

Rebuttal

We have carefully addressed all the comments below. The paper has substantially been changed according to the requested comments from the editors and reviewers. The answers are highlighted in blue.

As new experiments were performed and new results added the figures' names are shifted in the new version. In the first version we had 6 main figures. In the present version we have 7 main figures and 2 supplementary figures. Below we add an explanation about each change for the new figures:

- Figure 1: this figure now shows only the confocal microscopy of uninfected co-cultures. We add an experiment with tight junction staining to support the TEER value measurement, as requested by the reviewer
- Figure 2: the infected co-culture is now shown here with the ZO-1 staining (new experiment) showing in B the tight junction disruption, supporting the TEER measurement.
- Figure 3: we have improved the original figure 2, by showing only the second panel in which both macrophage transmigration and bacteria uptake are shown. We also highlighted for each time point bacteria uptake by macrophages, which are shown in separated channels, as requested by the reviewer.
- Figure 4: The figure 4 was initially figure 5. We decided to focus only in the co-culture for the confocal microscope images since this is the most important message for the readers. With that, we could separate the channels for PAO1-GFP, THP-1 and CFBE41o-. For the graphics showing the CFU, we changed only PAO1 (in the X axis) for abiotic substrate, to make it more clear for the reader that we are comparing the survival of bacteria in the co-culture and without co-culture (where bacteria are grown directly in the permeable support).
- Figure 5: This figure was originally the figure 4. No significant change was made except for the labels (PAO1 is corrected to PAO1-GFP).
- Figure 6: This figure was originally the figure 3. The standard deviation is now showed. In the process of reorganizing this figure, we realized that indeed some raw data in these graphics were changed in the first version. We apologize for such a mistake, and we, therefore, corrected the graphics accordingly.
- Figure 7: This figure was figure 6 before and changes for the axis and size of the letters were performed.
- Figure S1: To add important control experiments, this supplementary figure serves as an explanation for some methods assessed in the protocol. A detailed description is given in “figures and tables legends” and in the results part.
- Figure S2: This figure serves exclusively as a description for some control experiments in the protocol concerning cytotoxicity and cytokine release. Further information are given in “figures and tables legends” and the results part.

Editorial Comments:

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Thanks for this comment. An English native speaking scientist, Chelsea Thorn, thoroughly checked for it.

- **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have described as optimally as possible all the protocol steps as required. Moreover, the steps to be shown in the video are highlighted in yellow.

- **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

- 1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
- 2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
- 3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
- 4) Notes cannot be filmed and should be excluded from highlighting.

Thanks for this comment. The steps from our protocol are highlighted in yellow, according to the JoVE recommendations.

- **Discussion:** JoVE articles are focused on the methods and the protocol; thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Thank you, the discussion was changed accordingly. We have 6 paragraphs addressing each of those steps.

- **Figures:**

- 1) Fig 3: Please increase axis label and tick font sizes.
- 2) Fig 3,4, 5: Define error bars.
- 3) Fig 5A: define scale bars.

For all figures (graphics) we have increased axis label (3) and tick font sizes (Calibri 20 at least). Moreover, all error bars are now defined in the graphics. For the microscopies, the scale bars are also defined.

- **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are transwells, Transwell® 353181, LAS X
- 1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
 - 2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Many thanks for your comments and valuable tips to change the specific words in the manuscript. We replaced all commercial names by generical ones and indicated whenever needed (see table of materials) to draw the readers' attention. The trademark symbols in the table of materials are also removed.

- If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Not the case for our publication.

Comments from Peer-Reviewers:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if

the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Manuscript Summary:

While the authors demonstrate the ability to prepare co-culture models for infection, the biological relevance of this model is questionable. For example, there is significant damage to the epithelial barrier following infection with PAO1 as evidenced by low TEER values. Additional experiments need to be completed in order to show an intact barrier.

Clarity of presentation:

* This document needs significant editing for clarity, spelling, grammar, consistency, and proper English conventions.

Thank you for this comment. The paper has been carefully edited and proofread by an English native speaker.

* Overall clarity could be improved by adding one general description of experimental design/aims at the beginning of the protocol section, or introduction statements at the start of each of the seven subsections

Thank you for this valuable observation. We included the following at the beginning of the protocol section:

“This protocol combines two relevant cell types for inhalation therapy of the pulmonary airways: macrophages and CF bronchial epithelium. These cells are seeded on opposite sides of permeable support inserts, allowing cell exposure to air (a.k.a. ALI conditions). This co-culture of host cells is subsequently infected with *P. aeruginosa*. Both host cell lines are of human origin: the epithelial cells represent the cystic fibrosis bronchial epithelium, with a mutation on the CF channel (CFBE41o⁺), and the THP-1¹ cells are a well-characterized macrophage-like cell line. A confluent epithelial layer is first allowed to form on the upper side of well plate inserts before the macrophage-like cells are added to the opposite compartment. Once the co-culture is established at ALI, the system is inoculated with *P. aeruginosa* at the apical side. This infected co-culture system is then used to assess the efficacy of an antibiotic, e.g., tobramycin. The following end-points are analyzed: epithelial barrier integrity in terms of transepithelial electrical resistance (TEER), visualization of cell-cell and cell-bacteria interactions by confocal laser scanning microscopy (CLSM), bacterial survival by counting of colony-forming units (CFU), host cell survival (cytotoxicity) and cytokine release”.

