Mycobacteria–host interactions in human bronchiolar airway organoids

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Funding information
FRM "Amorçage Jeunes Equipes", Grant/ Award Number: AJE20151034460; ZonMw,

Abstract
Respiratory infections remain a major global health concern. Tuberculosis is one of the top 10 causes of death worldwide, while infections with Non-Tuberculous Mycobacteria are rising globally. Recent advances in human tissue modeling offer a unique opportunity to grow different human “organs” in vitro, including the human airway, that faithfully recapitulates lung architecture and function. Here, we have explored the potential of human airway organoids (AOs) as a novel system in which to assess the very early steps of mycobacterial infection. We reveal that Mycobacterium tuberculosis (Mtb) and Mycobacterium abscessus (Mabs) mainly reside as extracellular bacteria and infect epithelial cells with very low efficiency. While the AO microenvironment was able to control, but not eliminate Mtb, Mabs thrives. We demonstrate that AOs responded to infection by modulating cytokine, antimicrobial peptide, and mucin gene expression. Given the importance of myeloid cells in mycobacterial infection, we co-cultured infected AOs with human monocyte-derived macrophages and found that these cells interact with the organoid epithelium. We conclude that adult stem cell (ASC)-derived AOs can be used to decipher very early events of mycobacteria infection in human settings thus offering new avenues for fundamental and therapeutic research.

KEYWORDS
airways, infection, mycobacteria, organoids, tuberculosis

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1 | INTRODUCTION

Pulmonary diseases due to mycobacteria cause significant morbidity and mortality to human health. Although the global burden of tuberculosis (TB) has declined over the last decades, Mycobacterium tuberculosis is still one of the deadliest infectious agent worldwide with an estimated 10 million disease cases and 1.4 million deaths in 2019 (World Health Organization, 2020). By contrast, Nontuberculous Mycobacteria (NTM) infections are rising globally, especially in developed countries, causing opportunistic pulmonary infections affecting individuals who are immunocompromised and who have underlying health conditions (Ratnatunga et al., 2020; To et al., 2020). Among them, Mycobacterium abscessus is considered one of the most antibiotic-resistant mycobacteria and is associated with pulmonary disease such as cystic fibrosis (Johansen et al., 2020).

The lung is the main entry port for mycobacteria and the main site of disease. Bacteria-containing droplets or aerosols first navigate through the respiratory tract interacting with airway epithelium functions in order for mycobacteria to reach the alveolar space and thrive in alveolar macrophages (Corleis & Dorhoi, 2020; Leiva-Juárez et al., 2018; Torrelles & Schlesinger, 2017). However, how mycobacteria interact with the airway to establish a successful primary infection remains poorly understood. One of the challenges to address remains the complexity of host-pathogen interactions, particularly within human multicellular tissue.

Conventional 2D cell cultures of epithelial and immune cells, in vitro human-based 3D granuloma, and animal models have contributed to deciphering key host-pathogen mechanisms at play during mycobacterial infection (Bermudez et al., 2002; Bernut et al., 2014, 2015; Bielecka et al., 2017; Cohen et al., 2018; Elkington et al., 2019; Fonseca et al., 2017; Parasa et al., 2014; Puisségur et al., 2007; Rampacci et al., 2020; Silva-Miranda et al., 2015). More recently, in vitro lung models have been developed based on organ-on-chip and organoid technologies to mimic the structural and biological properties of the in vivo human lung environment to better replicate human disease and drug profiles (Cidem et al., 2020; Dutta & Clevers, 2017; Rossi et al., 2018). Lung-on-chip technology reconstructs the alveolar epithelium/endothelium interface and has deciphered the crucial role of surfactant in controlling Mtb replication (Thacker et al., 2020). We, and others, have applied organoid technology for respiratory infection modeling, including Cryptosporidium (Heo et al., 2018), RSV (Sachs et al., 2019), influenza viruses (Hui et al., 2018; Zhou et al., 2018), SARS-CoV-2 (Lamers et al., 2020, 2021), and Pseudomonas aeruginosa (Bagayoko et al., 2021) to decipher pathogen fitness, cell tropism, and host responses.

