrophages

Ines Boal-Carvalho, Beryl Mazel-Sanchez, Filo Silva, Laure Garnier, Soner Yildiz, Joao Pereira Bonifacio Lopes, Chengyue Niu, Nathalia Williams, Patrice Francois, Nicolaus Schwerk, Jennifer Schöning, Julia Carlens, Dorothee Viemann, Stephanie Hugues, and Mirco Schmolke
DOI: 10.15252/embr.202051421

Corresponding author(s): Mirco Schmolke (mirco.schmolke@unige.ch)

Review Timeline:

Submission Date:	12th Mar 20
Editorial Decision:	2nd Apr 20
Revision Received:	1st Sep 20
Editorial Decision:	29th Sep 20
Revision Received:	6th Oct 20
Accepted:	9th Oct 20

Editor: Achim Breiling

Transaction Report:

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

1st Editorial Decision 2nd Apr 2020

Dear Prof. Schmolke,

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

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

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

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

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

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

When submitting your revised manuscript, we will require:

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

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

We would like to publish the paper as Scientific Report. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do that for your manuscript.

For more details please refer to our guide to authors:
http://embor.embopress.org/authorguide#manuscriptpreparation

For a short report, we would require 5 main figures. I think it should be no problem to fit the main data you have now into 5 main figures and up to 5 EV figures. 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 name these figures following 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. Finally, please update all the figure callouts in the manuscript text.

Any additional supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends, and should include page numbers. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx throughout the text and also label the figures according to this nomenclature.

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

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

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

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

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

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

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

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

Data availability

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

- RNA-Seq data: Gene Expression Omnibus GSE46843

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

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

Moreover, I have these editorial requests:

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

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

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

9) Please provide the abstract written in present tense.

10) Please update the format of the references. See:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The study by Boal-Carvalho et al investigate the role of influenza H5N1 and H3N2 PB1-F2 in IL1b production by human macrophages. They showed in vitro using THP-1 cell line and human monocyte-derived macrophages that PB1-F2-deficient viruses induced higher levels of IL1b. They found increased NLRP3 activation and interaction with NEK7, indicating an increased inflammasome activation in absence of PB1-F2. The role of avian influenza PB1-F2 on inflammasome activation in macrophages is controversial, as some studies showed that PB1-F2 induced IL1b secretion (Pinar et al JBC 2017, MacAuley et al Plos Pathogens 2013) and others have shown the opposite effect (Schmolke et al Plos Pathogens 2011). The current study provides new mechanisms of PB1-F2 function in macrophages and support an inhibitory effect of PB1-F2 on IL1b production. While the data is convincing, some experiments are required to reinforce the findings.

Major comments:
1) In general, the data lack kinetic to look at protein levels in supernatants (24h), gene expression (8h) or inflammasome activation (45min). At least one additional time point should be used to confirm the current results. Additionally, in figure 1D a dose response should be performed.
2) In figure 2, please provide some FACS plot of cell death analysis. The cytotoxicity should also be measured at 24 hours post-infection using LDH assay. The results indicate that macrophages infected with the PB1-F2 deficient strain undergo more apoptosis after infection, and it is likely that double positive cells at 24 hours reflect secondary necrosis. Thus, I am questioning whether the increased IL1b production is caused by an early onset and increased magnitude of apoptosis leading to secondary necrosis rather than pyroptosis? Is there any increased in caspase 3 activation with PB1-F2-deficient strain?
3) Figure 3, as mentioned before please provide kinetic with earlier time points. It has been previously shown in macrophages infected with VN H5N1, that IL1b expression was induced (Schmolke et al Plos Pathogens 2011). Additionally, PB1-F2 deficiency led to increased IL1beta expression. Similarly, H5N1 strains induce IL1b expression in human macrophage (Sakabe et al J Gen Virol 2011). Here expression of IL1b is downregulated following infection with both parental and PB1-F2-deficient strains. What is the reason for that?
4) In figure 4A, the authors need to show cleavage of GasD in the cell lysate and in figure 4F, the authors need to show cleaved casp1 in cell lysate and pro-/cleaved-IL1b in both cell lysate and sup.
5) The immunoprecipitations were performed in 293T cells. This experiment needs to be performed in THP-1 cells infected with parental and PB1-F2-deficient strains (H5N1 and H3N2) to reproduce physiological infection process.