* Consistency/accuracy of text: mL not ml, 3 μ m not μ M, two or 2, o vs o-, PAO1 vs PAO1GFP, Figure 5 B/C Should the left hand side read "CFBE41o-"?, etc.

Thank you for this comment. We carefully checked for all inconsistent and not accurate text passages in the paper and changed them accordingly. Figure 5 is now figure 4. We reformulated this figure since we agreed that the message was not as clear as it should. We choose the more relevant picture (co-culture) to exemplify the survival of epithelial cells and macrophages as well as bacteria PAO1-GFP in the course of the infection and treatment. The graphics E and F are also corrected, in which we replaced the classification of "only PAO1" to abiotic substrate, to be more precise.

* Consistency for imaging: orientation/viewing angle of images, staining and demonstration of all single channels

Thanks for this comment. The figures are reformulated accordingly. We chose to show the cross-section to show the co-culture as a whole, including the macrophages migration (Fig Fig 1A, 2A and figure 3. The apical view was also chosen to show tight junctions staining (Figures 1B and 2B) or a representative figure of infected co-culture treated or not of Tobramycin (Figure 4A-D). As requested, single channels are shown in those figure 1-4.

* Please edit for clarity: Line 151-152, Line 175, Line 177-178, Line 192-195, Line 232, Line 234, Line 271

The lines are edited as follows:

➤ **Line 151-152 – current version lines 167-169:**

1.1.1. Count the cells with an automated cell counter or hemocytometer chamber. Seed cells with a density of 2×10^5 cells/well in 12 well plates with permeable supports (pore size of 3 μ m) (see table of materials).

➤ **Line 175 – current version lines 206-208:**

1.1.2. Differentiate the THP-1 cells. Centrifuge content of T75 at 300x g for 4 min, resuspend in fresh medium and put in new T75. Add 10 ng/mL Phorbol 12-myristate 13-acetate (PMA) incubated in RPMI for 48 h at 37 °C and 5% CO₂ atmosphere.

➤ **Line 177-178 – current version lines 220-222:**

1.2.3. After cell removal with accutase, re-suspend macrophage cells in 3 mL of THP-1 medium into a 15 mL-falcon tube, count the cells as described in 1.1.2. and incubate for a maximum 1 h at 37 °C, 5% CO₂ before setting up the co-culture.

➤ **Line 192-195 – current version lines 244-246:**

Note 2: CFBE410⁻ lung epithelial cells can be stained at this step. The procedure at 1.2.4. can be used; however, instead of a cell suspension, the dye solution in MEM is applied (500 µL apical side only) on the adhered cells on the permeable support.

➤ **Line 232** – this line was removed since it was not clear in the context

➤ **Line 234** – current version lines 285-287

3.6. Incubate the drug of interest 1 h after bacteria adhesion in the cells. For treatment experiments, add 500 µL apical of a drug solution diluted in cell medium (in this protocol tobramycin 6 µg/mL was used). Add 1500 µL cell medium on the basolateral side.

➤ **Line 271** – current version lines 361-364

6.1. The supernatant of infected cells containing bacteria (from 5.1) can be used for cell viability assessment for lactate dehydrogenase assay (LDH)². The supernatant is centrifuged at 21250 x g for 10 min to pellet the bacteria and eventually rest of the cells. The bacteria-free supernatant is then used to measure LDH release.

* Please edit for spelling/grammar/typos (e.g., Line 182 Correct to CellTrace™)

As CellTrace™ is a registered trademark, we replaced by cell viability dye based on the conversion of esterases (see table of material), as described in lines 227-229 below:

1.2.4. Stain macrophages with 10 µM of a cell viability dye based on the conversion of esterases (see table of material) in which 3 µL of the cell viability dye is applied to the cell suspension. Cells are incubated for 20 min at 37 °C, 5 % CO₂, then washed 1 X with PBS 37 °C to remove the dye.

* Consistency: E.g. Some graphs do not have THP-1 only data (Figure 5B/C), different colours across figures (some uninfected grey, some red).

The figure 5 is rearranged, and we decided to keep the data from co-culture only, as the most relevant indeed. For all other figures we re-checked to ensure consistency and clarity.

* Figure 2. It is unclear how A and B differ as both panels show macrophage cells moving across the membrane (transmigration) and engulfing bacteria (uptake). Please clarify

Thank you very much for this important observation. We agree with the reviewer that panel A and B indeed showed the same message regarding transmigration and bacteria uptake. Therefore we rearranged this figure by using only one panel, and separating the channels where we had THP-1 uptaking bacteria. Please note that figure 2 is now figure 3 in the reviewed manuscript.

* Need to follow JOVE guidelines for proper format of Figure legends and Representative Results section.

Thank you for this comment. The guidelines were followed and the text changed accordingly.

Scientific accuracy:

* Abstract: "THP-1 monocyte-derived macrophages" may be more accurate since the cells are monocytes that must be differentiated into macrophage cells

Thanks for the comment. We changed accordingly to this suggestion (line 85).