While the ongoing COVID-19 pandemic has very recently fueled the development of new human lung organoid models (Salahudeen et al., 2020; Youk et al., 2020), the first human lung organoid model described was derived from lung adult stem cells (ASC’s) and composed of a polarized, pseudostratified airway epithelium containing basal, secretory, and multi-ciliated cells, thus reproducing the bronchiolar part of the airway (Sachs et al., 2019). AOs display functional mucus secretion and ciliate beating (Sachs et al., 2019), therefore, constituting a human 3D system in which to model host-mycobacteria interactions (Iakobachvili & Peters, 2017).

Here, we have set out to use AOs as a model in which to study mycobacteria interaction with airway epithelial cells. Our data demonstrate that mycobacteria can be readily found in the lumen of AOs with some internalization by airway epithelial cells. While Mtb growth was overall controlled, Mabs readily replicated in the lumen of AOs. In response to Mtb infection, we showed that AOs induced the secretion of cytokines and antimicrobial peptides. Interestingly, both Mabs and Mtb inhibited the expression of mucins which function in pathogen clearance. The option to model innate cell recruitment by co-culturing human macrophages with bacteria-injected AOs was demonstrated in this work.

2 | RESULTS AND DISCUSSION

First of all, we evaluated the morphology and cell composition of AOs. As shown in Figure S1, AOs displayed a cystic structure (Figure S1a) and are composed of basal cells lined with ciliated and goblet cells on the lumen facing side (Figure S1b and Movie S1). AOs presented functional ciliate beating (Movies S2 and S3) and evident from the live-cell imaging is the functional mucociliary system where cilia beat secreted mucus and cell debris around the lumen (Movie S4).

Due to the innate cystic structure of AOs, where the pathogen-sensing apical side faces the lumen, DsRed-expressing H37Rv Mtb or dTomato-expressing Mabs were microinjected via a BSL-3- or a BSL-2-approved custom-made micro-injection system (Figure 1a). Mycobacteria could be found in the lumen of AOs and occasionally making contact with epithelial cells but without causing obvious alterations to organoid architecture and ultrastructure (Figures 1b,c, S2 and Movie S5–S7). We evaluated whether mycobacteria infection could trigger cell death and found neither Mtb nor Mabs infection
triggered additional cell death compared to uninfected AOs, which undergo regular cell turnover (Figure 1d).

*Mtb* is known to infect bronchial epithelial cells in 2D conditions (Reuschl et al., 2017), and pneumocytes in vitro (Ryndak et al., 2015) and in vivo (Cohen et al., 2018), but with low efficiency. To determine whether *Mtb* could infect airway epithelial cells, *Mtb*-infected AOs were dissociated and analyzed by flow cytometry to quantify cells positive for *Mtb* in an unbiased manner. Only 2% of the cells composing the organoid were positive for *Mtb* (Figure 1e). As mucociliary movement could minimize bacteria interaction with epithelial cells, to favor optimal contact between epithelial cells and bacteria, AOs were dissociated, and single cells were infected with *Mtb* and analyzed by flow cytometry. Approximately 13% and 15% of epithelial cells were found associated with bacteria after 4 and 24 hr of infection, respectively (Figure 1e), confirming that *Mtb* has a low tropism for epithelial cells as already described with primary bronchial cells (Reuschl et al., 2017). To evaluate bacterial internalization by epithelial cells, double positive cells sorted after flow cytometry were imaged by confocal microscopy. As shown in Figure 1f, epithelial cells containing *Mtb* could be found, suggesting cell invasion by a yet unknown mechanism and confirming that the majority of mycobacteria are extracellular in the lumen of AOs. The functioning mucociliary system within AOs is likely responsible for preventing mycobacterial contact with, and internalization by, epithelial cells (Leiva-Juárez et al., 2018; Whitsett & Alenghat, 2015).

