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An endometrial organoid model of interactions between *Chlamydia* and epithelial and immune cells

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MS TITLE: An endometrial organoid model of Chlamydia-epithelial and immune cell interactions

AUTHORS: Lee Dolat and Raphael Valdivia
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this report, Dolat and Valdivia describe the use of organoids derived from endometrial cells to monitor the infection by the intracellular pathogen Chlamydia trachomatis. They show that the organoids (EMOs) reproduce some key feature of the primary tissues, such as cell polarity and formation of a tight barrier, ciliation, mucus secretion, before investigating the process of Chlamydia infection in this model. One important improvement compared to previous published organoid models is that the infection is done at the apical side of the organoid, by microinjection. The authors monitored some of the key steps of Chlamydia infection: cytoskeleton reorganization, redistribution of the Golgi apparatus, protection against cell death, and convincingly showed that the model recapitulated observations done in non polarized cell lines. Using mutant strains previously characterized by their laboratory they showed that the different phenotypes of the mutants, previously described in cell lines, was verified in this model.
The Chlamydia field is currently struggling to find better models to study Chlamydia infection than tumor derived cell lines. The work by Dolat and Valdivia constitutes an important step in this direction, and the quality of the data and of the report are excellent.

Comments for the author

The relative weakness of the work is that no discovery is made using this new model. Whilet fulfils completely the expectations of the "Tools and Resources" section published by JCS, I wonder if the authors could not push the neutrophil/EMOs interaction a bit further. For instance, there are some reports of neutrophils engulfing infected cells, is this something that they could monitor in the TepP deletion mutant? Could EMOs be cleared of the infection (at least maybe the tepP mutant), which would support an autonomous role of neutrophils in clearing infected tissues? Another thing that is surprisingly not investigated is the behavior of the CPAF mutant in this model. If this is because there were interesting phenotypes that will be studied in a future study I would understand that it was not included, but if it is because there was no obvious phenotype, I think it would be worth saying so in the discussion (maybe in the discussion of the limitations, see below)
Since the ambition of this work is to propose a model that should be used by others in the field, I think it is important to describe the protocol in details, thus my genuine questions :

- Fig 1B nicely shows the growth of the EMOs. What was the right window of time to infect them?
- What is the rate of success (% of microinjected EMOs that could be analyzed 24h later to monitor infection?
- The methods mention 5E5-5E6 IFU, is that the amount of IFU injected per EMOs. Can the authors give an estimate of the number of cells/EMO to have an idea of the MOI?
- How does the infection evolve over time? Can one monitor reinfection of neighboring cells (some images suggests that, since one sees "patches" of infected cells, like in S1H, but this is a 24 infection time point, should not get such big secondary infections in this short interval…
- The authors did some time lapse, why are the movies not supplied?
- The discussion should include some comments regarding the limitations of the model.

Noting that the staining with specific antibodies have extended incubation times, does it mean that proteins have to be expressed at high level to be detected? (all those that were tracked in this report are cytoskeletal proteins or other highly expressed proteins). Another limitation seems to be image quantification, since only a few of the observations made were actually quantified (for instance : what percentage of the intracellular bacteria showed b-catenin co-staining, What percentage of inclusions displayed filamentous versus punctate actin etc?).
Other minor points:
Fig. 1A is not introduced in the text What do you call "isolated glands"?, how do they relate to the EMOs?
The panels of Fig. 2 are introduced with a wrong letter. I think Fig. 2C should read Fig. 2E, Fig. 2C and 2D are not introduced. 2E should read 2F etc.
The author refers several time to "tdTomato" as a marker, but this does not specify what marker that is. In Fig. 3 it is said to be a plasma membrane marker. The methods mention only one source of "tomato mice", i.e. expressing ACTB-tdTomato,-EGFP, I suppose this is Actin but this is not specified, and it is not clear which "tomato" data stains what in the end. And is it also green, in

which case it cannot be the one used in Fig. S1H for instance Fig3 B. What are time of infection observed?
Fig. 3C: is the repositioning of the Golgi different in C. trachomatis versus C. muridarum infected cells? It looks different, but it could be a matter of choice of the image.
Regarding the quantification of the Golgi distribution at the inclusion, do I understand correctly - from reading the method section- that what is plotted is the proportion of inclusion membrane that co-stain with Golgi marker? But the 2 markers do not co-localize on the images. What is not clear is what was considered as “Golgi signal at the inclusion membrane” with the segmented line tool. An image with the “segmented line” apparent would clarify this. Would it not make more sense to quantify Golgi staining at the inclusion periphery (in a defined distance to the inclusion staining) versus in random spots in the cytoplasm?