* In abstract and introduction: "biofilm-forming" *P. aeruginosa* is misleading. The authors did not specifically demonstrate biofilm formation in the model. The authors also used the term biofilm in the discussion when referring to their data (Line 396). Without specific demonstrations of biofilm extrapolymeric substances (EPS), e.g. pel or psl immunostaining, authors cannot claim that this is a biofilm. Please change terminology to cell aggregates or a similar term.

The reviewer is right. We don't have biofilm at this level. Therefore we removed the expression to be more precise.

* Line 160. What is the value (300ohms/cm²) based on? Provide reference, or confirm that this value is associated with an intact barrier by other means

We agree 300 ohms/cm² is an arbitrary value in the literature for intermediate intact barriers of cell layers from the lung and the gut (J Lab Autom. 2015 Apr; 20(2): 107–126.). We added ZO-1 stained cell layers from infected and non-infected epithelial cells to show an example of a tight-junction related protein contributing to cell barrier.

* Line 273. Correct to internalized bacteria. All adherent extracellular bacteria should be killed by treatment with tobramycin

Usually adherent bacteria are differentiated from internalized, either by using immunostaining (de Souza Carvalho et al; Cell Micro <https://doi.org/10.1111/j.1462-5822.2011.01615.x>), or when one use an antibiotic that don't permeate the mammalian cell membrane, and thereby kill only extracellular bacteria (like gentamycin; Dimer et al, Nanomedicine. 2019 Nov 18;24:102125. doi: 10.1016/j.nano.2019.102125). In the case of *P. aeruginosa*, this is known to be an extracellular pathogen, however we can not be 100% sure that no bacteria get internalized by epithelial cells. Nevertheless they are internalized by macrophages as we demonstrated in figure 3. Whether all internalized bacteria by macrophages are killed, remains to be investigated. Therefore we have to assume always when dealing with CFU of bacteria, those that are attached and/or internalized, mainly

because the concentration of tobramycin used here was not enough to kill 100% of the bacteria. As far our studies went we cannot differentiate, so far, which bacteria are adhered or intracellular, without another specific method of separation or further characterization of drug penetrance in the bacterial population. Therefore, we assume to have two fractions: free bacteria and adhered/internalized bacteria.

* Images: DAPI signal is very high in 6hpi monoculture and exists in areas where there is no red (macrophage) signal, suggesting epithelial cell cross contamination. Please address.

Indeed, depending on the view the snapshot was taken, DAPI and the red signal (coming from the viability dye) do not correlate. We chose the snapshots in an attempt to show the interactions, movement and morphology of the macrophage-like cell. The new inclusion of axis and angle views will help to understand that DAPI/red not always correlate.

* Line 458-460. None of the data is statistically significant, likely due to the study being underpower, so authors should not comment on the effect of tobramycin treatment on cytokine levels

Indeed, however we can still comment on trends if tobramycin samples presented less or more cytokines when comparing mono versus co-cultures during infection or infection/treatment.

* Line 462-463. This statement does not reflect the data. Figure 5C shows recovery of bacteria at 20hpi so the treatment does not "cure the infection"

Thank you. We changed to: "Tobramycin treatment decreases infection levels of PAO1-GFP but is not able to further influence inflammation".

* Protocol Step 3.5: Was the monolayer of epithelial cells on the transwell enumerated to calculate the MOI? Please clarify that the MOI is for PAO1:epithelial cells (not macrophages). Details are not clear enough to determine if this is truly a MOI of

No, the MOI was defined from the number of CFBE41o cells seeded at the beginning of each experiment. We did not define a specific number of the grown/established cells on the layer in the permeable support. We included: "This corresponds to a multiplicity of infection (MOI) of one bacterium to one epithelial cell" (lines 274-275) so that it becomes clear that epithelial cells were used to calculate the MOI.

* Inaccurate or unjustified information was presented. This was due to lack of references as well as improper grammar, leading to misinterpretations. (e.g., Line 108 - "the correction of the CFTR protein" makes it sound like *Pseudomonas aeruginosa* is correcting the CFTR mutation. Line 460-463 - "The response to tobramycin is crucial to establish a CF model,

especially for tobramycin being effective on inhibiting *P. aeruginosa* and even on biofilm mode of growth.")

Thank you for these comments. For line 108, now line 109, the sentence is changed to have more accurate information to "or assess the correction of the CFTR protein during infection". As we almost rewrote the whole discussion, the information in the lines 460-463 were deleted, since this information could not be justified.

Usefulness:

* The rationale for this model and its relevance to cystic fibrosis has not been made clear. It is unclear what the benefits of this model are in comparison to previous models. Please revise the introduction accordingly.