Minor comments.
Figure 2C is not listed in the manuscript
There is no figure 5E
I only have 6 figures, 7 are listed in the main manuscript, 6 in the figure legends. Please fix.

Referee #2:

Boal-Carvalho et al. describes a key finding that PB1-F2 (an influenza A viral accessory peptide) limits the activation of NLRP3 by locking it in a conformation which leads to non-availability of LRR surface for interaction with NEK7, eventually leading to inhibition of NLRP3 Inflammasome formation. I am convinced that this is appropriate to publish in EMBO Reports.

The finding reported are significant advancement of our understanding of host response to

Influenza A infection. The authors support their findings with series of experiments leading to the molecular dissection of interaction of PB1-F2 to NLRP3 interaction surface and concluded that the NLRP3 interaction with PB1-F2 is mutually exclusive to NEK7.

The findings are convincing, however, totally opposite to what McAuley et al., 2013 AND Pinar et al. 2017 reported. Authors discussed very briefly about this in discussion, they should discuss more with specific arguments as to why they got totally different results from earlier publications.

The scope of these results is appropriate for this journal audience including host-pathogen interactions, molecular biology of inflammasome activation. Experimental design is substantial to conclude these findings.

Minor comments:
Figure 5. Panel E is missing
Line 30-33: Not clear, should rephrase it
Line 243: Wrong figure citation (Fig 5C). Missing the blot for ASC western blot or referring to Fig. 6C?
Line 247: Should be Fig 5D instead of Fig. 6C
Line 248: Instead of Fig. 6D should be Fig. 5F (Fig. 5E is missing)
Line 249: Instead of Fig. 2C should be Fig. 5C.
Line 266: Should be Fig. 6C and D instead of Fig. 7
Line 266: Fig. 6C doesn't show interaction or competition with NEK7 instead of association of ASC with PB1-F2. Should discuss accordingly
Line 273: Should be Fig. 6E and F instead of Fig. 7
Line 278: Fig. 6G instead of Fig. 7G

Referee #3:

Overall, the study is interesting however the results in figure 1 which aim to show that IAV lacking PB1-F2 induces greater IL-1b secretion are far from convincing raising considerable uncertainty. There is also a lack of in vivo studies to confirm the findings.

Abstract:
"Pyroptosis is a fulminant form of macrophage cell death, contributing to an antiviral state by release of proinflammatory cytokines." There is no solid evidence that proptosis is antiviral. Indeed it is proinflammatory. This sentence needs rephrasing.

Introduction:
IL-1β directly induces an antiviral transcription program in dendritic cells (Aarreberg et al., 2018)." This study utilised the unusual neutrotropic stain WSN to examine responses in bone-marrow derived DCs. The authors need to include these important points. IL-1b is a well described activator of NFkB.

"lung resident macrophages and inflammatory monocytes, are infected in an abortive fashion" Infection of macrophages is generally abortive but it has been shown that H5 viruses can replicate.

There is no mention of PB1-F2 in the introduction and what is already known eg is has been shown to activate NLRP3 (PMID 27913620 26667784 23737748). This is included in the results but should

be in the introduction. In addition there is no mention that only full length PB1-F2 expressed by certain IAV strains activates. PB1-F2 has also been shown to induce cell death. This important points have not been mentioned.

Results:
Figure 1D and E. The error bars between VN WT and delta F2 are overlapping in both experiments suggesting there is no significance difference in IL-1b release. A t test is not an appropriate statistical test when comparing 3 groups but rather a one way anova. There is particularly no difference between VN WT and delta F2 in Fig 1E as all but 1 replicate overlap. The conclusion that there is higher secretion of IL-1b in the delta F2 group is therefore incorrect.