Reviewer 2

Advance summary and potential significance to field

The study by Lee Dolat and Raphael Valdivia entitled Â“An endometrial organoid model of Chlamydia-epithelial and immune cell interactionsÂ” investigated Chlamydia muridarum and Chlamydia trachomatis infection using mouse endometrial organoids (EMOs). Many of the host-pathogen interactions known to be modified by Chlamydia were reanalysed in these mouse endometrial organoids including invasion to egression, inclusion fusion, reorganization of the cytoskeleton and positioning of intracellular organelles and inhibition of cell death. Additionally, an interesting step forward is the co-culturing of the epithelial organoids with neutrophils which they used to provide new insights into the role of the Chlamydia TepP protein known to suppresses innate immunity. Here they provide evidence that TepP is involved in controlling the recruitment of neutrophils to infected cells as well as to chlamydial inclusions.

This is a nice and important study on the establishment of an endometrial organoid model for Chlamydia infection.

Comments for the author

The following points need to be addressed to strengthen some of the author's claims.

Major points:

1. Figure 1 mainly reproduced the endometrial organoid culturing and characterized some of the markers. Here they also tried to demonstrate the presence of ciliated cells, cells which are usually positive for acMT. However the cells shown in Fig. 1H seem to have no cilia projected and acMT is expressed within the cell. The authors should clarify if they have ciliated cells, e.g. by electron microscopy.

2. In Fig. 1I, they postulate that estrogen treatment promotes EMO growth.
However, the data provided do not allow this conclusion since no statistical analysis is provided. Is the difference in the area at 4 days vehicle and E2 treatment significant?

3. In the result section, the authors described an impact of progesterone, but no data are provided supporting their statement.

4. It is difficult to see C. muridarum in Figs. 2B and C, despite GFP expression. Maybe, co-staining for C. muridarum would help to better visualize the colocalization. It is also important to quantify the frequency of colocalization.

5. It is important to provide the complete organoid images for Figs. 2F-G (similar to 2A) to get an overview of the F-actin modulation in Ct L2 infected organoids. Particularly, the loss of F-actin from the apical surface can be better appreciated in such images.

6. In Fig. 2I, actMT was shown to be reorganized in Ct L2 infected cells. Since this is a marker for cilia and therefore a marker for ciliated cells, do the organoids lose the cilia upon infection? The

ciliated cells should be quantified and electron microscopy images should be provided to better see if microvilli and/or cilia are lost. See point 1.

7. Fig. 3G is missing in the pdf.

8. The images provided to demonstrate Golgi reorganization are not convincing at all. The structure of the Golgi in the non-infected and infected organoids look similar. The images provided to demonstrate similar recruitment of Golgi vesicles to the inclusion of Ct L2 and C. mu rather suggest different recruitment.

9. The authors investigate cytotoxic effects of the infection in figure 5.
Compared to the low infection rates deduced from the Figs. 5A and B obtained by 3D deconvolution microscopy and the massive PI staining in Fig. 5C M007 in the maximum projection image is surprising. What is the percentage of cells infected with CT L2 and M007? How many of the infected and noninfected cells are positive for PI?

10. The mouse endometrial organoids described here are certainly useful to study chlamydial infection. However, mouse and human cells differ dramatically in cell autonomous defense and both strains, Chlamydia muridarum and Chlamydia trachomatis are adapted to overcome this in their mouse and human host respectively. This point should be discussed to outline possible limits of the current model for C. trachomatis infections.

Minor point:

Abstract: “Collectively, our model details a system to study the cell biology …”
Consider rephrasing to: “Collectively, our model is useful to study the cell biology …”

First revision

Author response to reviewers' comments

Dear Editor,
We’d like to thank the reviewers for their comments on our manuscript. Based on their recommendations we added new experiments and provided more information to clarify the relevant sections. We address the specific issues raised below:

Reviewer 1 Advance Summary and Potential Significance to Field...

In this report, Dolat and Valdivia describe the use of organoids derived from endometrial cells to monitor the infection by the intracellular pathogen Chlamydia trachomatis. They show that the organoids (EMOs) reproduce some key feature of the primary tissues, such as cell polarity and formation of a tight barrier, ciliation, mucus secretion, before investigating the process of Chlamydia infection in this model. One important improvement compared to previous published organoid models is that the infection is done at the apical side of the organoid, by microinjection. The authors monitored some of the key steps of Chlamydia infection: cytoskeleton reorganization, redistribution of the Golgi apparatus, protection against cell death, and convincingly showed that the model recapitulated observations done in non-polarized cell lines. Using mutant strains previously characterized by their laboratory they showed that the different phenotypes of the mutants, previously described in cell lines, was verified in this model.