Thanks for this important observation. The introduction is revised to give consistent information on the relevance of this model. The present co-culture represents a CF lung barrier with an immunological component that can be high-throughput and can be further adapted to aerosol deposition since it is set up at air-liquid interface (ALI). The focus lies here on treating infection and simultaneously assess inflammatory response, epithelial barrier integrity, and macrophages transmigration towards the focus of infection. The use of a CF cell line is quite relevant here, since this cell (e.g.: CFBE41o-) has particular influence on *P. aeruginosa* as presented in the introduction and in the discussion part as well. The new information added in the introduction regarding the novelty of the present model can be found in lines (113-133):

"However, in the lung, the mucosal epithelium is exposed to air. Moreover, immune cells present in the airways, like tissue macrophages, play an essential role against inhaled pathogens or particles. Macrophages migrate through the different cell layers to reach the bronchial lumen and fight the infection. Furthermore, inhaled drugs also have to cope with the presence of mucus as an additional non-cellular element of the pulmonary air-blood barrier. Indeed, several complex three-dimensional (3D) *in vitro* models have been developed, aiming to increase the *in vivo* relevance. Co-culture systems not only increase the complexity of *in vitro* systems for drug discovery but also enable to study cell-cell interactions. Such complexity has been addressed in studies about macrophage migration, the release of antimicrobial peptides by neutrophils, the role of mucus in infection, and the epithelial cell reaction to excessive damage. However, a reliable CF infected *in vitro* model that features the genetic mutation in CF, that is exposed to the air (more physiological condition), and integrates immune cells is still lacking.

To close this gap, we here describe a protocol of a stable human 3D co-culture of the infected airways. The model is constituted of human CF bronchial epithelial cells and macrophages, infected with *P. aeruginosa* and capable of representing both a diffusional and immunological barrier. Aiming for testing anti-infectives at reasonably high throughput, this co-culture was established on the permeable filter membrane of well plate inserts, using two human cell lines: CFBE41o- and differentiated THP-1 macrophage. Moreover, to

eventually study the deposition of aerosolized anti-infectives³, the model was established at the air-liquid interface (ALI) rather than liquid covered conditions (LCC)".

* The level of detail in this protocol may not be appropriate for the novice user and more accurate vocabulary (i.e. more descriptive and less colloquial) would be better (e.g. feeding cells)

Thank you for this comment. The whole protocol has been thoroughly revised and accordingly changed, as requested.

* The recommended times should be less finite so that new tissue culture users understand that endpoints are based on culture characteristics rather than specific times.

Thanks for this comment. We have changed accordingly and added a note for the specific case of the protocol established here. Please see lines 182-188, in the protocol session:

1.1.4. To shift to the air-liquid interface (ALI) culture, on the third day after seeding, remove the medium from the basolateral side first, then from the apical side. Add in the basolateral side 500 µL of fresh MEM and change the medium every second day until cells form a confluent monolayer.

Note: for the conditions used in this protocol, the CFBE410⁻ cells usually are confluent after 7 days in culture.

* Alternate products or equipment could be suggested in case the specific items are not available. E.g. Line 150, 151, 164, 182, 218, 251-255, 284, 291-300

Thanks for this comment. We have included, wherever possible, a suggestion for products or equipment, as follows:

Line 150 (now line 167): "Count the cells with an automated cell counter or hemocytometer chamber"

Line 151 (now line 168): we added a note for possible replacement of the permeable filter support:

"Note 2: Permeable supports with a pore size of 0.4 µm could be used here; however, the macrophages, in this condition, should be added directly to the apical side, and their migration won't be assessed in this case. " (line 173-175)

Line 164 (now line 193-195): "in this case, where STX2 chopstick electrodes and EVOM are used to measure TEER, we did not add any alternative to that since this is the standard method to assess TEER on cells growing on permeable filters. Any other possibility should be tested and established by the user."

Line 182 (now line 217): a note was added in the line 220/221: “Note: macrophages can also be detached with trypsin–EDTA, 37 °C for 20 min. However, trypsin is harsher to macrophages than accutase. “

Line 218 (now 264-265): “Note: other strains of *P. aeruginosa* could also be used here, for instance, PAO1 wild type, PA14, or clinical strains.”

Lines 251-255 (now lines 316-318): imaging co-cultures on permeable filters is quite challenging with a normal epifluorescence microscope. Therefore the user of a confocal microscope is essential here. We nevertheless rewrote the previous information as following:

“4.3. Use a confocal microscope for imaging the stored membranes. Choose 25 x or 63 x water-immersion objectives and lasers at 405, 488, 505 or 633 nm for detection. Pictures should have a 1024 × 1024 pixel resolution.

Note: the lasers are chosen according to the staining used. “

Line 284 (now line 361-364): regarding the LDH assay, unfortunately, in case of infected samples, we don't have an alternative. The classical cell viability assays like MTT don't distinguish between the viability of cells and bacteria. However, in case of LDH, this is specifically for the enzyme release from the human cells, being therefore a more precise method to assess host cell viability.

Line 291-300 (now lines 374-385): here we have already described both possibility to detect cytokines, either via ELISA or cytometric bead array immunoassay. Still, we improved the text as following:

“7.1. For cytokine quantification, either ELISA or cytometric bead array immunoassay⁴ is used. For that, centrifuge supernatant from 5.1. at 21250 x g for 10 min and measure either immediately or store -80 °C for up to 15 days till analysis.

7.2. Supernatants are evaluated with a commercially available ELISA kit (see table of material). The procedure follows the manufacture instructions, which include the coating of plates with the capture antibody, addition of the samples (100 µL), incubation, washing, and addition of detection antibody to create a colorimetric measurement of cytokine presence.

7.3. When appropriate, flow cytometry is used to measure further cytokines secreted by uninfected or infected cells via commercially available kits (see table of materials), treated or not with tobramycin.”