It is not clear why PMBC macrophages and human BAL macrophages were infected with a different strain to the THP1 cells. This is confusing. Again the error bars in Fig 1J are overlapping and with only 3 replicates the data is not convincing. No responses in the human BAL macrophages were detected and it appears they weren't infected. The data should be repeated with the VN strain. If these strains don't infect macrophages then they may not be the appropriate cell type to study.

Figure 2 - studies have shown PB1-F2 induced cell death yet the authors show delta F2 virus induces more cell death. How do the authors explain this? Annexin V and LDH is not a readout of pyroptosis. GSDMD cleavage is the only real measure of pyroptosis. Not sure why this data is included.

Figure 3 - IL-1b secretion will induce NFKb/TNF/IL-6, pro-ilb mRNA upregulation. The experiments in figure 3 can not therefore determine if PB1-F2 provides signal 1 or signal 2. Previous studies have identified it provides signal 2 and not signal 1.

Figure 4 C - mock control is missing.
Figure 4F - WT virus did not induce caspase 1 cleavage yet IL-1b secretion and GSDMD cleavage was shown. How do the authors explain this result?

Figure 6 is incorrectly labelled in the text as figure 7.

EMBO Reports review for Boal-Carvalho et al

All answers are in italic.

Referee #1:

The study by Boal-Carvalho et al investigate the role of influenza H5N1 and H3N2 PB1-F2 in IL1b production by human macrophages. They showed in vitro using THP-1 cell line and human monocyte-derived macrophages that PB1-F2-deficient viruses induced higher levels of IL1b. They found increased NLRP3 activation and interaction with NEK7, indicating an increased inflammasome activation in absence of PB1-F2. The role of avian influenza PB1-F2 on inflammasome activation in macrophages is controversial, as some studies showed that PB1-F2 induced IL1b secretion (Pinar et al JBC 2017, MacAuley et al Plos Pathogens 2013) and others have shown the opposite effect (Schmolke et al Plos Pathogens 2011). The current study provides new mechanisms of PB1-F2 function in macrophages and support an inhibitory effect of PB1-F2 on IL1b production. While the data is convincing, some experiments are required to reinforce the findings.

We thank the reviewer for pointing out that our data are convincing and appreciate the comments to improve the manuscript.

Major comments:

1) In general, the data lack kinetic to look at protein levels in supernatants (24h), gene expression (8h) or inflammasome activation (45min (nigericin?)). At least one additional time point should be used to confirm the current results. Additionally, in figure 1D a dose response should be performed.

In order to address this valid point, we performed experiments at different time points and with lower infectios doses.
IL1beta secretion is also increased by VN delta F2 when applying 5MOI of virus (new Fig. EV1A).
IL1beta secretion is not yet increased by VN delta F2 after 12h (new Fig. EV1B). Since gasdermind D and caspase 1 cleavage was not observed ad this time point (new Fig. EV3A and B) we suspect that this time point was still to early.
Cytotoxicity was also increased for VN deltaF2 after 24h (new Fig. 2F)

2) In figure 2, please provide some FACS plot of cell death analysis. The cytotoxicity should also be measured at 24 hours post-infection using LDH assay. The results indicate that macrophages infected with the PB1-F2 deficient strain undergo more apoptosis after infection, and it is likely that double positive cells at 24 hours reflect secondary necrosis. Thus, I am questioning whether the increased IL1b production is caused by an early onset and increased magnitude of apoptosis leading to secondary necrosis rather than pyroptosis? Is there any increased in caspase 3 activation with PB1-F2-deficient strain?

LDH release also increased for VN deltaF2 after 24h (new Fig. 2F). Importantly, this difference in LDH release is abolished in Caspase 1-/- THP-1 both at 12h and 24h post infection (new Fig. EV3C).
Caspase 3 cleavage occurs after 24h with both VN Wt and VN delta F2. It appears that VN delta F2 does activate caspase 3 slightly more potent than VN Wt. Thus secondary effects by apoptosis might also play a role. This point is also discussed in line 287-296.
Representative FACS plots are presented in Figure EV2.

3) Figure 3, as mentioned before please provide kinetic with earlier time points. It has been previously shown in macrophages infected with VN H5N1, that IL1b expression was induced (Schmolke et al Plos Pathogens 2011).

