Journal of Visualized Experiments

P. aeruginosa Infected 3D Co-Culture of Bronchial Epithelial Cells and Macrophages at ALI for Preclinical Evaluation of Anti-Infectives

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61069R1
Full Title:	P. aeruginosa Infected 3D Co-Culture of Bronchial Epithelial Cells and Macrophages at ALI for Preclinical Evaluation of Anti-Infectives
Section/Category:	JoVE Immunology and Infection
Keywords:	biofilms, antibiotics, lung infections, cystic fibrosis, inflammation, interleukins, TEER, alternatives to animal testing
Corresponding Author:	Claus-Michael Lehr Helmholtz-Institut fur Pharmazeutische Forschung Saarland Saarbrücken, Saarland GERMANY
Corresponding Author's Institution:	Helmholtz-Institut fur Pharmazeutische Forschung Saarland
Corresponding Author E-Mail:	Claus-Michael.Lehr@helmholtz-hips.de;Cristiane.Carvalho@helmholtz-hips.de
Order of Authors:	Carlos Victor Montefusco-Pereira
	Justus C. Horstmann
	Thomas Ebensen
	Christoph Beisswenger
	Robert Bals
	Carlos A. Guzmán
	Nicole Schneider-Daum
	Cristiane de Souza Carvalho-Wodarz
	Claus-Michael Lehr
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Saarbrücken, Saarland, Germany

HIPS HELMHOLTZ Institute for Pharmaceutical Research Saarland

Helmholtz-Institute for Pharmaceutical Research Saarland | Department Drug Delivery |
Campus E8.1 | 66123 Saarbrücken | Germany

Dr. Jaydev Upponi
Science Editor
Immunology and Infection
JoVE

Dr. Cristiane de Souza Carvalho Wodarz

Department Drug Delivery

Phone +49 (0)681 988-06-1060
Fax +49 (0)681 988-06-1009

cristiane.carvalho@helmholtz-hips.de

Your Ref. Our Ref. Date
16 March 2020

Dear Dr. Upponi,

Please find enclosed the reviewed version of our manuscript for submission to **JoVE** with the title “Establishment of a Pseudomonas aeruginosa infected-3D co-culture of bronchial cells and macrophages for preclinical evaluation of anti-infectives”, by Carlos rictor Montefusco-Pereira, Justus C. Horstmann, Thomas Ebensen, Christoph Beisswenger, Robert Bals, Carlos A. Guzmán, Nicole Schneider-Daum, Cristiane de Souza Carvalho-Wodarz, Claus-Michael Lehr.

We are grateful for all suggestion from the reviewers and editors, which definitively contributed to improving the quality of our paper. We addressed each reviewer´s comments carefully in the rebuttal letter. Extra experiments were performed according to the reviewer's feedback, for instance, immunostaining for tight junctions proteins, with ZO-1. Moreover, helpful controls that were missing before are now included in this reviewed version; that consists of the survival of bacteria and mammalian cells after high-speed centrifugation, bacteria survival after freezing at -20 °C and LDH quantification upon infection for 20h. Moreover, the quality of the figures was improved; for instance, confocal microscope pictures as well as all the graphics, in which data were thoroughly checked for consistency and accuracy.

The manuscript has been thoroughly checked and modified for better clarity and proofed-read by an English native speaker. All the reviewer´s comments and suggestions were crucial for the

Helmholtz-Institute for Pharmaceutical Research Saarland
Campus E8.1
66123 Saarbrücken
Germany
www.helmholtz-hips.de

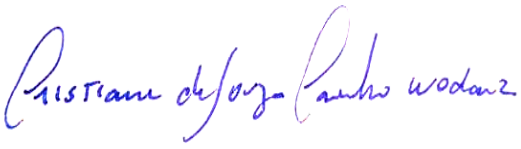
improvement of our manuscript, which we believe has reached the standard level for publication at JoVE.

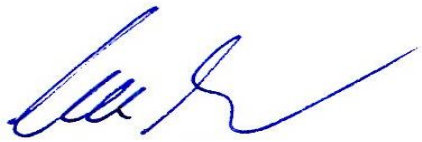
Given that the second author, Justus Hostmann, performed new experiments and contributed significantly to this reviewed version, we agreed that he should share the first co-authorship with Carlos Montefusco, as indicated in the paper.

We certify that this reviewed manuscript, or any part of it, has not been published and will not be submitted elsewhere for publication while being considered by the JoVE. All authors have read and approved the manuscript for submission.

Thank you in advance for your consideration of this manuscript.

Yours sincerely,





Dr. Cristiane de Souza Carvalho Wodarz Prof. Claus Michael Lehr

TITLE:

P. aeruginosa Infected 3D Co-Culture of Bronchial Epithelial Cells and Macrophages at Air-Liquid Interface for Preclinical Evaluation of Anti-Infectives

AUTHORS AND AFFILIATIONS:

Carlos Victor Montefusco-Pereira[1,2], Justus C. Horstmann[1,2], Thomas Ebensen[3], Christoph Beisswenger[4], Robert Bals[4], Carlos A. Guzmán[3], Nicole Schneider-Daum[1], Cristiane de Souza Carvalho-Wodarz[1], Claus-Michael Lehr[1,2]

[1]Department of Drug Delivery, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), 66123 Saarbrücken, Germany.
[2]Department of Pharmacy, Saarland University, 66123 Saarbrücken, Germany.
[3]Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Inhoffenstr 7, Braunschweig, 38124, Germany
[4]Department of Internal Medicine V – Pulmonology, Allergology, Respiratory Intensive Care Medicine, Saarland University Hospital, Kirrberger Straße 1, 66424 Homburg, Germany.

Corresponding Author:

Cristiane de Souza Carvalho-Wodarz (cristiane.carvalho@helmholtz-hips.de)
Claus-Michael Lehr (claus-michael.lehr@helmholtz-hips.de)

Email Addresses of Co-Authors:

carlos.montefusco@helmholtz-hips.de
Justus.horstmann@helmholtz-hips.de
thomas.ebensen@helmholtz-hzi.de
christoph.beisswenger@uks.eu
robert.bals@uks.eu
carlos.guzman@helmholtz-hzi.de
nicole.schneider-daum@helmholtz-hips.de

KEYWORDS:

Biofilms, antibiotics, lung infections, cystic fibrosis, inflammation, interleukins, TEER, alternatives to animal testing

SUMMARY:

We describe a protocol for a three-dimensional co-culture model of infected airways, using CFBE41o$^-$ cells, THP-1 macrophages, and *Pseudomonas aeruginosa*, established at the air-liquid interface. This model provides a new platform to simultaneously test antibiotic efficacy, epithelial barrier function, and inflammatory markers.

ABSTRACT:

Drug research for the treatment of lung infections is progressing towards predictive in vitro models of high complexity. The multifaceted presence of bacteria in lung models can re-adapt epithelial arrangement, while immune cells coordinate an inflammatory response against the

bacteria in the microenvironment. While in vivo models have been the choice for testing new anti-infectives in the context of cystic fibrosis, they still do not accurately mimic the in vivo conditions such diseases in humans and the treatment outcomes. Complex in vitro models of the infected airways based on human cells (bronchial epithelial and macrophages) and relevant pathogens could bridge this gap and facilitate the translation of new anti-infectives into the clinic. For such purposes, a co-culture model of the human cystic fibrosis bronchial epithelial cell line CFBE41o$^-$ and THP-1 monocyte-derived macrophages has been established, mimicking an infection of the human bronchial mucosa by *P. aeruginosa* at air-liquid interface (ALI) conditions. This model is set up in seven days, and the following parameters are simultaneously assessed: epithelial barrier integrity, macrophage transmigration, bacterial survival, and inflammation. The present protocol describes a robust and reproducible system for evaluating drug efficacy and host responses that could be relevant for discovering new anti-infectives and optimizing their aerosol delivery to the lungs.

INTRODUCTION:

Pseudomonas aeruginosa is a relevant pathogen in cystic fibrosis (CF) that contributes to lung tissue impairment[1]. The production of polysaccharides, such as alginate and other mucoid exopolysaccharides, coordinates the progress of the disease, which leads to tenacious bacterial adherence, limits the delivery of antibiotics to bacteria and protects the bacteria against the host immune system[2]. The transition of *P. aeruginosa* from the planktonic stage to biofilm formation is a critical issue in this context, also facilitating the occurrence of antibiotic tolerance.

In the context of CF, the mouse has primarily been used as a model. Mice, however, do not spontaneously develop this disease with the introduction of CF mutations[3]. Most of the bacterial biofilm development and drug susceptibility studies have been performed on abiotic surfaces, such as Petri dishes. However, this approach does not represent the in vivo complexity. For instance, important biological barriers are absent, including immune cells as well as the mucosal epithelium. Though *P. aeruginosa* is quite toxic to epithelial cells, some labs have managed to co-cultivate an earlier *P. aeruginosa* biofilm with human bronchial cells. These cells originated from cystic fibrosis patients with CFTR mutation (CFBE41o$^-$ cells)[4] and allowed to assess antibiotic efficacy[5] or assess the correction of the CFTR protein during infection[6]. Such a model was shown to improve the predictability of drug efficacy, in addition to enabling characterization of issues with drugs that failed in later phases of drug development[7].

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[8]. 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[9]. 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[10], the release of antimicrobial peptides by neutrophils[11], the role of mucus in infection[9], and the epithelial cell reaction to excessive damage[12]. 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 bridge this gap, we describe a protocol for 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. With the goal of 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 THP-1 monocyte-derived macrophage. Moreover, to eventually study the deposition of aerosolized anti-infectives[13], the model was established at the air-liquid interface (ALI) rather than liquid covered conditions (LCC).

As we report here, this model allows assessing not only bacterial survival upon an antibiotic treatment but also cell cytotoxicity, epithelial barrier integrity, macrophage transmigration, and inflammatory responses, which are essential parameters for drug development.

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 (called the air-liquid interface (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-1[14] 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.

PROTOCOL:

1. Growth and differentiation of cells in permeable support inserts

1.1. 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 with5 % CO_2 atmosphere. Add fresh medium to the cells every 2–3 days.

1.1.1. Detach the cells after reaching 70% confluency in the flask with 3 mL of trypsin-Ethylenediaminetetraacetic acid (EDTA) at 37 °C for 20 min. Add 7 mL of fresh MEM and centrifuge the cells at 300 x *g* for 4 min at room temperature (RT). Discard the supernatant and add new 10 mL of MEM while disrupting the clumps by gently pipetting up and down.