We next investigated mycobacterial survival in AOs. Bacteria-infected AOs were collected individually, lysed and analyzed for bacterial load by CFU assay. *Mtb* demonstrated a bi-phasic curve, with a significant decrease of bacterial load after 7 days followed by an increase at 21 days post-infection back to the initial load, without affecting AO viability (Figure 1d). This suggests an early stage of *Mtb* control by the AO microenvironment followed by bacterial adaptation and proliferation, with cell debris present in the lumen which could foster bacterial replication, as already shown in macrophages (Mahamed et al., 2017). By contrast, Mabs growth displayed a 2-day lag phase followed by significant bacterial replication and an increase in bacterial burden over 6 days (Figure 1g). These results also confirm that the airway constitutes a hospitable microenvironment for Mabs to thrive, compared to *Mtb* that establishes its replicative niche in alveolar macrophages (Corleis & Dorhoi, 2020; Johansen et al., 2020).

Next, we determined whether AOs mounted an inflammatory/antimicrobial response to myobacteria infection. *Mtb*- or Mabs-infected AOs were collected and subjected to RT-qPCR analysis to evaluate cytokine, antimicrobial peptide, and mucin gene expression (Figure 2a). *Mtb* induced significant gene expression of IL-8 (Figure 2b), a immune cell chemoattractant (Baggioni & Clark-Lewis, 1992), while IL-8 secretion did not reach significance (Figure 2c). Non-significant expression of IP-10, GM-CSF, and CCL5, cytokines mediating the accumulation of neutrophils and macrophages for optimal granuloma formation and control of *Mtb* (Domingo-Gonzalez et al., 2016) was observed. The antimicrobial peptides β-defensin-1 and 2, lactoferrin, hepcidin, and RNase7 were enhanced upon *Mtb* infection, being significant for only β-defensin-1 (Figure 2b). β-Defensin-1 and RNase7 may participate in *Mtb* restriction during early infection, promoting pore formation on the bacteria cell membrane (Arranz-Trullén et al., 2017; Torres-Juarez et al., 2018), while β-defensin-2 improves the chemoattraction of macrophages (Rivas-Santiago et al., 2005; Semple & Dorin, 2012). Interestingly, β-defensin-3 and 4, which are related to long-term control of mycobacterial infection (Dong et al., 2016) were downregulated. The upregulation of Hecpedin and Lactoferin could contribute to decrease the transmembrane transport of iron (Sow et al., 2007, 2009) and modulate its extracellular pool by hijacking it (Schaible et al., 2002), making the airway microenvironment more hostile for *Mtb* survival (Boelart et al., 2007).

By contrast, Mabs infection did not enhance the expression of cytokines and antimicrobial peptides (Figure 2a). This could be due to the presence of glycopolysaccharides on the surface of its cell wall (Johansen et al., 2020), which mask other lipids and prevent the interaction of Mabs with pattern recognition receptors expressed by host cells (Rhoades et al., 2009), resulting in overall low responsiveness of lung epithelial cells to bacterial challenge (Matsuyama et al., 2018). Surprisingly, both Mabs and *Mtb* significantly downregulated the expression of mucins, including MUC5B and MUC4 (Figure 2b). Mucin expression and secretion are normally enhanced in response to inflammatory challenge and form part of an efficient clearance system for pathogen removal from the airway (Leiva-Juárez et al., 2018; Ridley & Thornton, 2018; Whitsett & Alenghat, 2015). Our data also showed that mycobacterial infection is associated with downregulation of NFkβ-regulated genes such as IL-1b and IL-6 (Figure 2a), which could participate in mucin expression impairment as IL-1b is a powerful inducer of mucin expression in the airway (Chen...
et al., 2019). Pathogens such as *Helicobacter pylori* have been shown to downregulate the expression of mucin genes to promote stomach tissue colonization (Cooke et al., 2009). How downregulation of mucin expression by mycobacteria might interfere with mucociliary function and infection outcome remains to be elucidated.