We provide now data for 4h, 8h and 24h post infection (new Fig. EV2). The reviewer is right about the differences seen in BMDMs, however those were data obtained in mouse cells.

Additionally, PB1-F2 deficiency led to increased IL1beta expression. Similarly, H5N1 strains induce IL1b expression in human macrophage (Sakabe et al J Gen Virol 2011). Here expression of IL1b is downregulated following infection with both parental and PB1-F2-deficient strains. What is the reason for that?

Sakabe and colleagues used a lower MOI, which could result in more secondary effects by uninfected bystander cells. We might monitor with higher MOIs already host transcription shutoff in the infected macrophages as consequence of NS1 and PA-X action.

4) In figure 4A, the authors need to show cleavage of GasD in the cell lysate and in figure 4F, the authors need to show cleaved casp1 in cell lysate and pro-/cleaved-IL1b in both cell lysate and sup.

As suggested we tested gasdermin D cleavage from cell lysates but failed to detect the cleaved products after infection (Fig. EV3A). In correspondence with the company providing the antibody, we were told that generally it is more difficult to obtain the cleavage products from cells in late stages of pyroptosis.

5) The immunoprecipitations were performed in 293T cells. This experiment needs to be performed in THP-1 cells infected with parental and PB1-F2-deficient strains (H5N1 and H3N2) to reproduce physiological infection process.

We fully agree that the precipitation of endogenous NLRP3 would be a very important result. We tried numerous times to achieve this pulldown, but unfortunately failed. This is probably due to technical problems, since the NLRP3 pulldown is not very efficient and the custom anti-PB1-F2 serum generates a lot of background bands, so that a long term exposure of membranes is not feasible.
We included a sentence summarizing this point in line 314.

Minor comments.

Figure 2C is not listed in the manuscript

There is no figure 5E

I only have 6 figures, 7 are listed in the main manuscript, 6 in the figure legends. Please fix.

We corrected these mistakes in the revised version.

Referee #2:

Boal-Carvalho et al. describes a key finding that PB1-F2 (an influenza A viral accessory peptide) limits the activation of NLRP3 by locking it in a conformation which leads to non-availability of LRR surface for interaction with NEK7, eventually leading to inhibition of NLRP3 Inflammasome formation. I am convinced that this is appropriate to publish in EMBO Reports.

The finding reported are significant advancement of our understanding of host response to Influenza A infection. The authors support their findings with series of experiments leading to the molecular dissection of interaction of PB1-F2 to NLRP3 interaction surface and concluded that the NLRP3 interaction with PB1-F2 is mutually exclusive to NEK7.

The findings are convincing, however, totally opposite to what McAuley et al., 2013 AND Pinar et al.

2017 reported. Authors discussed very briefly about this in discussion, they should discuss more with specific arguments as to why they got totally different results from earlier publications.

> *The reviewer is right about the contradictory findings obtained by us versus previous studies. We see our findings as complementary to previous data obtained with different systems (peptides vs virus infection) or different viruses.*
> *Clearly PB-2 is a highly variable accessory protein of IAV (see sequence differences between PR/8 and the other strains (Fig. 4C and supplementary Figure 1). Moreover species specific effects might come into play, since we could not reproduce enhanced IL-1beta secretion in mouse BMDM infected with VN Wt and deltaF2 (new Fig EV1 C-T). It remains currently unclear, at which level of the inflammasome activation these species specific effects could occur. Of note, mouse and human NLRP3 are about 80% identical, which might partially explain the species specific effects). The new results and the current litarture were discussed in the newly formated manuscript.*

The scope of these results is appropriate for this journal audience including host-pathogen interactions, molecular biology of inflammasome activation. Experimental design is substantial to conclude these findings.

Minor comments:

Figure 5. Panel E is missing

Line 30-33: Not clear, should rephrase it

Line 243: Wrong figure citation (Fig 5C). Missing the blot for ASC western blot or referring to Fig. 6C?

Line 247: Should be Fig 5D instead of Fig. 6C

Line 248: Instead of Fig. 6D should be Fig. 5F (Fig. 5E is missing)

Line 249: Instead of Fig. 2C should be Fig. 5C.