* While CFBE41o-, form tight junctions and polarize under appropriate conditions (Illek et al. Cell Physiol Biochem 2008), they do not produce mucous or cilia (as discussed by the authors). Other cells types, such as primary immortalized cells, may be more representative,

e.g. CuFi cells (ATCC), UNCCF cells (Am J Physiol Lung Cell Mol Physiol. 2009 Jan;296(1):L82-91). Alternately, more than one immortalized cell line could be used to compensate for deficiencies of individual cell lines.

The reviewer is correct with this observation. While all the cells mentioned and others could be used in this model, they do not have the genetic mutation in the CFTR channel, as it is the case in cystic fibrosis disease. As we are aiming for an infected model of the CF lung, we consider it essential to have the epithelial cells with this mutation and thereby have a model as closest as possible to the *in vivo* scenario. As you describe in the discussion, there are essential characteristics related to the CFTR mutation that might play a role in the infection and test of drugs:

Lines 559-565: "The use of a CF cell line implements some of the characteristics of the disease in the model. The mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) is directly related to the dysregulation of epithelial fluid transport in the lungs. Furthermore, mutations in the CF gene, such as the $\Delta F508$, results in thick mucus, with inflammation and severe lung damage upon infection with *P. aeruginosa*⁵. These pathological manifestations caused by dysfunctional CFTR potentially involve autophagy impairment as an important cellular mechanism associated with the pathogenesis of CF lung disease⁶."

* Authors should briefly discuss caveats of TPH-1 cell line. E.g. Less responsive to LPS vs primary monocytes, low levels of CD14. Ann Transl Med. 2016 Nov; 4(21): 438.

Thanks for this suggestion. We included the following paragraph in the lines 603-611:

"As with any model, the infected co-culture also has some limitations; for instance, the integration of the macrophage-like cells. As for us, it was important to have macrophages on the basolateral side; however, the manipulation of the insert with a previously grown epithelial layer may have provided early damage and disturbances on the co-culture. Although, the permeable support model provided high-throughput characteristics, which has not been presented on previous co-cultures of the CF infected lung^{7,8}. With that, further experiments need to assess the limitations of using THP-1 as macrophage substitute. While this cell line is widely used, it is less responsive to LPS⁹ and it lacks full activation and not the entire population is differentiated from monocytes to macrophage-like cells¹⁰."

* Results show a significant drop in TEER following infection with PAO1. If the authors previously claimed that inserts with TEER <300 were not used (line 160) is that not an indication that the monolayer is too damaged to be considered biologically relevant? Specific examination of barrier (e.g., tight junctions) is recommended

Thanks for this comment. The threshold set for permeable supports <300 $\Omega \cdot \text{cm}^2$ is based on measurements with primary bronchial cells. Still, uninfected epithelial cells had a higher TEER (834 $\Omega \cdot \text{cm}^2$), which is reduced to 250 $\Omega \cdot \text{cm}^2$ after *P. aeruginosa* infection. Such reduction is not related to a previously damaged monolayer, as the reviewer suggested, but somewhat

related to the toxic characteristics of this bacteria to the cells. Extra experiments were performed to access the barrier, with immunostaining for ZO-1 protein (Figure 1B and 2B).

Compliance with research standards:

* Availability of materials: Catalog number for CFBE410- is given as Dr. Dieter C. Gruenert, for which there is no longer an active laboratory. Authors should provide an updated source. CFBE410- are commercially available from Sigma-Aldrich. Additionally, what is the recommendation of max 20 passages based on (Line 140)? Elaborate, provide reference, or remove.

Thanks for this comment. The CFBE410- cells were given to us as a gift from Dr. Dieter C. Gruenert. Now there is a program called: "GRUENERT CELL LINE DISTRIBUTION PROGRAM" to distribute the cells from Dr. Gruenert, via a MTA. We therefore included this source as well as the Sigma-Aldrich source in the table of material. For the recommendation of max 20 passages, this is indeed our internal procedure to keep the track on cell culture standards. However, this might differ from lab to lab and it is not essential information for the 3D co-culture protocol. Therefore we decided to remove this information from the paper.

Technical quality and efficiency:

* DAPI staining in co-culture samples is problematic as it stains both epithelial cells and THP-1 cells. Because of this, the blue channel overpowered the images, making it difficult to decipher the other fluorescent channels. A cell-specific staining protocol would be preferable.

Indeed we would need to further optimize our DAPI staining and we did use specific staining by using dyes separately before the establishment of the co-culture. In that way, we first stained CFBE410- in the permeable support, and in parallel the THP1 before its seeding on the support (figure 1A). A cell-specific staining would be great, but that would require cell transfection of both cell lines. We can work on that, but so far, the current combination was able to initially give us enough information.

* Lack of proper controls: Confirmation of cell lysis (Protocol Step 5.2), bacterial cell death due to freezing (Protocol Step 5.2), cell death due to high centrifugation speeds (Protocol Step 1.1.1 and 5.3), LDH at 20 hpi, and uninfected tobramycin-treat controls (line 234)

Thank you for this comment. All those experiments were additionally done and can be found in figures S1 and S2. Cell lysis was happening after treatment with water and scraping; bacterial viability was slightly reduced via freezing; there was no cell death after centrifugation of mammalian cells and only a small reduction in CFU after very high centrifugation speeds. Uninfected tobramycin-treated controls were done showing no toxicity and no further cytokine release in comparison to the control. We also showed that no LDH could be detected after 20 hour infection of pure medium initially supplemented with LDH, proving that LDH can only be detected after short incubation times and in longer

incubation times only with treatment, so that less bacteria are present. Overgrowing bacteria apparently destroy LDH.