The Chlamydia field is currently struggling to find better models to study Chlamydia infection than tumor derived cell lines. The work by Dolat and Valdivia constitutes an important step in this direction, and the quality of the data and of the report are excellent.

We thank the reviewer for commenting on the quality of the data and the report.

Reviewer 1 Comments for the Author

1. The relative weakness of the work is that no discovery is made using this new model. While it fulfils completely the expectations of the "Tools and Resources" section published by JCS, I wonder if the authors could not push the neutrophil/EMOs interaction a bit further. For instance, there are some reports of neutrophils engulfing infected cells, is this something that they could monitor in the TepP deletion mutant? Could EMOs be cleared of the infection (at least maybe the tepP mutant), which would support an autonomous role of neutrophils in clearing infected tissues?

We are reassured by the reviewer's assessment that our manuscript fulfils the expectations of the JCS "Tools and Resources" format. We agree that further investigation of neutrophil recruitment and/or dynamics will be of interest to the community, particularly with mutants that influence the interactions between these immune cell and the host. In the revised manuscript we further characterized neutrophil recruitment towards EMOs infected with tepp mutants using long-term, time-lapse microscopy. We observed two interesting behaviors that are absent in EMOs infected with wild-type *Chlamydia*: 1) PMNs swarming towards the EMO, transmigrating into the lumen, and aggregating when contacting luminal inclusions/EBs, and 2) neutrophils contacting an inclusion that is budding and then remaining in contact with the "bud." These new data further suggest that TepP dampens neutrophil recruitment and are now included in **Fig 7I-J**. At this time we are unable to determine how TepP suppresses neutrophil recruitment or if neutrophils can clear the infected EMO without establishing a more complex assay, such as re-infection of monolayers following co-culture and/or a 2D transmigration co-culture system. Development of these assays will require a significant amount of work that is beyond the scope of this "Tool and Resources" report and which frankly we would prefer to address mechanistically as a separate study.
We added the following discussion point as a limitation, "How TepP influences neutrophil behavior and whether neutrophils can more effectively clear TepP mutant in vivo remains to be determined and these studies will need to account for the likely possibility that *Chlamydia* employs redundant factors to limit neutrophil responses"…. We further add: "Our studies lay the foundation to investigate how TepP regulates neutrophil swarming and aggregation, behaviors that are dependent upon leukotriene signaling (Lämmermann et al., 2013). Of note, *C. trachomatis* is a human-adapted pathogen that differs from the rodent-adapted *C. muridarum* in its ability to counteract cell-autonomous immunity (Finethy and Coers, 2016). Future studies using *C. muridarum* mutants will be needed to better define the role of virulence factors in regulating immune responses."

2. Another thing that is surprisingly not investigated is the behavior of the CPAF mutant in this model. If this is because there were interesting phenotypes that will be studied in a future study I would understand that it was not included, but if it is because there was no obvious phenotype, I think it would be worth saying so in the discussion (maybe in the discussion of the limitations, see below).

We thank the reviewer for this suggestion. We now include data showing infection of EMOs with *Chlamydia cpaf* mutant. In these infected EMOs we observed enhanced neutrophil recruitment (**Fig 7A-B**), suggesting *Chlamydia* employs at least two factors to suppress neutrophil responses. In addition, we observed that the neutrophils that come in contact with *cpaf* mutant inclusions and/or extracellular EBs in the EMO lumen are morphologically distinct from those that come in contact with wild type *Chlamydia* (or *tepp* mutants) (**Fig 7C- E**). Because the *cpaf* mutant have been reported to induce neutrophil netosis, we also tested for the presence of nets using a DNA stain. Indeed, we found that neutrophils in contact with *cpaf* mutant exhibit diffuse staining with DNA dyes. We co-stained these infected EMOs with a fixable live/dead marker to show that neutrophils that have contacted cpaf mutant have lost membrane integrity (**Fig 7E**). These results are consistent with the published report by Rajeeve et al. Nat Micro, 2018.