Line 266: Should be Fig. 6C and D instead of Fig. 7

Line 266: Fig. 6C doesn't show interaction or competition with NEK7 instead of association of ASC with PB1-F2. Should discuss accordingly

Line 273: Should be Fig. 6E and F instead of Fig. 7

Line 278: Fig. 6G instead of Fig. 7G

> *We corrected these minor points.*

Referee #3:

Overall, the study is interesting however the results in figure 1 which aim to show that IAV lacking PB1-F2 induces greater IL-1b secretion are far from convincing raising considerable uncertainty. There is also a lack of in vivo studies to confirm the findings.

> *We thank the reviewer for his input and stating that the study is interesting. We performed as suggested appropriate statistical tests and still find significant and in our view biologically*

relevant differences in IL1-beta production after VN deltaF2 infection of macrophages as compared to VN Wt infected cells. We also included in vivo data from infected mice (new Fig. EV1 C-T). In mice we do not observe an increase in IL-1beta secretion in absence of PB1-F2, which might point to species-specific differences in the interference of PB1-F2 with NLRP3. Accordingly, we also did not find differences in immune cell invasion into the lungs, in clearance of the virus from the lungs or in susceptiblity to bacterial super infection, all phenotypes previously associated with PB1-F2 in vivo. This point is important, since virus strain-specific effects of PB1-F2 might apply when evaluating in vivo mouse data.

Abstract:
"Pyroptosis is a fulminant form of macrophage cell death, contributing to an antiviral state by release of proinflammatory cytokines." There is no solid evidence that proptosis is antiviral. Indeed it is proinflammatory. This sentence needs rephrasing.

We agree that the role of IL-1beta in the antiviral response is still udner debate (as discussed in our manuscript) and replaced this sentence by "Pyroptosis is a fulminant form of macrophage cell death, contributing to release of proinflammatory cytokines in context of viral infection."

Introduction:
IL-1β directly induces an antiviral transcription program in dendritic cells (Aarreberg et al., 2018)." This study utilised the unusual neutrotropic stain WSN to examine responses in bone-marrow derived DCs. The authors need to include these important points. IL-1b is a well described activator of NFkB.

We included this point in line 84.

"lung resident macrophages and inflammatory monocytes, are infected in an abortive fashion" Infection of macrophages is generally abortive but it has been shown that H5 viruses can replicate.

The reviewer is correct about this point, but it holds true solely for highly pathogenic H5 strains containing a multibasic cleavage site in the HA. The low pathogenic version we are using here does not replicate productively in THP-1, human or mouse BMDM.

There is no mention of PB1-F2 in the introduction and what is already known eg is has been shown to activate NLRP3 (PMID 27913620 26667784 23737748). This is included in the results but should be in the introduction. In addition there is no mention that only full length PB1-F2 expressed by certain IAV strains activates. PB1-F2 has also been shown to induce cell death. This important points have not been mentioned.

This was included in line 99-101.

Results:
Figure 1D and E. The error bars between VN WT and delta F2 are overlapping in both experiments suggesting there is no significance difference in IL-1b release. A t test is not an appropriate statistical test when comparing 3 groups but rather a one way anova. There is particularly no difference between VN WT and delta F2 in Fig 1E as all but 1 replicate overlap. The conclusion that there is higher secretion of IL-1b in the delta F2 group is therefore incorrect.

The reviewer is correct about the use of one-way ANOVA and we applied this test in the requested experiments.

It is not clear why PMBC macrophages and human BAL macrophages were infected with a different strain to the THP1 cells. This is confusing. Again the error bars in Fig 1J are overlapping and with only 3 replicates the data is not convincing. No responses in the human BAL macrophages were detected and it appears they weren't infected. The data should be repeated with the VN strain. If these strains don't infect macrophages then they may not be the appropriate cell type to study.