* Lacks statistical rigor: n values are low, description of statistical methods and results (e.g. error bars) are often lacking

Where applicable, statistical methods are described in the legends, error bars and n-values are all indicated after revision. For each experiment and variable (like time, treatment of not) we have 3 permeable supports, as biological replicate. Therefore now, by showing the number of biological replicates from at least 2 independent experiments, we could have n=6 (when 2 experiments were performed, for instance), and so on.

* Time in culture at ALI may be insufficient for epithelial cell differentiation. This could have a significant effect on the presence/absence of relevant structures (e.g. cilia), cell types (e.g. mucous producing cells), barrier/TEER (Tissue Eng Part B Rev. 2014 Feb;20(1):56-72). Baseline culture (i.e., uninfected at day 7 ALI) should be better characterized using histology and/or electron microscopy.

Thanks for this comment. As discussed in the paper, the CFBE410- fail to produce mucus or cilia even when culture for a period longer than 7 days. However, the TEER could be established in this short period of time, which was the reason we choose to set up the coculture after 7 days. For the purpose of the protocol developed here we do not see a need for further characterization of uninfected cells. Still, this suggestion is very much appreciated and we will definitively consider them for our future work on this and other models we are working on.

* The membranes of tissue culture inserts are often coated with extracellular matrix material (ECM) prior to epithelial culture. ECM can affect attachment, polarization, differentiation (Tissue Eng Part B Rev. 2014 Feb;20(1):56-72.) and response to drugs (Langhans. Front Pharmacol. 2018; 9: 6.) If this was not completed for a specific reason, the authors should discuss the discrepancy from common practice.

Thank you for this comment. Based on our previous work with these cells the coating is not necessary, which makes sense if these cells do not differentiate. We included this information in the discussion, to support our protocol (lines 616-620):

“Differently from what is observed by other cells, the tissue culture inserts coating with an extracellular matrix material (like fibronectin or collagen I) do not show a significant difference for CFBE410⁺, for instance in TEER. Therefore for this protocol, the permeable filters were not coated with an extracellular matrix material in this protocol”.

* re: Differentiation with PMA. What type of macrophage does this produce, why is it relevant to the model? Reference 14 (Schwende et al 1996) suggest that the cells will not proliferate post-PMA exposure, but this should not be considered common knowledge. This reference is not open access so some details should be provided.

Thank you for this comment. We included the following note on section 1.2.1.: “Note: After the differentiation with PMA cells do not proliferate anymore and attach to the flask.” Usually, after the PMA differentiation macrophages are expected to differentiate towards a M1 phenotype, what of course is relevant to the model. However, a better characterization with specific markers for M1 macrophages is needed that we can confirm that in the future.

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* Re: LDH assay, PAO1 + tobramycin <0% LDH- Assuming this is following the protocol in subsection 3 (line 234) then the apical volume will be 500ul of MEM. Is the LDH release lower here because the it has been diluted (vs. collecting 100ul apical in samples infected with PAO1, line 229)? Please clarify.

Thanks for this comment. As described in step 5.1., we added 500 µl of MEM in the apical side, and combined with 500 µl of MEM from the basolateral side, resulting in 1mL solution to read the LDH. Therefore there was no dilution that could explain the negative values. We indeed are facing this negative values in some LDH samples in other experiments in our group, for which we have so far not found a reasonable explanation. Still we don't see the negative values in our results as so problematic, since they are $\leq 5\%$. One possibility in the future is perhaps to determine the exact amount of LDH that is released instead of percentage.

General impact of article:

* Novelty: Other epithelial-macrophage co-culture models (1. NPJ Microgravity. 2017 Feb 28;3:10, 2. Sci Rep. 2017; 7: 45270, 3. ALTEX. 2018;35(2):211-222), but this appears to be the first with epithelial cells derived from patients with Cystic Fibrosis (CF).

Thanks for this comment. Yes this protocol is the first with a CF cell line derived from a patient, and set at ALI condition.

Reviewer #2:

Manuscript Summary:

The author describe a very nice modification and enhancement of a co-culture model of Pseudomonas and an airway epithelial cell line.

Major Concerns:

I have no major concerns.

Minor Concerns:

The text is full of very confusing and poorly written sentences. A few examples include:

Line 154. Lift to the air-liquid interface (ALI) on the third day after seeding by removing the medium from the basolateral side first, then from the apical side. The text should be "To shift to the air-liquid interface (ALI) culture, on the third day after seeding remove the medium from the basolateral side first, then from the apical side.

We thoroughly checked the whole manuscript and let it proof-read by a native speaker. The mentioned sentence is now corrected (lines 182-185):

1.1.5. To shift to the air-liquid interface (ALI) culture, on the third day after seeding, remove the medium from the basolateral side first, then from the apical side. Add in the basolateral side 500 μ L of fresh MEM and change the medium every second day until cells form a confluent monolayer.