Since the ambition of this work is to propose a model that should be used by others in the field, I think it is important to describe the protocol in detail, thus my genuine questions:

3. Fig 1B nicely shows the growth of the EMOs. What was the right window of time to infect them?

EMOs reach a size amenable for injection by 7-10 days post seeding and are chosen based on similar

sizes to achieve comparable infection rates among EMOs. Although EMOs can be passaged at 10-14 days, outgrowth is limited in the conditioned medium. We include this detail along with the additional text in Point 5 below.

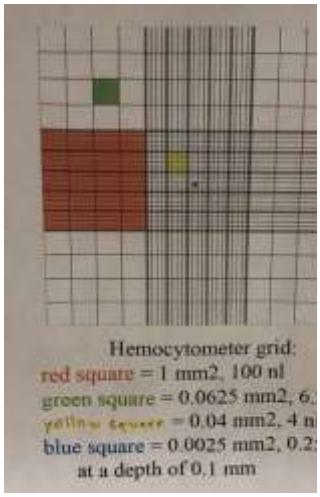
4. What is the rate of success (% of microinjected EMOs that could be analyzed 24h later to monitor infection? How does the infection evolve over time? Can one monitor reinfection of neighboring cells (some images suggests that, since one sees "patches" of infected cells, like in S1H, but this is a 24 infection time point, should not get such big secondary infections in this short interval…

To better determine infection efficiency, we microinjected 10 EMOs with either GFP-expressing *C. muridarum* or *C. trachomatis L2* and imaged at 1, 3, and 5 days post-infection. All EMOs contained inclusions at 1 dpi, indicating that all microinjections were successful, and we observed an increase in the number of inclusion over the course of five days, suggesting that cells within the organoid are being reinfected. We have included these data in **Fig 3G-I** and the following text, "To determine if new rounds of infection occurred after the initial challenge, we microinjected ten organoids with either GFP-expressing *C. muridarum* or *C. trachomatis* L2, imaged at 1, 3 or 5 d post-infection, and quantified the number of inclusions per organoid. We observed a significant increase in the number of inclusions for both strains, indicating that *Chlamydia* completes its lifecycle within the endometrial epithelia and released bacteria that infect other cells (Fig 3G-I)."

In Fig S1H, the EMO was infected and fixed at 24 hpi, and this patch is the result of a primary infection. The microinjection technique does not allow for absolute synchronization of the infection as EBs are freely moving around in the lumen, thus we cannot precisely control the MOI. We address this aspect of the model in the point below.

5. The methods mention 5E5-5E6 IFU, is that the amount of IFU injected per EMOs. Can the authors give an estimate of the number of cells/EMO to have an idea of the MOI?

To better define the number of IFUs used in our experiments, we microinjected buffer onto a hemocytometer grid, measured the area of the expelled volume, and compared the area to an area of known volume (100 nL) (see left image below; red square). We then divided the microinjected volume area by the known volume area (red square) and back calculated to determine the number of IFUs we employ in our experiments. On average, the microinjector expels 10s - 100s of IFUs (see table below). However, this is a methodological guidance as these parameters can vary based on the microinjection apparatus and microinjection needle width, which needs to be freshly broken at the tip. This is compounded by EMOs that contain different cell numbers, though we inject EMOs of roughly the same size. It will be important for anyone attempting to perform organoid microinjections to empirically test these parameters on their system.



				EB count			
Injection	Area	Fraction	Volume	5.00E+05	1.00E+06	5.00E+06	IFU
1	13355	0.066775	0.0066775	33.4	66.8	333.9	
2	10454	0.05227	0.005227	26.1	52.3	261.4	
3	5720	0.0286	0.00286	14.3	28.6	143.0	
4	9452	0.04726	0.004726	23.6	47.3	236.3	
5	9662	0.04831	0.004831	24.2	48.3	241.6	
Mean				**24.3**	**48.6**	**243.2**	

6. The authors did some time lapse, why are the movies not supplied?

During the preparation of the manuscript, we had limited access to our lab computers, which are

needed to open large time-lapse files. With our access restored, we now provide movies corresponding to figures listed below:

Figure 3
Video 1 Inclusion Fusion
Video 2 C.mu extrusion 1
Video 3 C.mu extrusion 2
Video 4 *Ct* – L2 basolateral extrusion

Figure 5
Video 5 cpoS lysis
Video 6 cpoS PI-positive

Figure 6
Video 7 *Ct* – L2 PMN recruitment

Figure 7
Video 8 *cpaf* mutant PMN recruitment
Video 9 *tepP* mutant PMN recruitment 1
Video 10 *tepP* mutant PMN recruitment 2

7. The discussion should include some comments regarding the limitations of the model. Noting that the staining with specific antibodies have extended incubation times, does it mean that proteins have to be expressed at high level to be detected? (all those that were tracked in this report are cytoskeletal proteins or other highly expressed proteins). Another limitation seems to be image quantification, since only a few of the observations made were actually quantified (for instance: what percentage of the intracellular bacteria showed b-catenin co-staining, what percentage of inclusions displayed filamentous versus punctate actin etc?).