Human alveolar macrophages were isolate in the pediatrics unit in Medical School Hannover, Germany, which does not have approval to work with H5N1 IAV under BSL2 conditions due to biosafety concerns. Additionally we would like to point out that BAL in non-infected patients is a rather rare procedure and the precious material gained from theses BALFs does not allow a lot of different experiments. Thus we decided to perform the experiments with the H3N2 strain. Of note PBMC were infected with H5N1 and H3N2 IAV (see Fig.1G and J) with comparable phenotypes for the delta F2 mutant. So we believe it is justified to only test the H3N2 strain under these conditions.

Figure 2 - studies have shown PB1-F2 induced cell death yet the authors show delta F2 virus induces more cell death. How do the authors explain this?

This is a key point raised also by the other reviewers. Partially this discrepancy stems from strain specific effects. As shown here in figure 4, we do not observe interaction of PR/8 PB1-F2 with NLRP3. PR/8 is probably the most widely used virus isolate to study PB1-F2 function. Additionally, the systems to study PB1-F2 might also contribute. Human macrophages respond differently than mouse macrophages upon infection in our hands. Some studies only used synthetic peptides to mimic the effect of PB1-F2 (Pinar et al 2017). In this case secondary signaling pathways might not be activated to the same extend as under infection conditions.
This is why we concentrated here on contemporary virus isolates and used a range of host cell models to confirm our findings.

Annexin V and LDH is not a readout of pyroptosis. GSDMD cleavage is the only real measure of pyroptosis. Not sure why this data is included.

We agree with the reviewers point. We would however like to stress that IAV infection triggers more than just pyroptosis in infected cells, as also pointed out by reviewer 1. Showing the kinetic of cell death induction and the activation of caspase 3 is thus a valuble point in our view. Additionally we now show that LDH release during IAV infection in absence of PB1-F2 depends on caspase 1. This is in our view linking LDH data to pyroptosis.

Figure 3 - IL-1b secretion will induce NFKb/TNF/IL-6, pro-ilb mRNA upregulation. The experiments in figure 3 can not therefore determine if PB1-F2 provides signal 1 or signal 2. Previous studies have identified it provides signal 2 and not signal 1.

The point we wanted to make with this figure is merely, that a differential regulation of components of the NLRP3 inflammasome does not occur on mRNA/transcriptional level after infection with Wt or deltaF2 virus. Earlier and later time points confirm this finding (new Fig. EV2).

Figure 4 C - mock control is missing.

We thank the reviewer for pointing out the mistake. The blot was included now including the mock lane (new Fig 3C).

Figure 4F - WT virus did not induce caspase 1 cleavage yet IL-1b secretion and GSDMD cleavage was shown. How do the authors explain this result?

We see a very faint band in the Wt infected cells, this is correct. We can only speculate that the sensitivity of the caspase 1 western blot is lower than that for GSDMD or the IL-1-beta ELISA.

Figure 6 is incorrectly labelled in the text as figure 7.

Thank you very much, this was corrected.

1st Revision - Editorial Decision 29th Sep 2020

Dear Prof. Schmolke,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, referees #1 and #2 now support the publication of your study. Referee #3 has remaining concerns, stating that the significance of the study is low and indicates that the manuscript should not be published in EMBO reports. However, after cross-commenting with the other referees (who indicate that they do not agree with the assessment of referee #3 and support publication) we decided to proceed with publication. Nevertheless, we ask you to discuss the limitations of the present report and discrepancies and differences to previous studies (mentioned by the referee) in detail in the discussion of the final revised version. Moreover, please provide a detailed point-by-point response addressing the remaining concerns of referee #3.

Moreover, I have these editorial requests:

- The title is presently too long. Please provide a title with not more than 100 characters (including spaces).

- Please restrict the key words to five.

- Please add a conflict of interest statement to the manuscript text.

- In the author contributions Chengyue Niu is missing, but an author CY is listed. Please check.

- Please order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - DAS - Acknowledgements - Author contributions - Conflict of interest - References - Figure legends - Expanded View Figure legends

- For Fig. 2, please call out the panels in a sequential manner (A->F) or change their order in the figure. There seems to be no callout for panel 2C.

- There is a callout to Fig. 4F but there is no such panel. Please check.

- There is no legend for Fig. 2G, or this is not indicated in the legend of Fig. 2. Please check.