Section 1.1.5. To assess the epithelial barrier properties, incubate CFBE41o⁻ cells with 500 μ L apical and 1.5 mL of MEM in the basolateral side for 1 hour. Use STX2 chopstick electrodes and an epithelial volt-ohmmeter for measurements. What day to measure TEER?

Thanks for the comment. The information is now added in the lines 190-198:

1.1.5 Assess the epithelial barrier properties on day 7 by incubating CFBE41o⁻ cells with 500 μ L cell medium in the apical side and 1.5 mL in the basolateral side for 1 h, at 37 °C, 5 % CO₂.

1.1.6 Measure barrier properties via transepithelial electrical resistance (TEER), with an STX2 chopstick electrode and an epithelial volt-ohmmeter, which after 7 days, is higher than 300 Ω *cm².

Section 1.2.3. Store the cell suspension in a cell incubator while waiting for the establishment of the co-culture. How long?

Thanks for this question. We incubated for 1 hour. The information is now added in the lines 220-222:

1.2.3. After cell removal with accutase, re-suspend macrophage cells in 3 mL of THP-1 medium into a 15 mL-falcon tube, count the cells as described in 1.1.2. and incubate for a maximum 1 h at 37 °C, 5% CO₂ before setting up the co-culture.

Section 2.1. Use the Transwell® inserts with CFBE41o-monolayers at ALI once they reach TEER > 300 $\Omega \cdot \text{cm}^2$. Remove the medium from the lower and upper chamber, respectively. In Ali there should be no fluid to remove if the cells are polarized.

Thanks for this observation. The reviewer is right. We corrected the text as follows (lines 235-238):

2.1. Use CFBE41o⁻ monolayers at ALI with TEER $\geq 300 \Omega \cdot \text{cm}^2$ (step 1.1.4.): remove the medium from the lower chamber, carefully turn the support up-side-down inside a sterile glass Petri dish (50 x 200 mm), and remove the cells overgrown through the membrane pores on the bottom side of the membrane using a cell scraper.

Line 192 Note: lung epithelia can be stained at this step and 12h before the addition of macrophages to the system. The protocol is described below (item 1.2.4.); however, instead of a cell suspension, the dye solution with CFBE medium should be applied (500 μL apical side only) on the adhered cells in the Transwell® insert. Confusing and this is described above

Thanks for this comment. We changed the text to improve clarity (lines 244-246):

Note 2: CFBE41o⁻ lung epithelial cells can be stained at this step. The procedure at 1.2.4. can be used; however, instead of a cell suspension, the dye solution in MEM is applied (500 μL apical side only) on the adhered cells on the permeable support.

Section 2.2. Remove the cells that are overgrown through the membrane pores in the basolateral bottom of the transwell. This is not clear.

Thanks for this observation. The new information is now on lines 237-238:

“and remove the cells overgrown through the membrane pores on the bottom side of the membrane using a cell scraper”.

Section 3.8. For treatment experiments, add 500 μL apical of tobramycin 6 $\mu\text{g}/\text{ml}$ in -MEM and 1.5mL of fresh medium in the basolateral side. How was this dose calculated and what was the purpose of the TB?

Here two solutions were prepared. One with tobramycin in cell medium and another of cell medium without tobramycin. Previously, tobramycin powder was weighted, diluted with PBS and this stock solution is at 1mg/mL. Then to obtain 10ml of 6 $\mu\text{g}/\text{ml}$ of tobramycin, we take 60 μL from the stock solution into 9.940 ml of cell medium. The purpose of tobramycin was to check the effect of bacterial reduction on cell mediated characteristics of the co-culture as explained. The concentration of tobramycin was chosen on previous considerations based on published information and in-house MIC tests that show for 6 $\mu\text{g}/\text{ml}$ a destruction of planktonic cells on plastic, nevertheless this situation is changed on cells (as shown in figure

4). Higher concentrations of tobramycin are necessary to completely eradicate these bacteria on cells. A general problem in comparing the *in vitro* from the *in vivo* situation is the correlation of a certain dose inhaled and the concentration of an antibiotic in a solution. This question is still not completely answered and should be addressed in future experiments.

Line 254. Acquire apical and cross-section views, plus zeta-stack mode (10-15 stacks) for the construction of a three-dimensional model using LAS X software. what is Las X? Leica?

Yes, LAS is from Leica. We removed this part due to JOVE guidelines.

Section 5. Measurement of bacterial proliferation by colony-formation units (CFU)-this section is very confusing.

We have modified the whole section now to the following (lines 325-357):

5. Measurement of bacterial proliferation via colony-forming units (CFU)

5.1. After infection, the apical and basolateral medium (containing bacteria) is collected to assess CFU of non-attached bacteria. 500 µL are withdrawn from the apical and basolateral side and pooled.

Note: Use this suspension directly to count bacteria (step 5.4) or centrifuge at 21250 x g for 10 min to evaluate LDH from the supernatant (step 6) and/or re-suspended bacteria in PBS to count (step 5.4).

5.2. The survival of bacteria attached and/or internalized in the cells is performed by adding 500 µL sterile deionized cold water in each compartment of the permeable support. The cells are incubated for 30 min at room temperature.

Note: the samples can either be plated on LB agar (see step 5.4) or frozen (as whole insert plate) at -20°C for plating later on.