1.1.2. Count the cells with an automated cell counter or hemocytometer chamber. Seed cells with a density of 2 x 10^5 cells/well in 12-well plates with permeable supports (pore size of 3 µm, see **Table of Materials**).

NOTE: The automated cell counter determines cell number, size distribution, and viability of the cells (see **Table of Materials**). 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 will not be assessed in this case.

1.1.3. Seed cells at liquid-liquid condition (LLC) by adding 500 µL of the cell suspension on the apical side of the permeable support and 1.5 mL of fresh medium in the basolateral side. Then incubate cells at 37 °C under 5% CO_2, for 72 h.

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. To the basolateral side, add 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 CFBE41o$^-$ cells usually are confluent after 7 days in culture.

1.1.5. Assess the epithelial barrier properties on day 7 by incubating CFBE41o$^-$ cells with 500 µL cell medium in the apical side and 1.5 mL in the basolateral side for 1 h, at 37 °C under 5% CO_2.

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

NOTE: Eventually, in some membrane inserts, the cells have low TEER. Therefore permeable inserts with TEER < 300 Ω·cm² are not used.

1.2. To cultivate THP-1 cells, grow them in a T75 flask using Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS, and incubate at 37 °C under 5% CO_2. Split cells every second day by seeding 2 x 10^6 cells/mL cells in a new T75 flask.

NOTE: Non-differentiated THP-1 cells are grown as monocytes in suspension.

1.2.1. Differentiate the THP-1 cells as follows. Centrifuge contents of a T75 at 300 x *g* for 4 min. Discard the supernatant, resuspend the pellet in fresh medium and put in a new T75. Add 10 ng/mL Phorbol 12-myristate 13-acetate (PMA) incubated in RPMI for 48 h at 37 °C and 5% CO_2 atmosphere[15].

NOTE: After the differentiation with PMA, cells do not proliferate anymore and attach to the flask.

1.2.2. To detach THP-1 macrophage-like cells, wash once with phosphate-buffered saline (PBS) at 37 °C and incubate with 3 mL of cell detachment solution (e.g., accutase) containing 0.5 mM EDTA for 10 min at room temperature.

1.2.3. Inspect the cells under an inverted microscope to look for cell detachment. Add 7 mL of fresh medium and centrifuge at 300 x *g* for 4 min at RT.

NOTE: Macrophages can also be detached with trypsin-EDTA, 37 °C for 20 min. However, trypsin is harsher to macrophages than the chosen cell detachment solution (see **Table of Materials**).

1.2.4. After cell removal, re-suspend macrophage cells in 3 mL of THP-1 medium into a 15 mL conical tube, count the cells as described in 1.1.2. and incubate for a maximum 1 h at 37 °C under 5% CO_2 before setting up the co-culture.

NOTE: THP-1 cells in suspension can be stained with viability dyes to image the co-culture further. At this step, use the procedure below (step 1.2.5).

1.2.5. Stain macrophages with 10 µM of a cell viability dye (based on the conversion of esterases, see **Table of Materials**) in which 3 µL of the cell viability dye is applied to the cell suspension. Incubate cells for 20 min at 37 °C, 5% CO_2, then wash 1x with PBS 37 °C to remove the dye.

NOTE: Centrifuge the cells to remove the dye at 300 x *g* for 4 min at room temperature (RT).

2. Establishment of an epithelial-macrophage co-culture on permeable supports

2.1. Use CFBE41o$^-$ monolayers at ALI with TEER ≥300 Ω·cm² (step 1.1.4.). Remove the medium from the lower chamber, carefully invert the support inside a sterile glass Petri dish (50 mm x 200 mm), and remove the cells overgrown through the membrane pores on the bottom side of the membrane using a cell scraper.

NOTE: Due to the pore size of 3 µm, epithelial cells tend to grow through the pores toward the basolateral side. Therefore, one needs to remove them before adding the macrophages on this side. CFBE41o$^-$ lung epithelial cells can be stained at this step. The procedure in step 1.2.5 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.

2.2. Use 2 x 10^5 cells/well (in 200 µL of RPMI) from the cell suspension of PMA-differentiated THP-1 macrophages and place the cells on the basolateral side of the inverted inserts.

2.3. Close the Petri dishes carefully and incubate for 2 h at 37 °C under 5% CO_2.

2.4. Place the inserts back into the 12-well microplates and add 500 µL of MEM medium in the basolateral side of the permeable insert to maintain ALI conditions. The cells are now ready for

infection.

3. Infection by *P. aeruginosa*

NOTE: All following steps from here must be done in a biosafety level 2 (BSL2) laboratory.

3.1. Inoculate 15 mL of lysogeny broth (LB) supplemented with 300 µg/mL ampicillin in an Erlenmeyer flask (50 mL) with a single colony of *P. aeruginosa* PAO1-GFP.

NOTE: Other strains of *P. aeruginosa* could also be used here, for instance, PAO1 wild type, PA14, or clinical strains.

3.2. Incubate the bacteria for 18 h at 37 °C, shaking at 180 rpm.

3.3. Transfer the contents after the 18 h to a 50 mL conical tube and centrifuge at 3850 x *g* for 5 min. Discard the supernatant and add 10 mL of sterile PBS at 37 °C.

3.4. Measure optical density on a spectrophotometer at wavelength 600 nm and adjust the concentration of bacteria using the cell culture medium to a final concentration of 2 x 10^5 CFU/mL. This corresponds to a multiplicity of infection (MOI) of one bacterium per epithelial cell.

3.5. Add 100 µL of bacterial suspension to the apical side of the permeable support (step 2.4.) and incubate at 37 °C under 5% CO_2 for 1 h, to allow bacteria attachment to the cells. Then, remove apical liquid carefully with a pipette to restore ALI conditions. Keep some samples uninfected as a control.

NOTE: At this stage, the bacteria attached should be plated in LB agar (see steps 5.4/5.5) to determine the initial bacteria inoculum.

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

NOTE: Instead of instilling the drugs as a solution, the model can also be adapted to aerosol deposition. For such purposes, the cells at ALI are fed from the basolateral side with 500 µL of cell medium. The drug is then first nebulized and allowed to deposit in the apical compartment by an appropriate device (not described here). The infected and treated sample can be checked for the endpoints outlined in the sections 4–7. From this step on, permeable supports can be used to create either images (section 4) or to get results of bacteria growth and mammalian cell viability, amongst others (sections 5–7).

4. Sample preparation for confocal laser-scanning microscopy (CLSM)

4.1. After the establishment of the co-culture, infection and drug treatment, remove all medium

from the apical and basolateral side. Wash 1x with PBS at 37 °C. and then fix the cells with 3% paraformaldehyde (PFA) for 1 h at RT (300 µL on apical/600 µL on basolateral). Cell nuclei are stained with 5 µg/mL of DAPI-PBS for 30 min at room temperature.

CAUTION: PFA is hazardous.

4.2. Cut the membranes using a scalpel and place them between two 12 mm microscopy cover slides using a mounting medium (see **Table of Materials**). Let it dry inside the flow bench for 30 min before storage at 4 °C. Visualize by confocal scanning microscopy.

NOTE: After the co-culture, tight junctions immunostaining can be performed. For that, cells are fixed with paraformaldehyde 3% for 30 min, washed again with PBS, and permeabilized with saponin 0.05%/BSA 1% in PBS. In this protocol, the zonula occludens protein (ZO-1) was detected via mouse anti-human ZO-1 antibody (1:400, incubation at 4 °C overnight). The samples were then incubated for 2 h at RT with goat anti-mouse IgG antibody Alexa Fluor 633 (1:2000 in red). Nuclei were stained with DAPI (1 µg/mL) and mounted with mounting medium on coverslips.

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

NOTE: The lasers are chosen according to the stain used.

4.4. Acquire apical and cross-section views, and use zeta-stack mode (10–15 stacks) for the construction of a three-dimensional model using imaging software.

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

5.1. Collect the apical and basolateral medium (containing bacteria) to assess CFU of non-attached bacteria. Withdraw 500 µL from the apical and basolateral sides and pool them.

NOTE: Use this suspension directly to count bacteria (step 5.4) or centrifuge at 21,250 x g for 10 min to evaluate lactate dehydrogenase (LDH) from the supernatant (section 6) and/or re-suspended bacteria in PBS to count (step 5.4).

5.2. Assess survival of bacteria attached and/or internalized in the cells by adding 500 µL of sterile deionized cold water in each compartment of the permeable support. Incubate cells 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 are 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: A temperature of 30 °C at the time of plate incubation is essential for treated-samples and to observe delayed-growth of colonies.

6. Evaluation of cell cytotoxicity via lactate dehydrogenase assay

6.1. Use the supernatant of infected cells containing bacteria (from step 5.1) for cell viability assessment for LDH assay[16]. Centrifuge the supernatant at 21,250 x *g* for 10 min to pellet the bacteria and eventually rest of the cells. Use the bacteria-free supernatant to measure LDH release.

NOTE: The supernatant should not be frozen before measuring LDH by this assay.

6.2. Transfer 100 µL of the supernatant to a 96-well plate, and add 100 µL of the LDH assay solution (see the **Table of Materials**). Incubate at room temperature for 5 min in the dark, then read absorbance at 492 nm.

7. Assessing the release of human cytokines

7.1. For cytokine quantification, use either ELISA or cytometric bead array immunoassay[17]. For this, centrifuge supernatant from step 5.1 at 21,250 x *g* for 10 min and measure either immediately or store -80 °C for up to 15 days till analysis.

7.2. Evaluate supernatants with a commercially available ELISA kit.

NOTE: 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 provide a colorimetric measurement of cytokine presence.

7.3. When appropriate, use flow cytometry to measure further cytokines secreted by uninfected or infected cells via commercially available kits (see the **Table of Materials**), treated with tobramycin or untreated.

REPRESENTATIVE RESULTS:

Figure 1A shows the morphology of the resulting co-culture of human bronchial epithelial cells and macrophages after growing both for 24 h on the apical and basolateral side of permeable supports, respectively. The epithelial barrier integrity is shown by higher TEER (834 $\Omega \cdot cm^2$) and CLSM by immunostaining for the tight junction protein ZO-1 (**Figure 1B**). The same results observed in terms of barrier integrity of uninfected CFBE41o$^-$ monoculture could be seen in the uninfected epithelial-macrophage co-cultures.