In the discussion section, we address the limitations of this model in determining the mechanism by which TepP influences PMN recruitment as written in point 1. We also include a discussion point addressing the differences in cell-autonomous immunity between *C. trachomatis* and *C. muridarum*, as suggested by Reviewer 2. We focused our quantifications on previously described phenotypes relevant to *Chlamydia* mutants while describing other observations. However, we now include the quantification of β-catenin recruitment to the nascent inclusion in **Fig 2B**, further supporting the notion that our model is suitable for quantitative microscopy.

With respect to antibody staining, we do not find these incubations times to be significantly longer than what is performed in 2D cell culture as we routinely incubate primary antibodies for 1.5 - 2 hours. However, we chose to use slightly longer incubations (2-3 hours) for antibodies because the EMOs are labeled in a 0.5 mL suspension, as opposed to lying down on flat coverslip, and to ensure the staining works as generating samples from 3D cultures requires more work than simple passaging of 2D cells onto coverslips.

Other minor points:
1. Fig. 1A is not introduced in the text. What do you call "isolated glands"?, how do they relate to the EMOs?

We modified the first sentence of the Results sections to state, "Isolated endometrial epithelia glands, which contain stem cells that regenerate the luminal epithelia during the estrous cycle (Gargett et al., 2016; Jin, 2019), were isolated and cultured in a three-dimensional Matrigel matrix in the presence of conditioned medium from L-WRN cells (Fig 1A)."

2. The panels of Fig. 2 are introduced with a wrong letter. I think Fig. 2C should read Fig. 2E, Fig. 2C and 2D are not introduced. 2E should read 2F etc.

We apologize for this oversight. We have fixed the text.

3. The author refers several times to "tdTomato" as a marker, but this does not specify what marker that is. In Fig. 3 it is said to be a plasma membrane marker. The methods mention only one

source of "tomato mice", i.e. expressing ACTB-tdTomato,-EGFP, I suppose this is Actin but this is not specified, and it is not clear which "tomato" data stains what in the end. And is it also green, in which case it cannot be the one used in Fig. S1H for instance

In the results section referring to Fig 3 Panel C-E where we specify, "We next monitored inclusions exit dynamics using EMOs derived from ROSA^{mTmG} mice that express a membrane-targeted tdTomato allowing for visualization of the plasma membrane of individual cells." In addition, the full mouse strain label and stock number for the commercially available mice from The Jackson Laboratory are provided in the methods section: "EMO generation from the mouse endometrium and hormone stimulation: "…B6.129(Cg)- Gt(ROSA)26SOR$^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$ (Strain no. 007676) mouse strain was purchased from The Jackson Laboratory." The membrane-targeted TdTomato is expressed using the β-actin promoter. However, it is flanked by loxP sites as it is used frequently as a reporter for cre recombinase, which turns on GFP expression. Because there is no cre recombinase in our experiments, the membrane-targeted TdTomato is expressed constitutively and localizes to the plasma membrane.