Upon *Mtb* infection, macrophages mount an inflammatory response that modulates the lung microenvironment (Fonseca et al., 2017; Lastrucci et al., 2015). AOs were stimulated with the supernatant of *Mtb*-infected human macrophages (cmMTB) and analyzed for gene expression compared to stimulation with the supernatant of non-infected macrophages (cmCTR). As shown in Figure 2d, the expression of IL-8 and GM-CSF, major cytokines for macrophage control of *Mtb* infection, were significantly enhanced in cmMTB-stimulated AOs compared to those treated with cmCTR which
mimics the paracrine macrophage-epithelial signaling naturally occurring during lung Mtb infection (Reuschl et al., 2017).

Finally, due to the essentiality of macrophages in TB disease (Cadena et al., 2016; Corleis & Dorhoi, 2020), we co-cultured human monocyte-derived macrophages, alongside mycobacteria-injected organoids, and observed hourly by confocal microscopy over the course of 4 days. Due to the complex nature of this experiment, it was optimized and set up under BSL-2 conditions using Mycobacterium bovis BCG. Human macrophages were found to migrate within the collagen matrix and in some instances, moved towards organoids containing mycobacteria (Movie S8). While we found no evidence of macrophages being able to traverse the basal side and enter the organoid lumen to clear mycobacteria, we did observe macrophages migrating to and interacting with the basolateral edge of the AO, capturing and ingesting bacteria (Movie S8). This resembles the natural process of peri-bronchiolar macrophage migration to the site of infection and phagocytosis of pathogenic material (Cambier et al., 2014).

Using AOs as a human 3D system to study the early steps of mycobacterial infection, we have shown that Mtb remains viable for up to 21 days within the lumen of AOs, while Mabs actively replicated over 7 days (Figure 1g). During this timeframe, while AO integrity remained uncompromised (Figures 1b,c, S2 and Movies S5 and S6), molecular interactions began as early as 48 hr after injection with the modulation of cytokines and antimicrobial peptides, and the inhibition of mucin expression (Figure 2b–d). Within 72 hr, innate immune cells can be recruited to the surface of infected AOs (Movie S8). Together, these data indicate that AOs offer a valuable 3D system to assess early interactions of mycobacteria with the human airway.

The ability to model these early timeframes in a responsive, multicellular and functionally similar system to the human airway, but without the complications, monetary and ethical restrictions of animal research, constitutes a major advance for the field. Organoid technology provides a complementary strategy to model in vitro human tissues, and infection by human-related pathogens. Organoids based on lung ASC’s are advantageous as they generate AOs via a one-step proliferation and differentiation protocol and naturally self-organize in 3D to recapitulate the complexity of the lung epithelium. The resulting epithelium comprises all the different cell types and related secreted products (mucus, surfactant proteins), thus providing a reductionist system to assess host-pathogen interactions at the tissue level. Due to the self-renewal of ASC’s, one of the most important advantages of organoids is that they are genetically stable during long-term culture, allowing for their cryopreservation in biobanks.

One of the main limitations of the organoid system comes from the absence of air-liquid-mucosa interface due to their closed, cystic structure. Very recent advances in bioprinting and microfluidic approaches allowed intestinal organoid-based tissue engineering to create tubelike epithelia and a continuous lumen (Nikolaev et al., 2020), with the potential of extending this approach to airway models. Lung-on-a-chip technology also constitutes an alternative approach to reproduce the air-liquid-mucosa interface and model mycobacterial infection (Huh et al., 2013; Thacker et al., 2020). However, lung-on-chips are generally developed using induced Pluripotent Stem Cells (iPSC) or epithelial cell lines, requiring complex expertise in both iPSC cultures based on long stepwise differentiation protocols, and in nano-micro fabrication for chip devices. Therefore, organoids and on-chip organs are complementary approaches that provide alternatives to animal experimentation thus complying with the 3R’s—Replacement, Reduction, Refinement—which call for in vitro or a alternatives to model functional organs and pathologies (Gkatzis et al., 2018).