To model a bacterial infection, *P. aeruginosa* was inoculated at a multiplicity of infection (MOI) of 1:1 on CFBE41o$^-$ cells. Six hours after infection (**Figure 2A**), macrophages were observed on the apical side of the co-culture. After the infection, the TEER dropped from 834 to 250 $\Omega \cdot cm^2$, indicating a compromised epithelial barrier, as also visualized by ZO-1 staining (**Figure 2B**).

Figure 3 shows macrophage transmigration through the permeable filter pores and bacteria uptake by THP-1 cells on the apical side. The samples were fixed at 1, 3, and 6 h post-incubation. In the THP-1 monocultures (**Figure 3A–C**), macrophages migration was observed as early as 1 h, while in the co-culture (**Figure 3D–F**), this was seen after 3 h infection. Bacteria uptake in THP-1 was observed after 3 h of infection, in both monoculture and co-culture. No bacterial uptake by CFBE41o- could be seen. Cross-sectional views were placed such that the permeable membrane support was in the middle as a separation of the apical and basolateral compartment.

Figure 4 shows confocal scanning laser microscopy pictures of infected co-cultures (CFBE41o$^-$ + THP-1) treated with or without tobramycin for 6 h (**Figure 4A, B**) or 20 h (**Figure 4C–D**). Without treatment, either the epithelial cells or macrophages died after 20 h of infection (**Figure 4C**). However, upon tobramycin treatment, the host cells are preserved after 20 h; still, some bacteria can be observed in the culture. Despite being seen after 6 h of treatment in the microscopy pictures (**Figure 4B**), the bacteria did not proliferate as observed in CFU assays in **Figure 4E**. Nevertheless, after 20 h treatment, the bacteria recovered the proliferation capability, as seen by the colonies in the CFU assay (**Figure 4F**). The cell lysis protocol with cold water and scraping can release bacteria attached and possibly internalized in cells. At the same time, the cells are destroyed (**Supplementary Figure S1A, B**). The centrifugation steps used in this paper for epithelial cells (300 x *g*) or bacteria (21,250 x *g*) did not hamper the viability of both (**Supplementary Figures S1C, D**). All CFU assays were performed by freezing the samples at -20 °C, followed by thawing and plating. This procedure reduced the number of bacteria by 2-logs, compared to fresh samples (**Supplementary Figure S1E**). As this procedure is done simultaneously for all experimental groups (treated and untreated) at different time points, this reduction will be incorporated in the final results (**Supplementary Figure S1E**). Moreover, the concentration of tobramycin used here showed no toxicity for the uninfected cells (**Supplementary Figure S2A**) and also no further inflammatory response (**Supplementary Figure S2B**). Howver, it was within the range of the minimum inhibitory concentration to kill *P. aeruginosa*.

Figure 5 shows the transepithelial electrical resistance (TEER) and cell viability. **Figure 5A–B** illustrates the TEER of monocultures and co-cultures. The co-culture of CFBE41o$^-$ cells with THP-1 did not induce any change in the epithelial barrier integrity compared to the monoculture (red bars). Upon the infection, the TEER value dropped (green bar). After 1 h of infection, some samples were treated with the antibiotic tobramycin (blue bar), for 6 or 20 h. The treatment preserved the epithelial barrier integrity, as observed by the higher TEER. **Figure 5C** shows the percentage of LDH release as an indication of cell toxicity upon infection and tobramycin treatment after 6 h. The co-culture itself induced a release of LDH, which was the same for the infected cells (around 20%). After 20 h of infection, no signal of LDH could be detected. To prove LDH reliability for long-term infection, PAO1-GFP was incubated in medium with and without LDH 1 U/mL compared to respective uninfected controls (**Supplementary Figure S2C**). The LDH signal was lost after prolonged incubation (20 h) with *P. aeruginosa*, indicating that LDH is only stable in shorter incubation times in infected cultures.

Figure 6 shows the kinetics of pro-inflammatory cytokines detected via ELISA. The advantage of an infected co-culture of CFBE41o- and THP-1 cells was observed with the higher secretions of pro-inflammatory cytokines**.** The secretion of some pro-inflammatory cytokines was either similar (IL-6) or higher (IL-8, TNF-α, IL-1β) in the infected co-culture (**Figure 6C**) than in the corresponding monocultures (**Figures 6A,B**). Unexpectedly, some cytokines in THP-1 monocultures (**Figure 6B**) are downregulated in infected samples (Il-8, TNFα, IL-1β).

Figure 7 demonstrates the release of cytokines in mono- and co-cultures upon infection and treatment with tobramycin measured via fluorescence activated cell sorting (FACS). The secretion of the pro-inflammatory cytokine IL-8 (**Figure 7A**) and the anti-inflammatory cytokine IL-10 (**Figure 7F**) was higher in the co-cultures of epithelial cells and macrophages, compared to the monocultures. However, for all other cytokines (IL-1 α, IL-12p40, IL-23 and GM-CSF) (**Figures 7B–E**), the levels of cytokine secretion were not higher in the co-culture that in the respective monocultures.

FIGURE AND TABLE LEGENDS:

Figure 1: Cross-sections and apical views of the uninfected epithelial-macrophage co-culture. (**A**) Cross views of uninfected 24 h epithelial-macrophage co-culture. CFBE41o$^-$ stained red (CellTrace), THP-1 macrophages yellow (CellTrace) and nuclei blue (DAPI). (**B**) Apical views of the uninfected CFBE41o- monolayer immunostained for ZO-1 (red). DAPI: nuclei. Scale bars: 50 µm.

Figure 2: Cross-sections and apical views of the infected epithelial-macrophage co-culture. (**A**) Cross views and (**B**) apical view of epithelial-macrophage co-culture at 6 h post-infection (hpi) with *P. aeruginosa* PAO1-GFP. CFBE41o$^-$ stained in red (CellTrace), THP-1 macrophages in yellow (CellTrace), nuclei in blue (DAPI) and *P. aeruginosa* PAO1-GFP in green. (**B**) Apical views of the 6 h infected CFBE41o- monolayer. Scale bars: 50 µm.

Figure 3: Kinetics of macrophage transmigration and bacteria uptake visualized by cross-section of the 3D model. PAO1-GFP infection kinetics in monocultures of (**A–C**) THP-1

macrophages or (**D–F**) co-culture. THP-1macrophages (CellTrace Far Red), nuclei of epithelial cells (blue: DAPI), and *P. aeruginosa* (green: GFP). Each figure is divided into apical and basolateral sides, the space in between is considered to be the membrane, which is empty or occupied by the CFBE41o$^-$ confluent layer (D–F). Inserts in the figures show bacteria uptake by macrophages at different times (A–F). Scale bars: 50 µm.

Figure 4: Characterization of PAO1-GFP survival in tobramycin-treated co-culture. (A–D) Confocal micrographs co-cultures with and without treatment. (**A**) Untreated co-culture after 6 h of infection. (**B**) Infected co-culture treated with tobramycin 6 µg/mL (Tob) for 6 h. (**C**) Untreated co-culture 20 h post-infection, (**D**) infected co-culture treated with tobramycin 6 µg/mL for 20 h. Nuclei stained with DAPI (blue), macrophages by CellTrace (red) and *P. aeruginosa* GFP (green). (**E**) Colony-forming units (CFU) of adherent/internalized bacteria after 6 and (**F**) 20 h with tobramycin 6 µg/mL treatment. Empty membrane insert was used as an abiotic substrate to grow PAO1-GFP. Two-way ANOVA with Tukey's multiple comparisons test (# no colonies)was used, *p < 0.05; ***p < 0.001; ****p < 0.0001; ns: not significant). Error bars indicate standard deviation, n = 9–27 replicates of 3–9 independent experiments.

Figure 5: Barrier integrity and evaluation of the viability of mono- and co-culture. The following co-culture conditions were assessed: uninfected (gray bars), infected (green bars), or infected and treated with tobramycin (blue bars). (**A**) Transepithelial electrical resistance after 6 h and (**B**) 20 h of infection in mono-cultures (CFBE41o$^-$ and THP-1) and co-culture. (**C**) Cytotoxicity of mono- and co-culture measured via LDH release 6 h post-infection. Two-way ANOVA with Tukey's multiple comparisons test was used; *p < 0.05; ****p < 0.0001; ns: not significant. Error bars indicate standard deviation; n = 9 replicates of three independent experiments.

Figure 6: Kinetics of cytokine release of uninfected and infected mono- and co-culture supernatants assessed via ELISA. ELISA was done according to the kit manufacturer's protocol. (**A**) CFBE41o-, (**B**) THP-1, and (**C**) co-culture releasing IL-8, TNF-α, IL-1β, and IL-6. Error bars indicate standard deviation. n = 6 replicates of 2 independent experiments.

Figure 7: Supernatant results of cytokine panel measured via FACS with and without tobramycin 6 µg/mL for 6 h post-infection. Supernatants of mono- and co-culture after 6 h post-infection used to analyze the respective cytokines IL-8 (**A**), IL-1α (**B**), IL12p40 (**C**), IL-23 (**D**), GM-CSF (**E**) and IL-10 (**F**). Error bar indicates standard deviation, n = 9 replicates of 3 independent experiments.

Supplementary Figure S1: Control experiments for critical steps of the protocol. (**A**, **B**) Micrographs of CFBE41o- cells in 24-well plates grown for 2 days at density of 2 x 10^5 cells/well. (**A**) CFBE41o- cells in PBS for 30 min, and (**B**) water-treated cells after 30 min after scraping with a pipette. (**C**) Viability of mammalian cells after centrifugation. CFBE41o- cells were removed from T75 cell culture flask as described in step 1.1 and 1.1.1. 100 µL of resulting cell suspension was analyzed in 10 mL isotonic solution. An automated cell counter was used to assess the viability of single cells. Then, respective cell suspensions were centrifuged at 300 x *g* for 4 min, resuspended and counted again. Error bars indicate standard deviation, n = 6 different flasks of

2 individual experiments. (**D**) Viability of PAO1-GFP after centrifugation. PAO1-GFP bacteria were diluted to OD = 0.01 in cell medium. CFU was assessed via a 10-fold dilution row and LB plates incubated overnight at 30 °C. Respective plastic tubes were centrifuged at 21,250 x *g* for 10 min and resuspended in medium. CFU was assessed accordingly again. Two-tailed student's t-test, * $p < 0.033$. Error bar indicates standard deviation, n = 6 of 2 experiments. (**E**) Viability of bacteria after freezing. PAO1-GFP bacteria were prepared as in (D) and CFU was analyzed, then plastic tubes were frozen for one day at -20 °C and thawed to assess CFU again. Two-tailed student´s t-test, *** $p < 0.001$. Error bars indicate standard deviation, n = 6 of two experiments.