4. Fig3 B. What are time of infection observed?

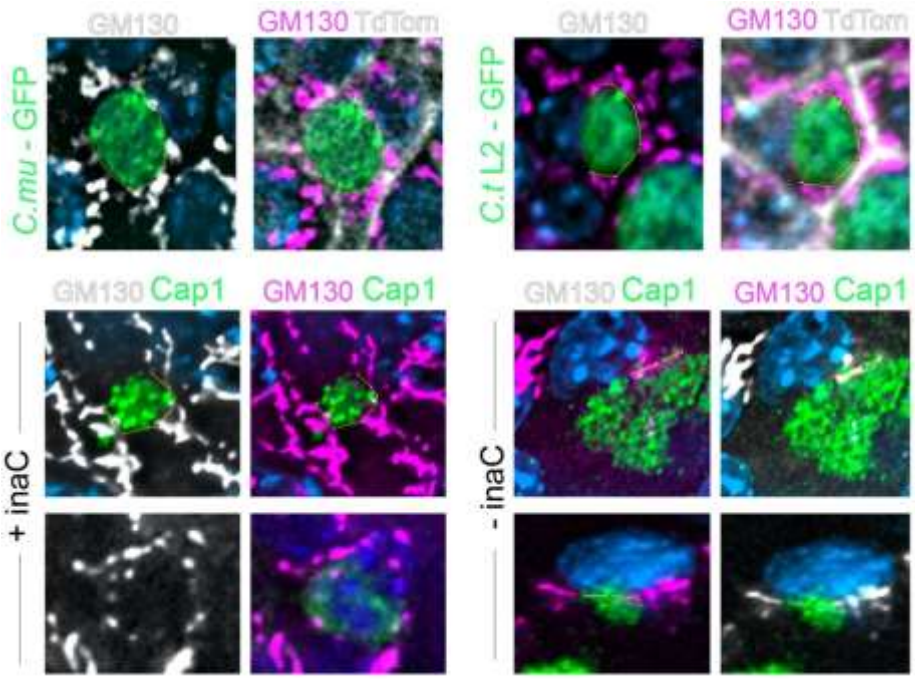
The organoids were imaged at 48 hpi. We have clarified this in the Fig 3 legend, "Maximum projections of EMOs infected for 48 hours and imaged by 3D widefield deconvolution microscopy."

5. Fig. 4C: is the repositioning of the Golgi different in C. trachomatis versus C. muridarum infected cells? It looks different, but it could be a matter of choice of the image.

The Golgi repositioning is not different. We now provide a new image of an EMO infected with C.t L2-GFP showing an inclusion of more comparable size to the C.mu-GFP inclusion directly above.

Regarding the quantification of the Golgi distribution at the inclusion, do I understand correctly - from reading the method section- that what is plotted is the proportion of inclusion membrane that co-stain with Golgi marker? But the 2 markers do not co-localize on the images. What is not clear is what was considered as "Golgi signal at the inclusion membrane" with the segmented line tool. An image with the "segmented line" apparent would clarify this. Would it not make more sense to quantify Golgi staining at the inclusion periphery (in a defined distance to the inclusion staining) versus in random spots in the cytoplasm?

We quantified GM130-positive Golgi stacks that are only directly adjacent to the inclusion. Below we provide examples of the line segmentation method. Using the segmentation line tool in ImageJ, we select GM130-positive Golgi stacks that are directly adjacent to the inclusion, using the plasma membrane-bound TdTomato as a reference of the cell boundaries. We measured the length of the segmented line and divided this length by the length of the inclusion perimeter to identify the percentage of Golgi around inclusion. We have published this analytical method in Pruneda et al. Nat Microbiology, 2018 and used similar criteria in Kokes et al CHM 2015. We updated the text in Image Analysis methods section to clarify this point, "Using the segmented line tool, the Golgi signal directly adjacent to the inclusion was also traced to measure its length," as well as cite the primary publication in which this analysis is derived.



1) Reviewer 2 Advance Summary and Potential Significance to Field...

The study by Lee Dolat and Raphael Valdivia entitled "An endometrial organoid model of Chlamydia-epithelial and immune cell interactions" investigated Chlamydia muridarum and Chlamydia trachomatis infection using mouse endometrial organoids (EMOs). Many of the host-pathogen interactions known to be modified by Chlamydia were reanalysed in these mouse endometrial organoids including invasion to egression, inclusion fusion, reorganization of the cytoskeleton and positioning of intracellular organelles and inhibition of cell death. Additionally, an interesting step forward is the co-culturing of the epithelial organoids with neutrophils which they used to provide new insights into the role of the Chlamydia TepP protein known to suppresses innate immunity. Here they provide evidence that TepP is involved in controlling the recruitment of neutrophils to infected cells as well as to chlamydial inclusions.

This is a nice and important study on the establishment of an endometrial organoid model for Chlamydia infection.

We thank the reviewer for highlighting the importance of this study in establishing new 3D epithelial models of *Chlamydia* infection.

1. Figure 1 mainly reproduced the endometrial organoid culturing and characterized some of the markers. Here they also tried to demonstrate the presence of ciliated cells, cells which are usually positive for acMT. However, the cells shown in Fig. 1H seem to have no cilia projected and acMT is expressed within the cell. The authors should clarify if they have ciliated cells, e.g. by electron microscopy.