As mycobacteria, especially Mtb, establish their replicative niche in the alveolar part of the lung, another limitation comes from the absence of pneumocytes in the AO model. Recent advances in modeling the human alveolar part of the human lung as organoids (Salahudeen et al., 2020; Youk et al., 2020) will allow further modeling of the mycobacterial replicative niche. Finally, the ability to introduce human macrophages allows functional modeling of a key cell type and its cellular network, overcoming another limitation of organoid systems. Extension of the BALO technology, combining connected airway with alveoli and engraftment of macrophages (Vazquez-Armendariz et al., 2020), to human stem cells could open new venues for mycobacterial infection modeling. We believe that this work forms a starting point for modeling a wide range of human respiratory pathogens using lung organoids, as exemplified with SARS-CoV-2, (Lamers et al., 2020, 2021; Salahudeen et al., 2020; Si et al., 2021; Thacker et al., 2021), but also respiratory infections driven by host disorders such as cystic fibrosis.

3 | METHODS

3.1 | Ethic statements

The collection of patient data and tissue for AO generation was performed according to the guidelines of the European Network of Research Ethics Committees following European and national law. In the Netherlands and France, the responsible accredited ethical committees reviewed and approved this study in accordance with the Medical Research Involving Human Subjects Act. Human lung tissue was provided by the Primary Lung Culture Facility (PLUC) at MUMC+, Maastricht, The Netherlands. Collection, storage, use of tissue and patient data were performed in agreement with the “Code for Proper Secondary Use of Human Tissue in the Netherlands” (http://www.fmwv.nl). The scientific board of the Maastricht Pathology Tissue Collection approved the use of materials for this study under MPTC2010-019 and formal permission was obtained from the local Medical Ethical Committee (code 2017-087). The CHU of Toulouse and CNRS approved protocol CHU 19 244 C and Ref CNRS 205782. All patients participating in this study consented to scientific use of their material; patients can withdraw their consent at any time, leading to the prompt disposal of their tissue and any derived material.

Human buffy coats were obtained from volunteers with informed consent via Sanquin (NVT0355.01) or établissement français du sang (Agreement 21PLER2017-0035AV02).
3.2 | Organoid culture

Healthy tissue from five independent donors receiving surgical treatment for lung cancer were used to derive organoids. AOs were characterized as described (Sachs et al., 2019).

3.3 | Bacterial culture and microinjection

DsRed-WT H37Rv Mtb strains were obtained by complementation with the pMRF plasmid containing a DsRed cassette, and were cultured in the continuous presence of 20 μg/ml of the selective...
antibiotic hygromycin under BSL-3 conditions (Simeone et al., 2015). Mtb strains (BSL-3) and GFP expressing M. bovis BCG (BSL-2) were grown under the selection of 20 µg/ml Kanamycin in BSL-2 conditions. M. abscessus sensu stricto (Mabs) strain CIP104536T (ATCC199777) morphotype Smooth (S) carrying pASTA3 plasmid (Addgene, plasmid 24,657) that express red fluorescent protein (tdTomato), was cultured.

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For microscopy, injected organoids were seeded in IBIDI 4 well chamber (IBIDI) and stained with CellMask™ Green Plasma Membrane Stain (1/1000, Molecular Probes) for 30 min at 37°C. Organoids were washed and fresh medium containing TOPRO-3 Iodide (1/1000, Molecular Probes) was added. Organoids were imaged using a FEI CorrSight at Maastricht University (BSL-2) or Andor/Olympus Spinning Disk Confocal CSU-X1 (10x Air 0.4 NA, 3.1 mm WD) at IPBS (BSL-3). Z-stacks were acquired every hour for the duration of experiments and data analyzed using Fiji/Image J.