Supplementary Figure S2: Control experiments to assess LDH behavior and influence of tobramycin. (**A**) Control experiment to assess cytotoxicity after 20 h of incubation with 6 µg/mL tobramycin. Mono- and co-culture was done as described in the protocol, but cells were grown for 2 days on 24-well plates and THP-1 cells were seeded apically. Cells with 6 µg/mL tobramycin or controls were incubated for 20 h. One-Way ANOVA, Tukey's multiple comparisons test, *** $p < 0.001$. Error bar indicates standard deviation, n = 6 of 2 experiments (CFBE41o-), n = 3 of one experiment (THP-1 and co-culture). (**B**) Control-ELISA of supernatants of mono- and co-culture with/without tobramycin. Cell culture was done according to (A) to show no cytokine release compared to controls for all conditions. ELISA was done in step 7.1 and 7.2, 10 µg/mL (LPS) was added as control, IL-8 release for LPS-treated controls containing THP-1 was higher than detectable. Two-Way ANOVA, Tukey's multiple comparisons test, ns $p > 0.12$; * $p < 0.033$; *** $p < 0.001$. Error bars indicate standard deviation, n = 6 of two experiments, n = 3 of one experiment (LPS control). (**C**) LDH degradation due to excessive PAO1-GFP proliferation. LDH was added at concentration of 1 U/mL to the MEM Medium. Either LDH medium and control medium were used to dilute cells to OD = 0.01 (corresponds to 1 x 10^8 CFU/mL) and then incubate for 20 h. LDH assay was done as described in section 6. One-Way ANOVA, Tukey´s multiple comparisons test, ns $p > 0.12$; ***$p < 0.001$. Error bar indicates standard deviation, n = 8–9 of three individual experiments.

DISCUSSION:

This paper describes a protocol for a 3D co-culture of the infected airways, constituted by the human cystic fibrosis bronchial epithelial cell line CFBE41o- and the human monocyte-derived macrophage cell line THP-1. The protocol allows the assessment of epithelial barrier integrity, macrophage transmigration, bacteria survival, and inflammation, which are important parameters when testing drug efficacy and host-responses simultaneously. The novelty in the model lies within the incorporation of epithelial cells (i.e., human CF cell line and macrophages) with acute bacterial infection (i.e., *P. aeruginosa)*. The acute infection in the epithelial cells is demonstrated to be controlled by an antibiotic (i.e., tobramycin). Besides the use of a human CF cell line, the entire model is set up at ALI conditions, which is considerably closer to the physiological conditions in CF. 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 ΔF508, result in thick mucus, with inflammation and severe lung damage upon infection with *P. aeruginosa*[4]. These pathological manifestations caused by dysfunctional CFTR potentially involve autophagy impairment as an

important cellular mechanism associated with the pathogenesis of CF lung disease[18]. However, the CFBE41o$^-$ fails to secret mucus, which is a limitation of this cell line. If it is intended to study the role of mucus more specifically, the protocol can be adapted by using other bronchial cell lines (e.g., Calu-3).

One critical step to set up this protocol is the combination of epithelial and immune cells and the subsequent infection with *P. aeruginosa* at ALI. The infection of CFBE41o$^-$ by *P. aeruginosa* in vitro has already been described, mainly using a flow-cell chamber, supplemented with arginine in the culture media, to improve epithelial cell survival and support biofilm formation[19]. The present protocol aimed for a new model using only human cells, which moreover, could be grown at ALI on permeable well plate inserts for higher sample throughput. The inclusion of THP-1 differentiated macrophages as a human immortalized cell line, instead of being dependent on obtaining reproducible primary cells from donors, is another advantage of our model. By adding these macrophages to the basolateral side of permeable membrane support, it was observed that macrophages protruded and eventually transmigrated to the apical side of the filter-grown epithelial barrier. A variation of this protocol could be the addition of macrophages directly on the apical side on top of the epithelial cells, as described by Kletting *et al*.[14]. The co-culture of non-human immune and lung cells has already been described before. Ding *et al.*[10] used mouse Lewis lung carcinoma cells on permeable insert supports in combination with macrophages on the basolateral side and infected with *S. aureus*, another critical pathogen of chronic infection in CF patients. However, in this study, there was no focus on cystic fibrosis or to use the co-culture as a platform for the evaluation of drug efficacy. Our protocol can be adapted for other bacterial infections, such as *Staphylococcus aureus*, *Mycobacterium abscessus,* or *Burkholderia cepacia*—important pathogens in CF lung.

Another critical step is the addition of THP-1 macrophages to the cells by flipping the permeable inserts upside-down (section 2). This is crucial to assess macrophage transmigration through the well to the side of infection. The later imaging process from the 3D models with z-stacks, and cross-section view, can be performed to observe the inside of macrophages and detect bacteria uptake (**Figure 3**)**.** At 1 hours post infection (hpi), bacteria applied on the apical side migrate through the membrane, while macrophage migration and uptake only take place at 3 hpi. Therefore after 1 h of infection, it was appropriate to start the treatment with tobramycin and have the possibility to address both host cell and bacteria survival for a long period (20 h). In the course of the protocol, maintaining sterility is a critical issue due to the multitude of steps that each carry the risk of contamination. Nevertheless, experienced cell-culture personnel will be able to follow this protocol after appropriate preparation and training. Cell medium should be regularly checked for contamination, preferably after all critical steps.

As with any model, the infected co-culture also has some limitations; for instance, the integration of the macrophage-like cells. Here, 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 to the co-culture. Although, the permeable support model provided high-throughput characteristics, which has not been observed in previous co-cultures of the CF infected lung[20,21]. 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 LPS[22] and it lacks full activation and the entire population is not differentiated from monocytes to macrophage-like cells[23]. Another limitation is the lack of other key components in CF infection and drug delivery. The CFBE41o$^-$ cell line does not possess cilia nor does it produce mucus, which usually happens 20-30 days of cell culture at ALI. As this was not the case for CFBE41o$^-$ cell line, we used the cells after seven days when a tight epithelial barrier was formed. Mucociliary clearance alters the residence conditions for either microbes[24] or drug particles[9,25] and in vitro models assessing lung deposition should take this into account. 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 CFBE41o$^-$, for instance in TEER[26]. Therefore, the permeable filters were not coated with an extracellular matrix material in this protocol.

With the protocol described here, mono and co-cultures after 6 h infection provide sufficient cytokine release to be used as a measurement in future drug testing. The co-culture brings an advantage of cell cooperation in modeling immune response. The inefficacy of tobramycin in reducing inflammation was expected since not all bacteria were eliminated during the treatment (**Figure 4E, F**). Nevertheless, modeling the response to tobramycin in a CF model is crucial, as tobramycin (in higher concentrations) can be effective in *P. aeruginosa* inhibition, even on biofilm[19,27]. One possibility for further use of this protocol is to integrate anti-inflammatory drugs in the treatment. The overall recommendation regarding inflammatory responses would be to use the short duration treatment (6 h), which still has the host cell and bacteria present. After this time point, the host cells are destroyed in untreated samples. Both ELISA or FACS could be used to measure the release of cytokines. Finally, if the samples are stored longer than 15 days at -80 °C, it is recommended to check the reliability of the cytokines by using, for instance, positive control of fresh samples (e.g., cells stimulated with LPS).

Some modifications of the protocol are possible. For example, the current protocol can be expanded to the application of nebulized drugs (step 3.6). This is necessary to model pulmonary drug delivery via oral inhalation. Nebulization of water-soluble drugs, like tobramycin, or nano-carriers thereof, such as liposomal colistin, is relatively straight forward by commercially available devices routinely used in the clinic. Also, there are several commercially available devices to deposit aerosols onto cell culture inserts. In addition, as the model described here is based on permeable membrane supports, it could also be adopted to some contemporary microfluidic (e.g., “lung on a chip”) devices, for example, to study the influence of breathing and the related mechanical stretching and changes in the airflow. Moreover, this protocol could be modified by the addition of mucus or replacement by primary cells depending on the scientific question to be addressed. Another interesting next step would be the testing of nanomedicines, especially as nanotechnology is making progress in the development of novel anti-infectives[28], CF correctors[29] and co-delivery of antibiotics and pathoblockers[30]. Overall, the current protocol may be perceived as useful in assessing bacterial survival upon antibiotic treatment in a complex system, together with some host-related readouts: cell cytotoxicity, epithelial barrier integrity, macrophage transmigration, and inflammatory response. These are essential parameters for drug development.

ACKNOWLEDGMENTS:

This work received funding from the European Union's HORIZON 2020 Program for research, technological development, and demonstration under grant agreement no. 642028 H2020-MSCA-ITN-2014, NABBA - Design, and Development of advanced Nanomedicines to overcome Biological Barriers and to treat severe diseases. We thank Dr. Ana Costa and Dr. Jenny Juntke for the great support on the development of the co-culture, Olga Hartwig, for the scientific illustration, Anja Honecker, for ELISA assays, Petra König, Jana Westhues and Dr. Chiara De Rossi for the support on cell culture, analytics, and microscopy. We also thank Chelsea Thorn for proofreading our manuscript.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Cutting, G. R. Cystic fibrosis genetics: from molecular understanding to clinical application. *Nature Reviews Genetics.* **16** (1), 45–56 (2015).
2. Lyczak, J. B., Cannon, C. L., Pier, G. B. Establishment of Pseudomonas aeruginosa infection: Lessons from a versatile opportunist. *Microbes and Infection.* **2** (9), 1051–1060 (2000).
3. Wilke, M., Buijs-Offerman, R. M. et al. Mouse models of cystic fibrosis: Phenotypic analysis and research applications. *Journal of Cystic Fibrosis.* **10** (SUPPL. 2), S152–S171 (2011).
4. Moreau-Marquis, S., Stanton, B. A., O'Toole, G. A. Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulmonary Pharmacology and Therapeutics.* **21**, (4), 595–599 (2008).
5. Yu, Q. et al. In vitro evaluation of tobramycin and aztreonam versus Pseudomonas aeruginosa biofilms on cystic fibrosis-derived human airway epithelial cells. *Journal of Antimicrobial Chemotherapy.* **67**, (11), 2673–2681 (2012).
6. Moreau-Marquis, S., Redelman, C. V, Stanton, B. a, Anderson, G. G. Co-culture models of Pseudomonas aeruginosa biofilms grown on live human airway cells. *Journal of visualized experiments : JoVE* (44), 2–5 (2010).doi:10.3791/2186
7. Stanton, B. A., Coutermarsh, B., Barnaby, R., Hogan, D. Pseudomonas aeruginosa reduces VX-809 stimulated F508del-CFTR chloride secretion by airway epithelial cells. *PLoS ONE* **10** (5), 1–13 (2015).
8. Lambrecht, B. N., Prins, J., Hoogsteden, H. C. Lung dendritic cells and host immunity to infection. *European Respiratory Journal*. (18), 692–704 (2001).
9. Murgia, X., De Souza Carvalho, C., Lehr, C. M. Overcoming the pulmonary barrier: New insights to improve the efficiency of inhaled therapeutics. *European Journal of Nanomedicine.* **6**, (3), 157–169 (2014).
10. Ding, P., Wu, H., Fang, L., Wu, M., Liu, R. Transmigration and phagocytosis of macrophages in an airway infection model using four-dimensional techniques. *American Journal of Respiratory Cell and Molecular Biology.* **51** (1), 1–10 (2014).
11. Hartl, D., Tirouvanziam, R. et al. Innate Immunity of the Lung: From Basic Mechanisms to Translational Medicine. *Journal of Innate Immunity.* **10** (5–6), 487–501 (2018).
12. Esposito, S. et al. Manipulating proteostasis to repair the F508del-CFTR defect in cystic

659 fibrosis. *Molecular and cellular pediatrics.* **3** (1), 13 (2016).
660 13. Hein, S., Bur, M., Schaefer, U. F., Lehr, C. M. A new Pharmaceutical Aerosol Deposition
661 Device on Cell Cultures (PADDOCC) to evaluate pulmonary drug absorption for metered dose dry
662 powder formulations. *European Journal of Pharmaceutics and Biopharmaceutics.* **77** (1), 132–138
663 (2011).
664 14. Kletting, S., Barthold, S. et al. Co-culture of human alveolar epithelial (hAELVi) and
665 macrophage (THP-1) cell lines. *Altex* **35**, (2) 211–222 (2018).
666 15. Schwende, H., Fitzke, E., Ambs, P., Dieter, P. Differences in the state of differentiation of
667 THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *Journal of leukocyte biology.*
668 **59** (4) 555–561 (1996).
669 16. Castoldi, A., Empting, M. et al. Aspherical and Spherical InvA497-Functionalized
670 Nanocarriers for Intracellular Delivery of Anti-Infective Agents. *Pharmaceutical Research.* **36**, (1),
671 1–13 (2019).
672 17. Ebensen, T., Delandre, S., Prochnow, B., Guzmán, C. A., Schulze, K. The Combination
673 Vaccine Adjuvant System Alum/c-di-AMP Results in Quantitative and Qualitative Enhanced
674 Immune Responses Post Immunization. *Frontiers in cellular and infection microbiology* **9**,
675 (February), 31 (2019).
676 18. Brockman, S. M., Bodas, M., Silverberg, D., Sharma, A., Vij, N. Dendrimer-based selective
677 autophagy-induction rescues δF508-CFTR and inhibits Pseudomonas aeruginosa infection in
678 cystic fibrosis. *PLoS ONE.* **12**, (9), 1–17 (2017).
679 19. Anderson, G. G., Moreau-Marquis, S., Stanton, B. A., O'Toole, G. A. In vitro analysis of
680 tobramycin-treated Pseudomonas aeruginosa biofilms on cystic fibrosis-derived airway epithelial
681 cells. *Infection and Immunity.* **76** (4), 1423–1433 (2008).
682 20. Moreau-Marquis, S., Bomberger, J. M. et al. The DeltaF508-CFTR mutation results in
683 increased biofilm formation by Pseudomonas aeruginosa by increasing iron availability. *American*
684 *journal of physiology. Lung cellular and molecular physiology.* **295**, L25–L37 (2008).
685 21. Braakhuis, H. M., Kloet, S. K. et al. Progress and future of in vitro models to study
686 translocation of nanoparticles. *Archives of Toxicology* **89**, (9), 1469–1495 (2015).
687 22. Bosshart, H., Heinzelmann, M. THP-1 cells as a model for human monocytes. *Annals of*
688 *Translational Medicine.* **4**, (21), 4–7 (2016).
689 23. Daigneault, M., Preston, J. a., Marriott, H. M., Whyte, M. K. B., Dockrell, D. H. The
690 identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and
691 monocyte-derived macrophages. *PLoS ONE.* **5** (1), (2010).
692 24. Bismarck, P. V., Schneppenheim, R., Schumacher, U. Successful treatment of
693 pseudomonas aeruginosa respiratory tract infection with a sugar solution - A case report on a
694 lectin based therapeutic principle. *Klinische Padiatrie.* **213**, (5), 285–287 (2001).
695 25. Klinger-Strobel, M., Lautenschläger, C. et al. Aspects of pulmonary drug delivery strategies
696 for infections in cystic fibrosis – where do we stand? *Expert Opinion on Drug Delivery* **5247**,
697 (November), 1–24 (2015).
698 26. Ehrhardt, C., Collnot, E.-M. et al. Towards an in vitro model of cystic fibrosis small airway
699 epithelium: characterisation of the human bronchial epithelial cell line CFBE41o-. *Cell and tissue*
700 *research.* **323**, (3), 405–415 (2006).
701 27. Anderson, G. G., Kenney, T. F., Macleod, D. L., Henig, N. R., O'Toole, G. A. Eradication of
702 Pseudomonas aeruginosa biofilms on cultured airway cells by a fosfomycin/tobramycin antibiotic

combination. *Pathogens and Disease.* **67** (1), 39–45 (2013).
28. Cavalieri, F., Tortora, M., Stringaro, A., Colone, M., Baldassarri, L. Nanomedicines for antimicrobial interventions. *Journal of Hospital Infection.* **88**, (4), 183–190 (2014).
29. Savla, R., Minko, T. Nanotechnology approaches for inhalation treatment of fibrosis. *Journal of Drug Targeting.* **21** (10), 914–925 (2013).
30. Ho, D.-K. et al. Challenges and strategies in drug delivery systems for treatment of pulmonary infections. *European Journal of Pharmaceutics and Biopharmaceutics.* **144** (August) 110–124 (2019).