We provide better evidence of ciliated cells in the mouse EMOs by increasing the estrogen concentration (20 nM), which is comparable to the concentrations used in Turco et al. Nat Cell Biol, 2017,. We now show unequivocal evidence of ciliated cells in Fig 1 Panel H; however, multi-ciliated cells are not very abundant and are only found in very large organoids. Unfortunately, this precludes our ability to test whether *Chlamydia* infection can affect the frequency of ciliated cells (see point 6) as observed in Kessler et al. Nat Comm, 2018. We have included the following in the discussion pointing out this limitation in the model "While some aspects of this complexity can be reconstituted in EMOs, how hormonal changes impact the cell biology of *Chlamydia*-host cell interactions in the UGT is largely unexplored".

2. In Fig. 1I, they postulate that estrogen treatment promotes EMO growth. However, the data provided do not allow this conclusion since no statistical analysis is provided. Is the difference in the area at 4 days vehicle and E2 treatment significant?

We repeated the experiment using the higher estrogen levels (see previous point) and compared the fold change in EMO size between treatments from three independent experiments. We have updated the graph in Fig 1 Panel I and tested for significance (p = 0.0027).

3. In the result section, the authors described an impact of progesterone, but no data are provided supporting their statement.

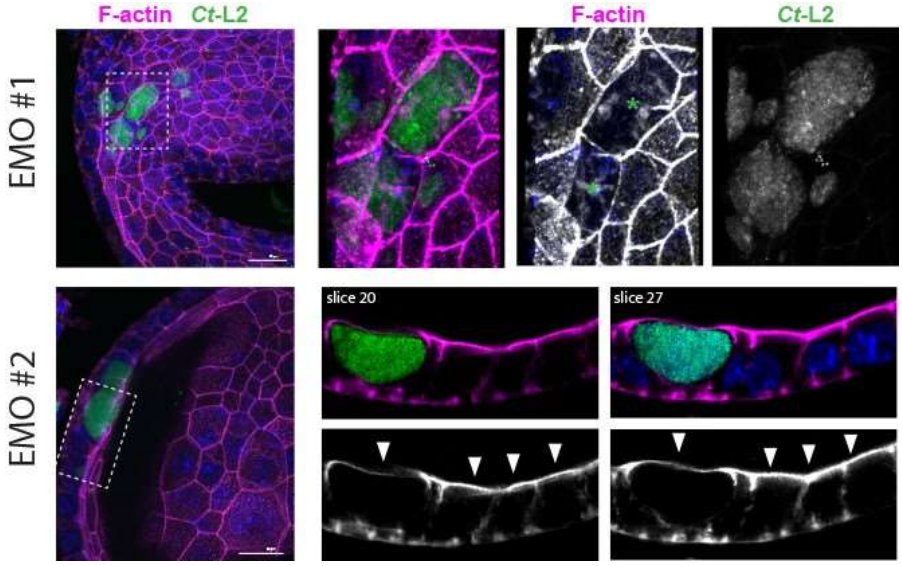
We removed progesterone from text.

4. It is difficult to see C. muridarum in Figs. 2B and C, despite GFP expression. Maybe, co-staining for C. muridarum would help to better visualize the colocalization. It is also important to quantify the frequency of colocalization.

We separated the panels to show fluorescent *C. muridarum* more clearly in Fig 2 Panel B and removed the fluorescence line scan analysis. We also include the quantification of ß-catenin recruitment to *C.mu* and *C.t* inclusions at 8 hours post-infection in Fig 2 Panel C.

5. It is important to provide the complete organoid images for Figs. 2F-G (similar to 2A) to get an overview of the F-actin modulation in Ct L2 infected organoids. Particularly, the loss of F-actin from the apical surface can be better appreciated in such images.

We reorganized Fig. 2F-G showing the two independent organoids infected with C.t-GFP and stained for F-actin (EMO #1-2). In contrast to Fig 2A, we used a 40x water objective coupled with an Airyscan detector in Fig 2F- G, which greatly increases the resolution but does not have enough width/depth to image the entire organoid, though we captured large sections (shown below). While we think the difference in cortical actin organization is clear in the Fig 2 Panel F inset between the infected and uninfected, we also show a 3D reconstruction of EMO #1 below (EMO #1 inset, infected cells demarcated with a green asterisk). In the original submission, we showed the loss of actin at the cortex in EMO #2, however we provide individual confocal sections of the same region below. We can provide the images below in a supplemental figure if deemed necessary by the editors.