| TABLE 1 | List of primers used for RT-qPCR experiments on airway organoids |
| --- | --- |
| Gene | Primers 5’−3’ | Reference |
| Cytokines and chemokines | | In-house |
| CCL5 (NM_002985) | F: CTCATTGCTACTGCTCTTCTC | PrimerBank |
| GM-CSF (NM_000758) | F: CTTGACACCTGACTGAAGACTCTC | In-house |
| IL-1β (NM_000576) | F: AGCTAGGACACCTGGACACCAC | In-house |
| IL-6 (NM_000600.5) | F: ACTCACCTTCTTCAAGAAGATTTG | PrimerBank |
| IL-8 (NM_000584) | F: TACTCACAATACCTTACCAACCC | In-house |
| IP-10 (NM_001565) | F: GTGGCATTCAACAGTACTCTC | In-house |
| IL-18 | | In-house |
| IL-23 | | In-house |
| IL-17 | | In-house |
| IL-22 | | In-house |
| IL-1α | | In-house |
| IL-4 | | In-house |
| Antimicrobial peptides | | In-house |
| β-Defensin 1 (NM_005218) | F: ATGGCCTCAGGTGTAACCTTTC | In-house |
| β-Defensin 2 (NM_004942) | F: ATAGGCGATCCTGTTACCTGC | In-house |
| β-Defensin 3 (NM_018661) | F: TGGGGTGAAAGCTTACGAC | In-house |
| β-Defensin 4 (NM_080389.3) | F: TGCCCGGAAGAAATGTCGCA | In-house |
| Cathelicidin LL37 (NM_004345) | F: ATGCTACCTCAGGCTCACCTTG | In-house |
| Heparin (NM_021157) | F: CTGTCTTCAAGTCGCTCGCC | In-house |
| Lactoferrin (NM_002343) | F: CAGACCTTGCCGACTTTCAG | In-house |
| RNAse 7 (NM_032572) | F: GCCGACATAGCAGAAGACAC | In-house |
| | | In-house |
| Mucins | | In-house |
| MUC4 (NM_018406.7) | F: CTCAGTACGCCTCAGGAC | PrimerBank |
| MUC5AC (NM_001303459.2) | F: AGCCCTTGGCCGATGTCGAC | In-house |
| MUC5B (NM_002458.3) | F: GCCCACATCTCAGCAGGATCG | In-house |
| | | In-house |
| Housekeeping | | In-house |
| GAPDH (NM_002046) | F: TCTCCAAAATCAAGTGGGGGAGATG | In-house |

plates, and cultured in complete AO medium for 2-21 days. At the relevant timepoint, organoids were lysed in 100 µl of 10% Triton X100 in water, serial dilutions were plated on 7H11 agar plates for Mtb or LB agar plates for Mabs and cultured for 3 weeks or 6 days respectively at 37°C.
TABLE 2 Summary of fluorescent markers used for flow cytometry and microscopy

| Dye                  | Concentration/Dilution | Marker of                      | Flow cytometry or microscopy |
|----------------------|------------------------|--------------------------------|------------------------------|
| DAPI                 | 1 µg/ml                | Nuclei                         | Microscopy                   |
| CellMask deep red    | 1/30000                | Cell membranes                 | Microscopy and Flow Cytometry |
| CellMask Green       | 1/1000                 | Cell membranes                 | Microscopy                   |
| TOPRO-3 iodide       | 1/1000                 | Dead cells                     | Microscopy                   |
| CellTracker CMAC blue| 20 µM                  | Cell cytosol                   | Microscopy                   |

3.6 | RT-qPCR

Uninfected control and Mtb- or Mabs-infected AOs (15 per condition) were collected at 48 hr post-infection, lysed in 1 ml of TRIzol Reagent (Invitrogen) and stored at −80°C for 2 days. As positive controls, AOs were stimulated with 0.02 µg/ml of human IL-1β (Invivogen) or 0.1 µg/ml of IFNγ (PeproTech) for 48 hr. Total RNA was extracted using the RNeasy mini kit (Qiagen) and retrotranscribed (150 ng) using the Verso cDNA Synthesis Kit (Thermo Scientific). mRNA expression was assessed with an ABI 7,500 real-time PCR system (Applied Biosystems) and the SYBR® Select Master Mix (ThermoScientific). Relative quantification was determined using the 2−ΔΔCt method and normalized to GAPDH. Primer sequences are provided in Table 1.