Figure 1

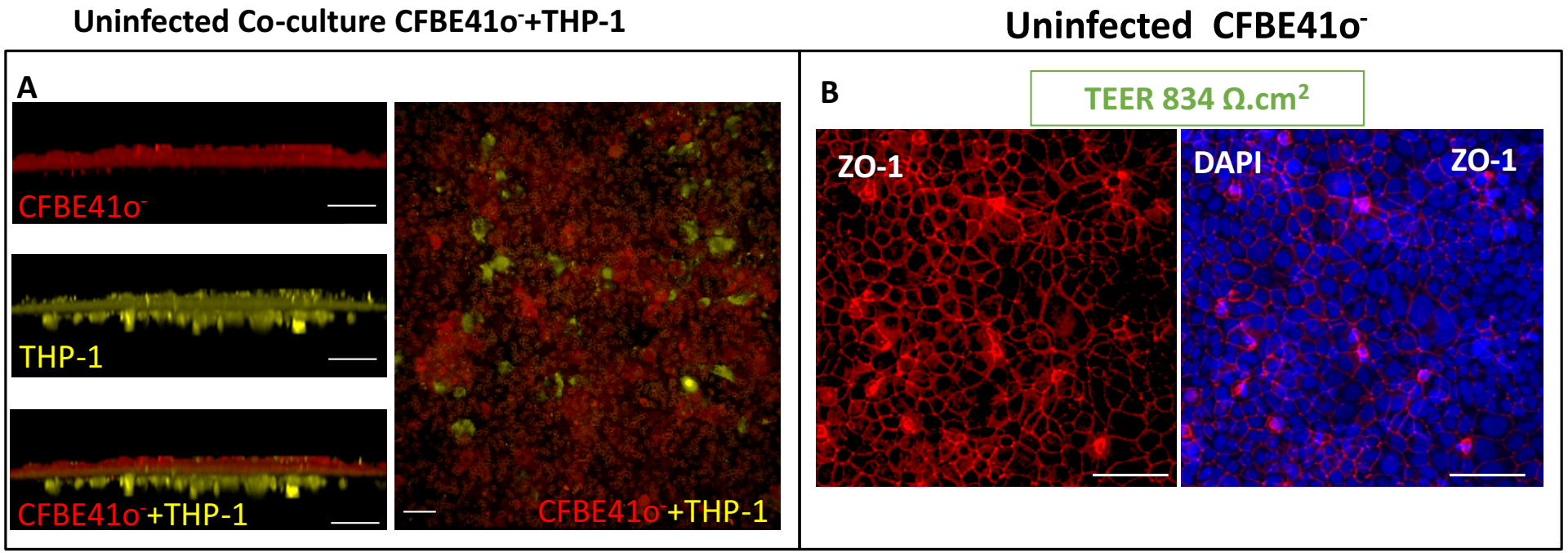


Figure 2

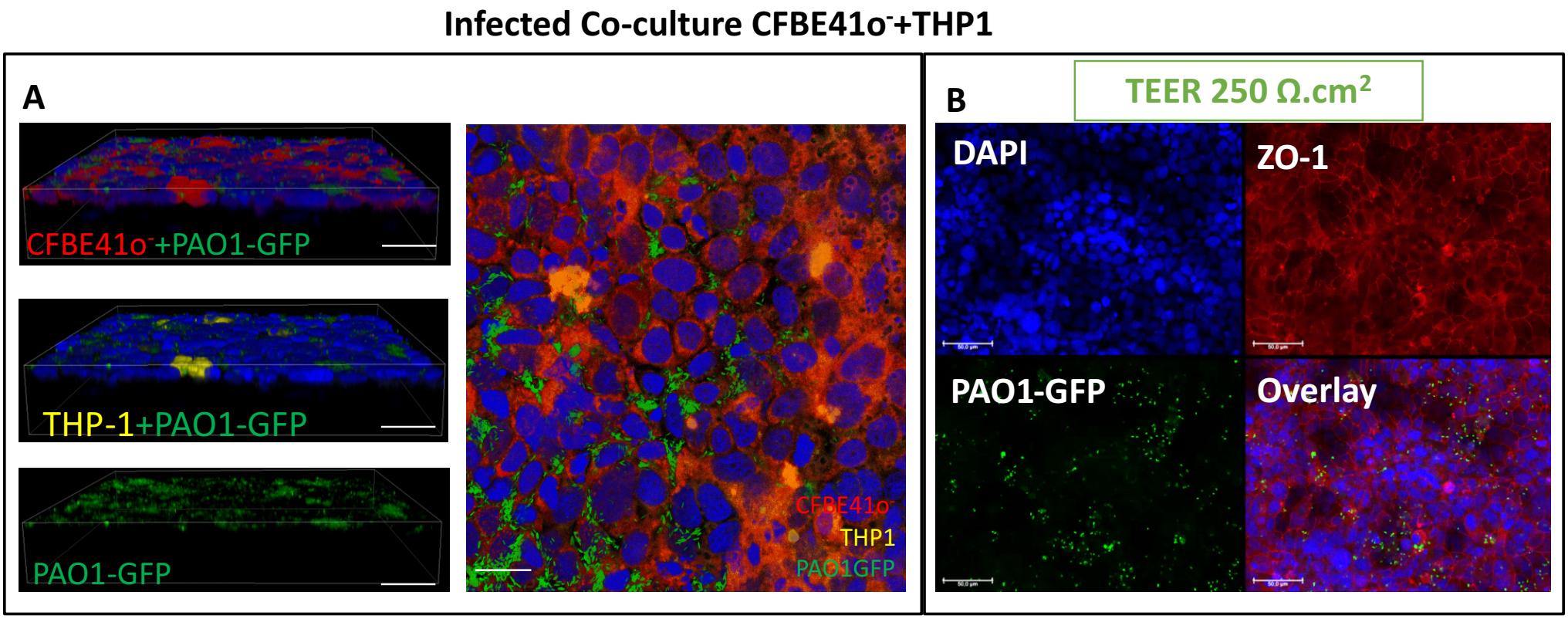


Figure 3

Click here to access/download;Figure;Figure 3.pdf

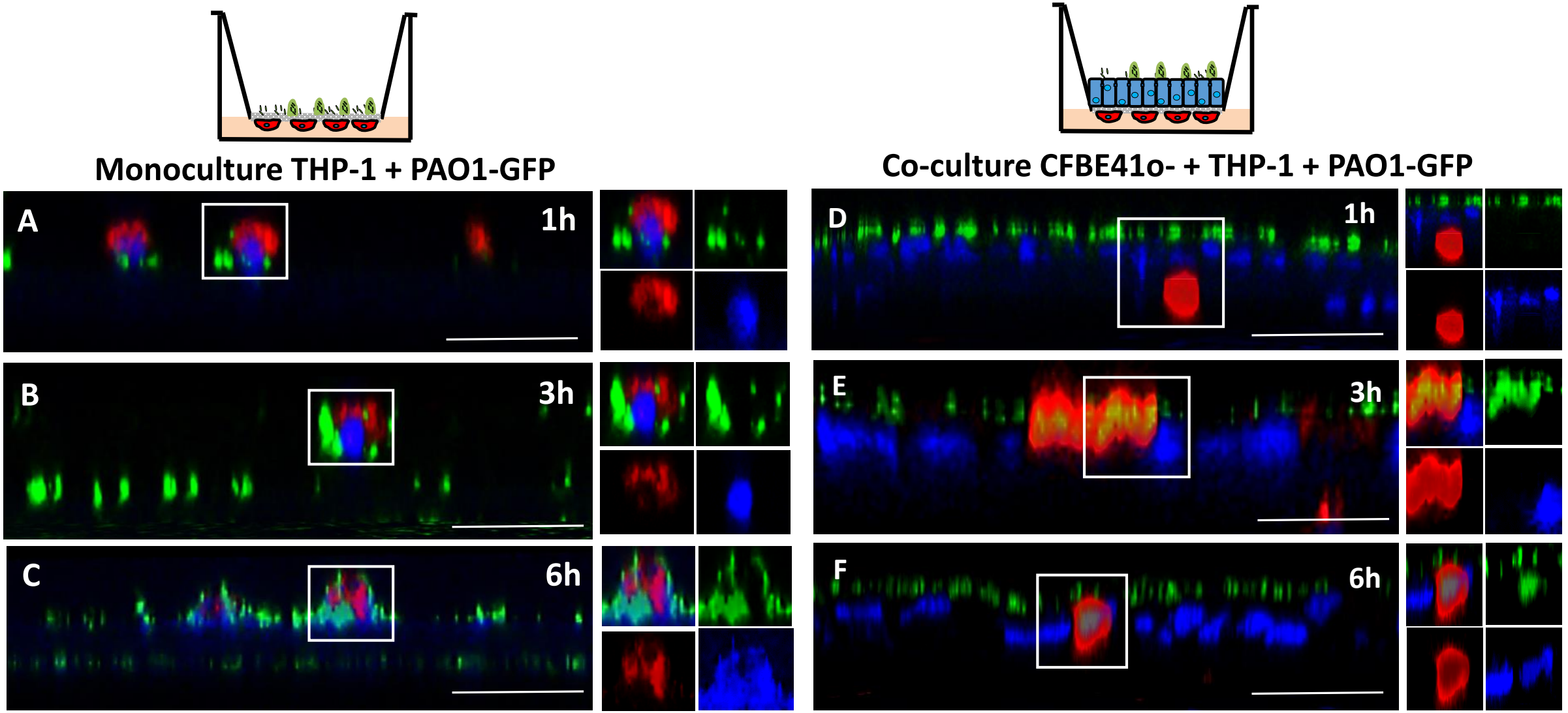
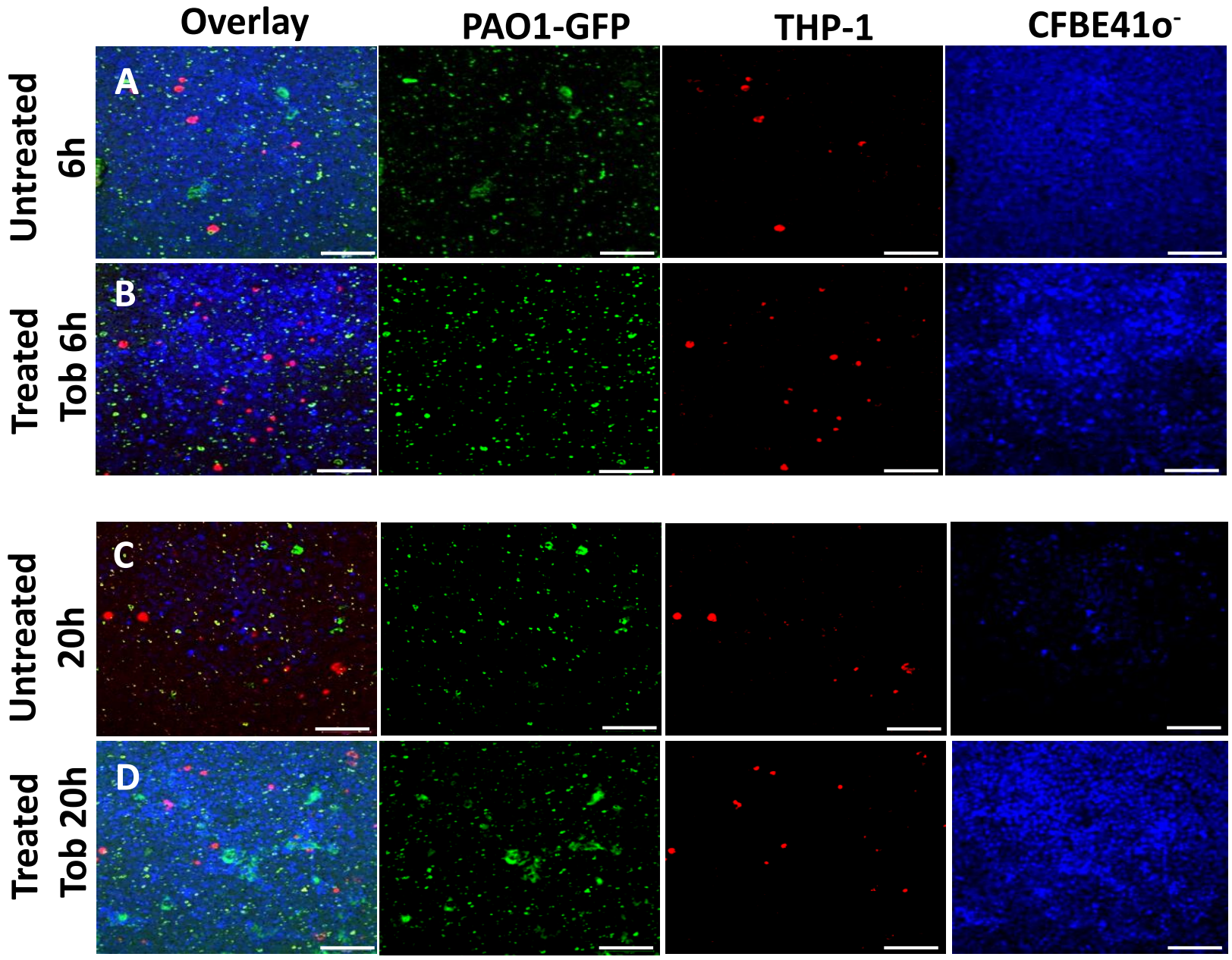
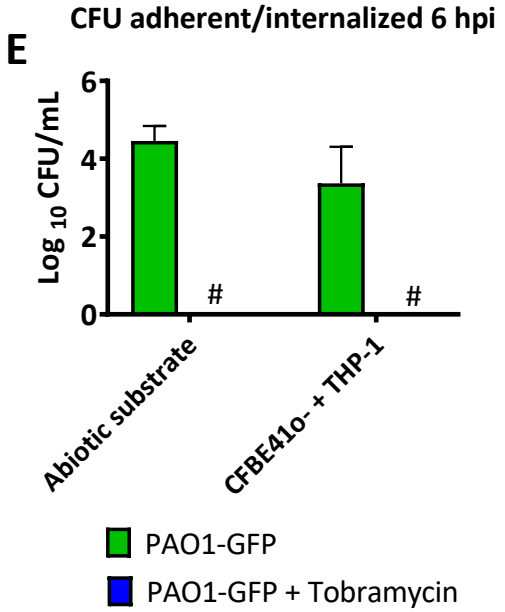