6. In Fig. 2I, actMT was shown to be reorganized in Ct L2 infected cells. Since this is a marker for cilia and therefore a marker for ciliated cells, do the organoids lose the cilia upon infection? The ciliated cells should be quantified and electron microscopy images should be provided to better see if microvilli and/or cilia are lost. See point 1.

Please see point 1.

7. Fig. 3G is missing in the pdf.

We apologize for the oversight. We had removed this panel from the manuscript prior to submission. It has been replaced with suggested experiments from Reviewer 1 showing re-infection occurs within the EMO.

8. The images provided to demonstrate Golgi reorganization are not convincing at all. The structure of the Golgi in the non-infected and infected organoids look similar. The images provided to demonstrate similar recruitment of Golgi vesicles to the inclusion of Ct L2 and C. mu rather suggest different recruitment.

In contrast to transformed 2D cells where the Golgi is generally packed closely to the nucleus, the Golgi is inherently more dispersed in polarized epithelia. Indeed, this makes distinguishing the difference in Golgi recruitment between uninfected and infected cells less obvious. We provide a new image of an EMO infected with *C.t* L2-GFP showing an inclusion of more comparable size to the *C.mu*-GFP inclusion directly above (**Fig 4C**). We believe this to be a better representative Golgi dispersion around the inclusion perimeter. We used the InaC mutant, which fails to induce Golgi dispersion in 2D cultures, to quantitatively measure the degree of Golgi dispersion in EMO epithelia. In our analysis, we measure Golgi stacks that are only directly adjacent to the inclusion membrane and not localizing elsewhere in the cytoplasm. This point was also clarified for Reviewer 1 point #5.

9. The authors investigate cytotoxic effects of the infection in Figure 5. Compared to the low infection rates deduced from the Figs. 5A and B obtained by 3D deconvolution microscopy and the massive PI staining in Fig. 5C M007 in the maximum projection image is surprising. What is the percentage of cells infected with CT L2 and M007? How many of the infected and noninfected cells are positive for PI?

The reviewer is correct that Figure 5C is a maximum projection while the images in Fig 5A and B are single sections, so we would expect to see a difference in the number of PI puncta between the two. We cannot determine the number of cells infected out of the total, unlabeled cell population. Propidium iodide is not fixable, so we needed to count PI-positive cells in live EMOs. To improve upon our analyses, we quantified the number of PI- positive inclusions in similarly sized EMOs (**Fig 5D**). In addition, we provide time-lapse microscopy of an EMO infected with a GFP-expressing cpoS mutant (M007) cultured in the presence of propidium iodide (**Fig 5E**) and show: 1) lysis of the inclusion/infected cell and the manner in which it become PI-positive, and 2) an inclusion gradually becoming PI-positive.

10. The mouse endometrial organoids described here are certainly useful to study chlamydial infection. However, mouse and human cells differ dramatically in cell autonomous defense and both strains, Chlamydia muridarum and Chlamydia trachomatis are adapted to overcome this in their mouse and human host, respectively. This point should be discussed to outline possible limits of the current model for C. trachomatis infections.

We have included the following limitation in the discussion, “Our studies lay the foundation to investigate how TepP regulates neutrophil swarming and aggregation, behaviors that are dependent upon leukotriene signaling (Lämmermann et al., 2013). Of note, *C. trachomatis* is a human-adapted pathogen that differs from the rodent- adapted *C. muridarum* in its ability to counteract cell-autonomous immunity (Finethy and Coers, 2016). Future studies using *C. muridarum* mutants will be needed to better define the role of virulence factors in regulating immune responses.”

Add text in discussion.
Minor point:

Abstract: “Collectively, our model details a system to study the cell biology …”

Consider rephrasing to: “Collectively, our model is useful to study the cell biology …”

We amended the abstract as “Collectively, our model can be applied to study the cell biology of *Chlamydia* infections in three dimensional structures that better reflect the diversity of cell types and polarity encountered by *Chlamydia* upon infection of their animal hosts.”

Second decision letter

MS ID#: JOCES/2020/252403

MS TITLE: An endometrial organoid model of Chlamydia-epithelial and immune cell interactions

AUTHORS: Lee Dolat and Raphael H Valdivia
ARTICLE TYPE: Tools and Resources

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed all my comments. This is a great paper.

Comments for the author

N/A

Reviewer 2

Advance summary and potential significance to field

This paper shows for the first time the establishment and use of an endometrial organoid model to study the interaction of Chlamydia with epithelial cells and neutrophils.

Comments for the author

The authors have adequately responded to all my points.