5.3. For assessing CFU of adherent/internalized bacteria thaw samples at 37 °C for 10 min (if frozen). Using pipette tips for each well, scrape the membrane surface and pipette up and down to remove all adhered content.

Note: at this step, all the epithelial cells are lysed and adherent/internalized bacteria available as a suspension to be plated.

5.4. With the bacterial suspension from both fractions, perform a 1/10 serial dilution using PBS/Tween 80 0.05% and plate the bacteria on LB agar plates.

Note: Dilutions between 1 to 10 are recommended. The bacteria should be counted in the highest dilution, where single colonies are first identified.

5.5. Incubate agar plates at 30 °C for 16-72 h to count colonies, and calculate CFU accordingly.

Note: The variation in the time of plate incubation at 30 °C is essential for treated-samples and to observe delayed-growth of colonies.

Section 4.1. After co-culture establishment and infection, remove all liquid and fix the Transwell® inserts with paraformaldehyde 3% for 1 h at room temperature (300 µL apical/600 µL basolateral), then use 5 µg/mL of DAPI-PBS staining for the epithelial layer for 30 minutes, at room temperature. How long?

For 30 minutes.

Section 5.2. Add sterile Milli-Q water in the apical and basolateral sides (500 µL each) of the Transwell® inserts for cell lysis of 15 minutes at room temperature and freeze it at -20°C. Why? How does this relate to CFU described in this section?

In order to obtain internalized bacteria, we need to produce cell lysis, to ensure that bacteria attached and eventually internalized are released. For that, we used a method to increase the cell's turgor pressure with water. After 30 minutes (error from previous version, 15 min changed to 30 min), we have a mixture of a suspension of cell debris, adherent and internalized bacteria. To ensure all the cells are detached even after the lysis with water, wells are scraped with a pipette tip. With this procedure no cells are visible any more (Figure S1 A/B). The freezing procedure is due to the fact that we often have a big set of experiments with different time points that should be comparable. If the plating is done in different days for each time, for instance, it is hard to compare. As the samples are diluted, we tried to keep the same solutions, and the same procedure during the plating. Nevertheless, variation by handling might happens. Therefore we decided to freeze the samples and plate them afterwards together (for each single experiment) to avoid such variation. The bacteria freezing for late CFU assay has indeed been well established and used for other bacteria, like *Mycobacterium* spp., *S. aureus* (Ribeiro et al 2017, doi: 10.1186/s12866-017-1102-7; Dimer et al 2019 <https://doi.org/10.1016/j.nano.2019.102125>; Therefore here, we froze the samples containing *P. aeruginosa* and plated afterwards. These bacteria are described as "CFU adhered/internalized" (Figure 4 E/F).

Reviewer #3:

Manuscript Summary:

The manuscript is an original description of a co-culture model of the human cystic fibrosis bronchial epithelial cell line CFBE41o- and the human monocyte-derived macrophage cell line THP-1 to mimic pulmonary infection by biofilm-forming *P. aeruginosa* under air-liquid interface (ALI) conditions. This model allows to simultaneously assess epithelial barrier integrity, macrophage transmigration, bacteria survival, and inflammation. This is very

important issue for assessing drug efficacy and host responses that could be relevant for discovering new anti-infectives.

Major Concerns:

Line 273: How to distinguish adherent from internalized bacterial cells ?

Thanks for this question. Usually adherent bacteria are differentiated from internalized, either by using immunostaining (de Souza Carvalho et al; Cell Micro <https://doi.org/10.1111/j.1462-5822.2011.01615.x>), or when one uses an antibiotic that don't permeate the mammalian cell membrane, and thereby kill only extracellular bacteria (like gentamycin; Dimer et al, Nanomedicine. 2019 Nov 18;24:102125. doi: 10.1016/j.nano.2019.102125). In the case of *P. aeruginosa*, this is known to be an extracellular pathogen, however we can not be 100% sure that no bacteria get internalized by epithelial cells. Nevertheless they are internalized by macrophages as we demonstrated in figure 3. Whether all internalized bacteria by macrophages are killed, remains to be investigated. Therefore we have to assume always when dealing with CFU of bacteria, those that are attached and/or internalized, mainly because the concentration of tobramycin used here was not enough to kill 100% of the bacteria.

Line 307: a MOI of only 1:1 seems to be very low. Take a look at manuscript PMID: 27671059. A higher density of bacterial cells would be recommended

Although MOI 1:1 is low, our previous experiments showed no difference on bacterial proliferation or for increased formation of bacterial aggregates resembling biofilm when we used 1:20 (data not shown). Therefore, we wanted to preserve the viability of both cells lines in order to increase the experimental window. A higher infection would decrease the time of experiments and this in turn the co-culture as unavailable to the understanding of cell-to-cell interaction or response to tobramycin.

Minor Concerns:

Line 141: remove medium

Thank you. It is removed. The new sentence is on line 158-160:

“Cultivate CFBE41o⁻ in a T75 flask with Minimum Essential Medium (MEM) containing 10 % fetal calf serum (FCS), 1 % non-essential amino acids and 600 mg/L glucose at 37 °C with 5 % CO₂ atmosphere. Add fresh medium to the cells every 2-3 days”.

292: Do not use personal verbs

Thanks for this comment. All personal verbs were altered.