- Thank you very much for providing the source data for the Western blots of Fig. 1. As also the remaining the Western blots shown are often significantly cropped, could you please provide the source data for all the blots (main and EV figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number and send one PDF file per figure.

- Please add page numbers to the Appendix file and the TOC. We need a table of contents (TOC) on the first page with page numbers. Appendix Figure S1 needs a title and the legend below the figure.

- Please name the Appendix items Appendix Figure Sx or Appendix Table Sx in the Appendix file and use these names for the callouts. Please make sure all the Appendix items ARE called out in the manuscript text.

- Please provide a complete author checklist, also with section C (reagents) filled in.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

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

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

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors have addressed the vast majority of my concerns.

Referee #2:

This reviewer is satisfied with the revision.

Referee #3:

The study contradicts the study by McAuley et al 2013 Plos Pathogens where they used peptides and PB1-F2 deficient virus to show PB1-F2 results in NLRP3 activation, caspase 1 cleavage and Il-1b secretions in vitro and in vivo. The authors have suggested their results in THP1 cells differ as peptides were used by McAuley et al however PB1-F2 deficient virus, as well as peptides was used in the previous study.
The authors have now demonstrated that their results in PBA-treated THP-1 cells cannot be replicated in vivo. This has significantly reduced the novelty of their findings. The authors were also not able to replicate their findings in primary human alveolar macrophages suggesting it is not a

species difference.
Overall the significance is now diminished.

Figure 1G. Samples were obtained from 3 donors yet there are 4 data points on the graph.

Referee #1:

The authors have addressed the vast majority of my concerns.

Referee #2:

This reviewer is satisfied with the revision.

We thank the reviewer and for their constructive feedback, which we believe helped to improve the manuscript.

Referee #3:

The study contradicts the study by McAuley et al 2013 Plos Pathogens where they used peptides and PB1-F2 deficient virus to show PB1-F2 results in NLRP3 activation, caspase 1 cleavage and Il-1b secretions in vitro and in vivo. The authors have suggested their results in THP1 cells differ as peptides were used by McAuley et al however PB1-F2 deficient virus, as well as peptides was used in the previous study.

We thank reviewer three for his concerns, which we would like to discuss as follows:
Reviewer 3 is right about the contradiction in phenotype compared to McAuley et al 2013. For clarification, throughout their study they use PB1-F2 from PR8 (a mouse adapted laboratory strain from 1934), which in our hands does not bind to NLRP3. A control H3N2 virus does not provide the same NLRP3 activating phenotype.

In contrast, we show that in recent isolates of influenza A virus (we provide data for two viruses and their isogenic delta PB1-2 mutant and we additionally provide a third over expression system) Pb1-F2 inhibits the NLRP3 inflammasome, presumably by binding to NLRP3 and locking it in its closed confirmation.

Now reviewer 3 argues that since PR8 PB1-F2 activates NLRP3 all other PB1-F2s should behave the same way, thus our data are wrong, not novel and insignificant.
On the contrary we suggest, that by using recent and human relevant viruses, we describe the more abundant phenotype provoked by PB1-F2 and we support these data in human PBMC. Unfortunately, this is currently as close as we can get to a human in vivo model.

The authors have now demonstrated that their results in PBA-treated THP-1 cells cannot be replicated in vivo. This has significantly reduced the novelty of their findings. The authors were also not able to replicate their findings in

primary human alveolar macrophages suggesting it is not a species difference.

We would like to stress that alveolar human macrophages were not susceptible to infection (as described before with low pathogenic influenza A viruses). Consequently it is not surprising that no IL1beta secretion was observed with either virus tested.

Overall the significance is now diminished.

We here provide data in human and mouse monocyte derived macrophages showing clearly a species specific effect of PB1-F2 on NLRP3, which is present in human cells and absent in murine cells. Consequently the mouse model does not display downstream phenotypes related to IL1beta secretion when infected with PB1-F2 deficient virus.
The reviewer is fully right, when stating, that a confirmatory animal model would improve significance. Currently, besides the mouse, ferrets and guinea pigs are frequently used to study influenza A virus pathology. In both genetic tools to eleminate NLRP3 are rather limited.