Figure 4

Click here to access/download;Figure;Figure 4.pdf





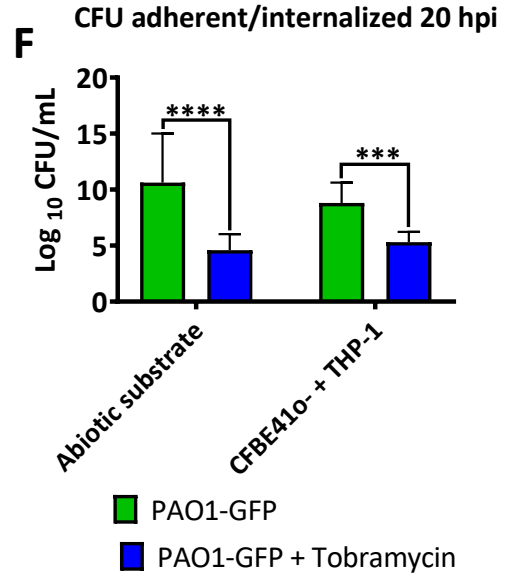


Figure 5

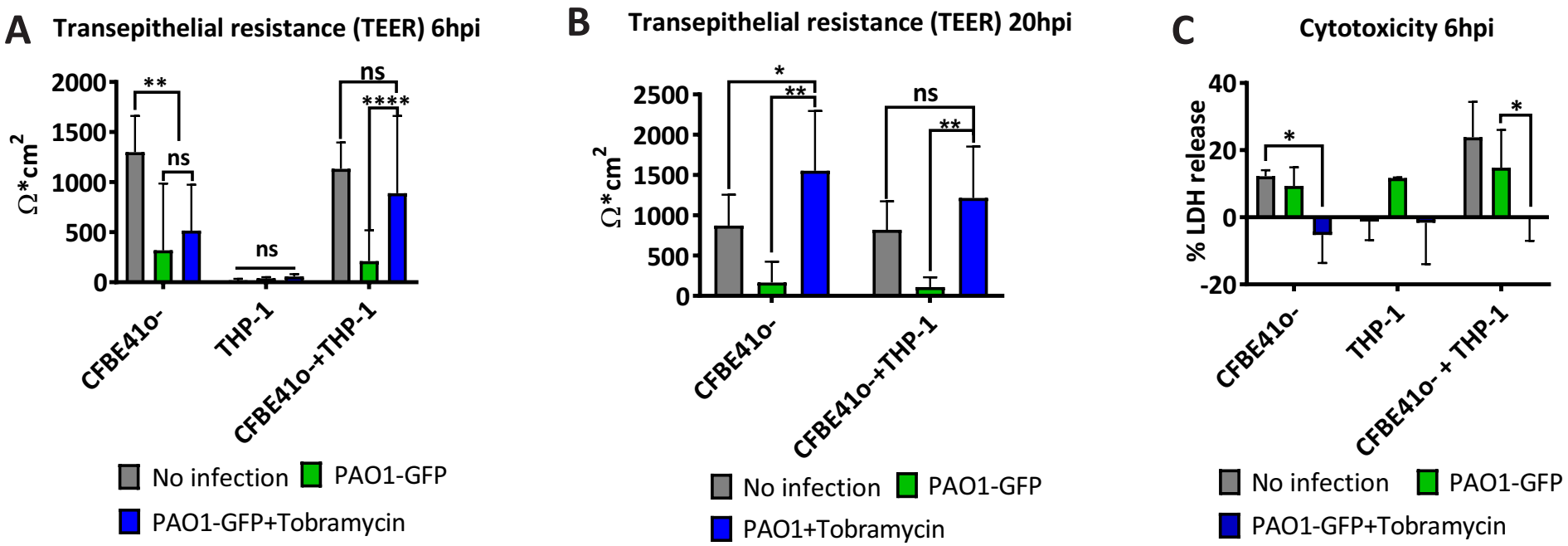


Figure 6

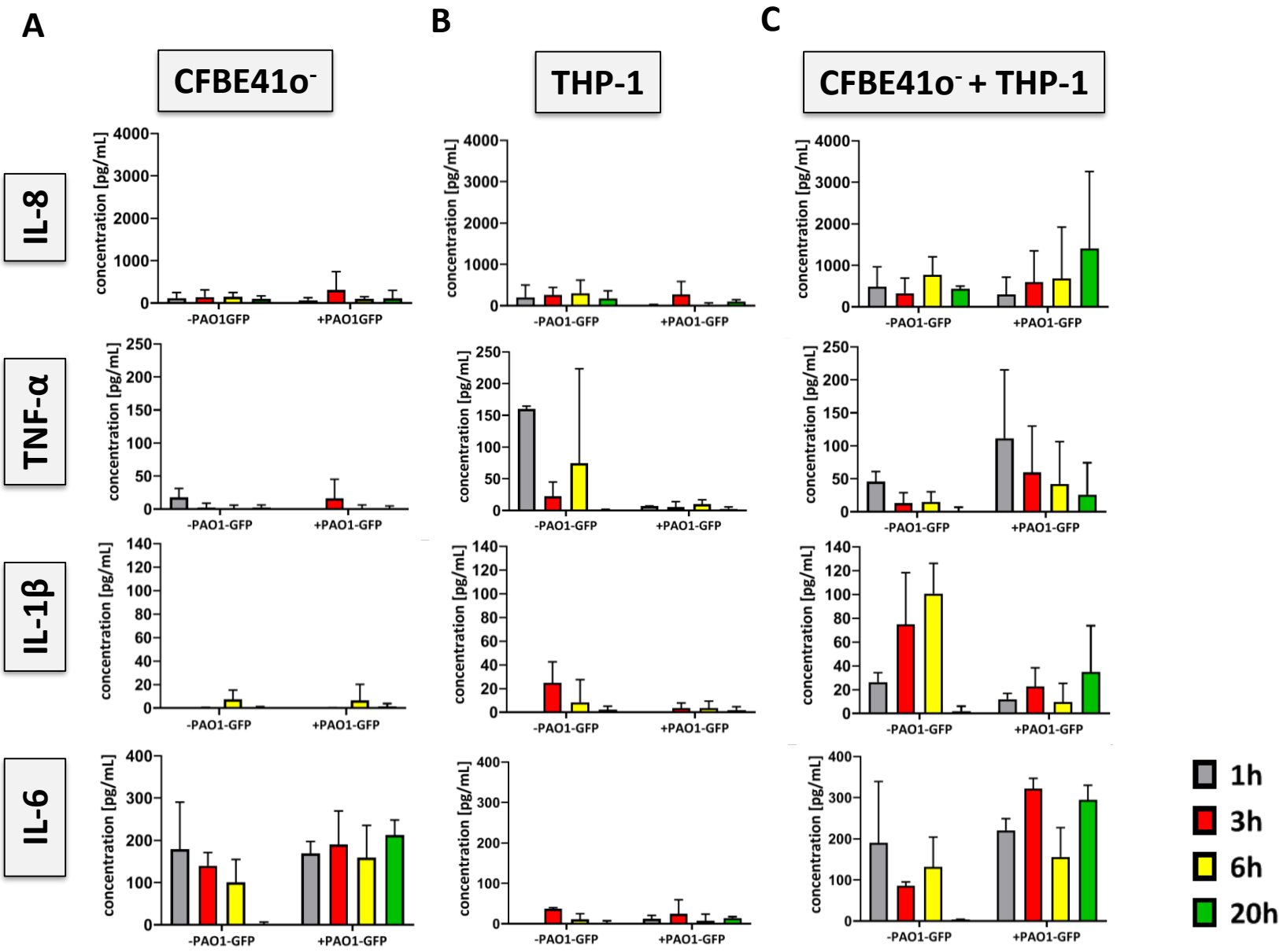


Figure 7

Click here to access/download;Figure;Figure 7.pdf

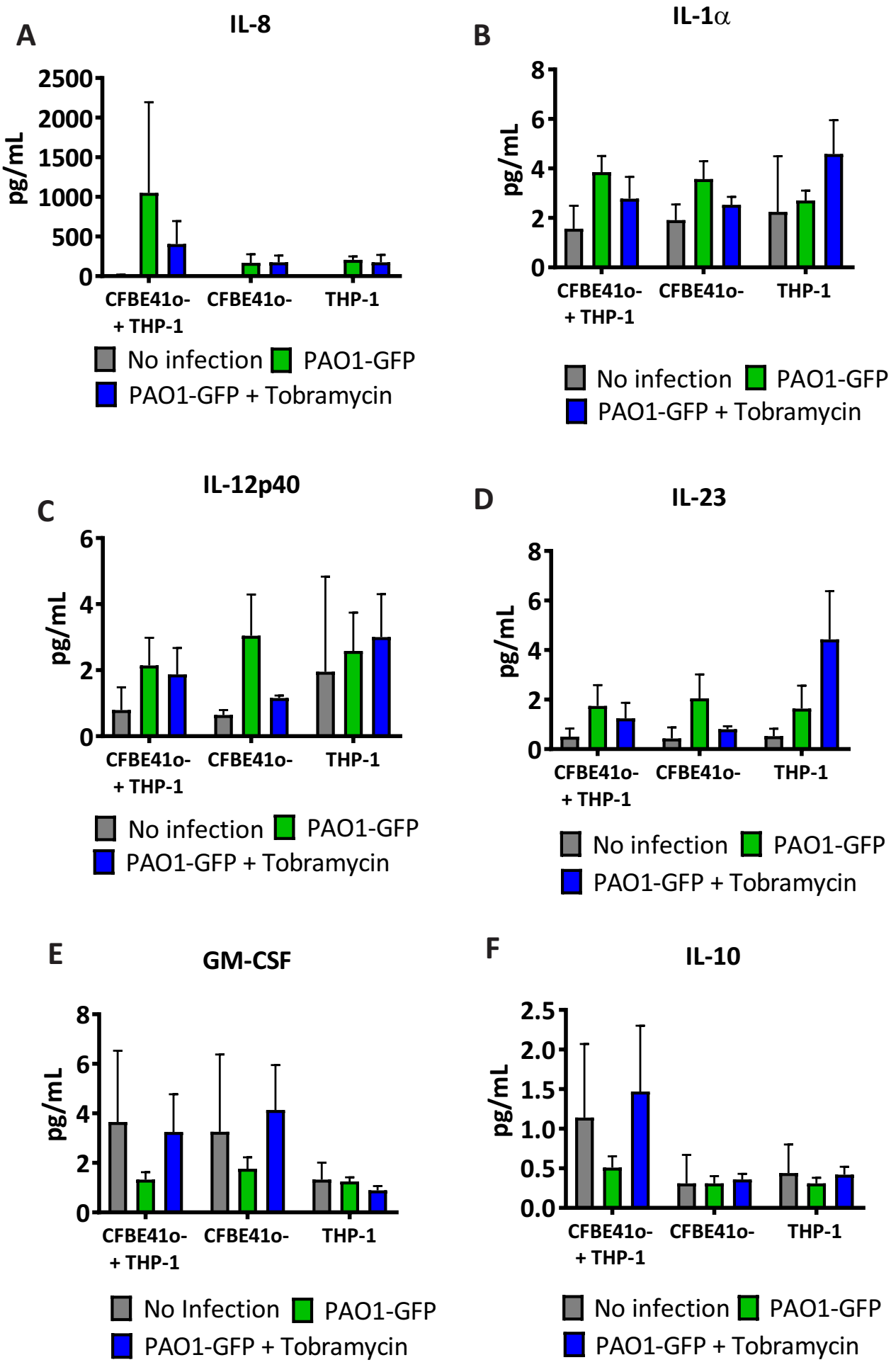


Table of Materials

Name of Material/Equipment
Accutase
Ampicillin
CASY TT Cell Counter and Analyzer
CellTrace Far Red
Centrifuge Universal 320R
CFBE41o^{-} cells
Chopstick Electrode Set for EVOM2, 4mm
Confocal Laser-Scanning Microscope CLSM
Cytokines ELISA Ready-SET-Go kits
Cytokines Panel I and II
Cytotoxicity Detection Kit (LDH)
D-(+) Glucose
Dako Fluorescence Mounting Medium
DAPI (4',6-diamidino-2-phenylindole)
Epithelial voltohmmeter
Falcon Permeable Support for 12 Well Plate with 3.0µm Transparent PET Membrane, Sterile
Fetal calf serum
Goat anti-mouse (H+L) Cross-adsorbed secondary Antibody, Alexa Fluor 633
L-Lactate Dehydrogenase (LDH), rabbit muscle
LB broth
MEM (Minimum Essential Medium)
Non-Essential Amino Acids Solution (100X)
P. aeruginosa strain PAO1
P. aeruginosa strain PAO1-GFP
Paraformaldehyde Aqueous Solution -16%
Phosphate buffer solution buffer
Petri dishes
Phorbol 12-myristate 13-acetate (PMA)
Precision Cover Glasses
Purified Mouse anti-human ZO-1 IgG antibody
Roswell Park Memorial Institute (RPMI) 1640 medium
Soda-lime glass Petri dish, 50 x 200 mm (height x outside diameter)
Saponin
T75 culture flasks
THP-1 cells
Tobramycin sulfate salt
Trypsin-EDTA 0.05%
Tween80

Company
Accutase
Carl Roth, Germany
OLS Omni Life Sciences
Thermo Fischer
Hettich, Germany
1. Gruenert Cell Line Distribution Program 2. Sigma-Aldrich
World Precision Instruments, Sarasota, USA
Leica, Mannheim, Germany
Affymetrix eBioscience, USA
LEGENDplex Immunoassay (Biolegend, USA).
Roche
Merck
DAKO
Thermo Fischer
World Precision Instruments, Sarasota, USA
Corning, Amsterdam, Netherlands
Lonza, Basel, Switzerland
Invitrogen
Roche, Mannheim, Germany
Sigma-Aldrich, Germany
Gibco Thermo Fisher Scientific Inc.
Gibco Thermo Fisher Scientific Inc.
American Type Culture Collection
American Type Culture Collection
EMS DIASUM
Thermo Fischer
Greiner
Sigma, Germany
ThorLabs
BD Transduction Laboratories
Gibco by Lifetechnologies, Paisley, UK
Normax, Portugal
Sigma-Aldrich, Germany
Thermo Fischer
Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany)
Sigma
Thermo Fischer
Sigma-Aldrich, Germany

Catalog Number
AT104
HP62.1
-
C34564
1406
1. gift from Dr. Dieter C. Gruenert 2. SCC151
STX2
TCS SP 8
15541037
740102
11644793001
47829
S3023
D1306
EVOM2
353181
DE14-801F
A-21050
10127230001
L2897-1KG
11095072
11140050
47085
10145GFP
15710-S
10010023
664102
P8139-1MG
CG15KH
610966
11875093
5058561
S4521
156499
No. ACC-16
T1783-500MG
25300054
P1754

Manuscript JoVE61069

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-1[1] 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 um not uM, 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 x 10^5 cells/well in 12 well plates with permeable supports (pore size of 3 µm) (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% CO_2 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% CO_2 before setting up the co-culture.

- **Line 192-195 – current version 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.

- **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 CellTraceTM)

As CellTraceTM 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 % CO_2, 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/cm^2 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-infectives[3], 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 CFBE41o$^-$ 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 asses 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 immunoassay[4] is used. For that, centrifuge supernatant from 5.1. at 21250 x g for 10 min and measure either immediately or store -80 ^{o}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 ΔF508, 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 disease[6]."

* 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 LPS[9] and it lacks full activation and not the entire population is differentiated from monocytes to macrophage-like cells[10]."

* 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 Ω.cm^2 is based on measurements with primary bronchial cells. Still, uninfected epithelial cells had a higher TEER (834 Ω*cm^2), which is reduced to 250 Ω*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 CFBE41o- is given as Dr. Dieter C. Gruenert, for which there is no longer an active laboratory. Authors should provide an updated source. CFBE41o- 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 CFBE41o- 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 CFBE41o- 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 independents 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 CFBE41o- 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 CFBE41o^{-}, 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.

.

* 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 ≤ 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 µL 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 µl apical and1.5 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 µL cell medium in the apical side and 1.5 mL in the basolateral side for 1 h, at 37 °C, 5 % CO_2.

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% CO_2 before setting up the co-culture.

Section 2.1. Use the Transwell® inserts with CFBE41o-monolayers at ALI once they reach TEER > 300 190 Ω*cm². 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 ≥300 Ω*cm² (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 (500µL 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 µg/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 µg/ml of tobramycin, we take 60µl 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 6µg/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 platting 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 stablished 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 is 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 % CO_2 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.

Figure S1

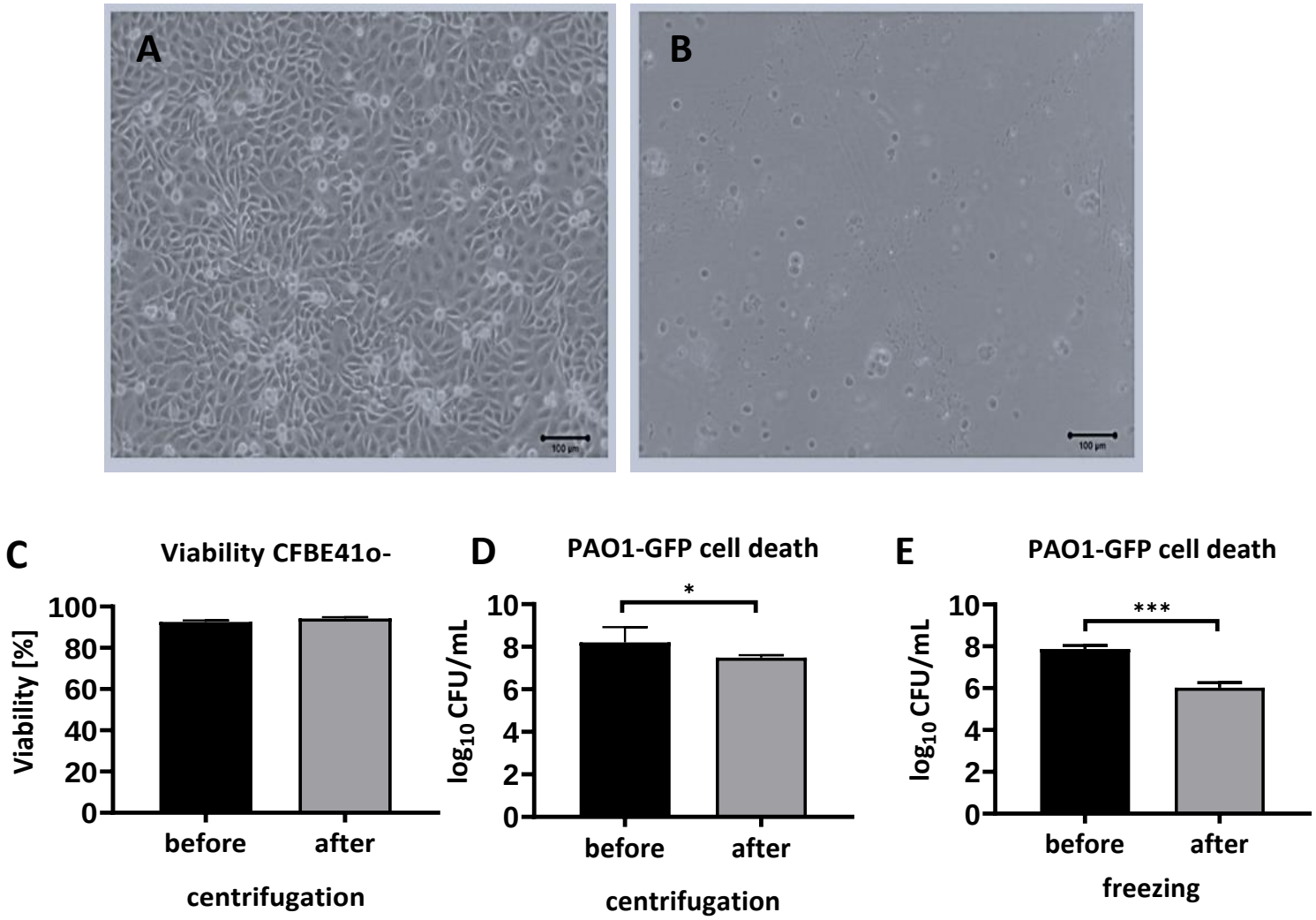


Figure S2