Figure 1G. Samples were obtained from 3 donors yet there are 4 data points on the graph.

We thank the reviewer for pointing this out and corrected the figure legend.

2nd Revision - Editorial Decision 9th Oct 2020

Prof. Mirco Schmolke
University of Geneva
Microbiology and Molecular Medicine
Rue Michel Servet 1
Geneva, Geneva 1211
Switzerland

Dear Prof. Schmolke,

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.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to

our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-50421V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mirco Schmolke

Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-50421V1

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.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	sample sizes were estimated based on Gpower analysis for viral titers from previous experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We see no differences between the two groups we would like to compare. It would require quite a lot more mice to show that both groups are indeed not significantly different. Since this is not the main point of the manuscript, we find it ethically not justified to sacrifice so many animals.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	exclusion criteria were not applied
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.	no formal randomization was applied
For animal studies, include a statement about randomization even if no randomization was used.	no formal randomization was applied
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	no blinding was applied
4.b. For animal studies, include a statement about blinding even if no blinding was done	no blinding was applied
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.	was not applied
Is there an estimate of variation within each group of data?	no

Is the variance similar between the groups that are being statistically compared?	no

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).	mouse monoclonal anti-actin-HRP (Sigma-Aldrich, Cat# A5441), mouse monoclonal anti-tubulin-HRP (Cell signaling, Cat# 12351), rabbit monoclonal anti-NEK7 (Abcam, Cat# ab133514), rabbit monoclonal anti caspase 1 (Cell signaling, Cat# 3866S), mouse monoclonal anti-caspase-1 (Adipogen, Cat# AG 20B-0048) rabbit monoclonal anti-ASC (Cell signaling, Cat# 13833), rabbit monoclonal anti-NLRP3 (Cell signaling, Cat# 15101), rabbit polyclonal anti-Gasdermin D (Novus, NBP2-33422), rabbit monoclonal anti-Gasdermin D (Abcam, ab210070) mouse anti-Flag-HRP (Sigma; Cat# A8592), mouse anti-V5 (Invitrogen; R960-25), goat polyclonal anti-mouse-HRP (Sigma-Aldrich Cat# A5278), goat polyclonal anti rabbit HRP (Sigma-Aldrich, Cat# A0545), rabbit polyclonal anti-influenza virus NP (Invitrogen, Cat# PA5-32242), mouse monoclonal antibody anti-influenza virus M1 (Bio-Rad; Cat# MCA40), and rabbit polyclonal anti-H5N1 PB1-F2 clone 9947, described elsewhere (Schmolke et al., 2011).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T, MDCK and THP1 are from ATCC and frequently tested for absence of mycoplasma. CRISPR/Cas modified THP-1 (kindly provided by Dr. Veit Hornung LMU, Munich, Germany) (Schmid-Burgk et al., 2015)

* 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.	C57/Bl/6J mice housed under strict 12h/12h day night cycle under BSL2 SPF conditions with enriched cages. Animals were provided by Charles River France.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were in accordance with federal regulations of the Bundesamt für Lebensmittelsicherheit und Veterenärwesen (BLV), Switzerland (Tierschutzgesetz) and approved by direction de l'expérimentation animale and the cantonal authorities of the canton Geneve (license number GE/44/17). Embryonated chicken eggs were obtained from the University of Geneva Animalerie d'Arare and infected on day 10 of embryonic development.e
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.	We complied with ARRIVE guidelines, with exception to blinding and formal randomization.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All human donors of monocytes provided informed consent in written form.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All human donors of monocytes provided informed consent in written form.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	does not apply
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	no restrictions apply
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	does not apply
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.	does not apply
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.	does not apply

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	does not apply
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).	does not apply
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).	does not apply
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.	does not apply

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.	The H5N1 strain used in this experiment is a low pathogenic variant of A/Viet Nam/1203/2004 H5N1 influenza A virus. Deletion of the PB1-F2 ORF does not appear to enhance its replication, pathogenicity in vivo and to our best knowledge should not affect host range, tropism or transmissiblity.