3.7 | Enzyme-linked immunosorbent assay

Between 20 and 30 organoids were embedded in fresh BME Cultrex and cultured with 800 µl complete media. After 48 hr, the supernatant was collected, sterilized through double 0.22 µm filters and stored at −80°C until analysis. IL-8 ELISA was performed according to manufacturer instructions (Qiagen).

3.8 | Flow cytometry and cell sorting

Organoids were washed out of Matrigel and dissociated into single cells using TrypLE for 5 min at 37°C. A minimum of 5 x 10^5 cells/ml were incubated with dsRED expressing Mtb at an MOI of 10 in complete organoid media. After 4 or 24 hr for single cells, or 7 days for whole intact organoids, samples were washed with PBS, stained with CellMask Deep Red (1:30,000) to highlight all cells composing the organoid and fixed in 4% paraformaldehyde overnight at 4°C. Cells were pelleted and resuspended in PBS supplemented with 2% FCS. Samples were filtered just before analysis and sorted using a BD FACS ARIA Fusion. Cells were selected on their FSC-A/SSC-A profile, doublets were excluded on FSC-W/FSC-H and SSC-W/SSC-H parameters. Finally, double positive cells were gated based on Cell Mask Deep Red staining and DsRED-expressing Mtb. The population of cells from PS (positive for GFP/bacteria and APC/organoid cells) was pooled and imaged by confocal microscopy to determine if bacteria was intracellular. The fluorescent dyes used are summarized in Table 2.

3.9 | CmMTB preparation and macrophage co-cultures

Peripheral blood mononuclear cells were enriched from buffy coat using RosetteSep human monocyte enrichment cocktail according to the manufacturer’s instructions (Stem Cell Technologies). Monocytes were purified by density gradient centrifugation using Lymphoprep (Stem Cell Technologies) according to the manufacturer’s protocol. Monocytes were differentiated into macrophages by addition of 5 ng/ml macrophage colony-stimulating factor (Sigma Aldrich) for 6 days. cmCTR and cmMTB were prepared and used as previously described (Lastrucci et al., 2015). Organoids were stained with CellMask Deep Red plasma membrane dye as previously described, and macrophages stained with 20 µM CellTracker Blue CMAC dye (ThermoFisher Scientific) for 1 hr in serum-free media. Microinjected organoids and macrophages were resuspended in freshly prepared Rat Tail Collagen type 1 (Thermofisher, 1 mg/ml) and polymerized in a 4-well, glass-bottom µ-slide (Ibidi) at 37°C for 30 min and imaged for 96 hr under a FEI CorrSight microscope.

ACKNOWLEDGMENTS

Authors acknowledge C. Kuo (Stanford University, USA) for the stable expressing Rspo1-Fc cell line, and the Hubrecht Institute for the stable expressing Noggin cell line; Genotoul TRI-IPBS core facility for flow cytometry and imaging, in particular E. Näsér and E. Vega; IPBS BSL-3 facilities, in particular C. Verollet for technical support. Authors also acknowledge the Microscopy CORE lab and PLUC facility at Maastricht University. This work was supported by grants from Campus France PHC Van Gogh (40577Z to GL-V), the Agence Nationale de la Recherche (ANR-15-CE15-0012 (MMI-TB)) to GL-V, FRM “Amorçage Jeunes Equipes” (AJE20151034460 to E.M), ERC StG 693 (INFLAME 804249 to EM), ATIP to EM, ZonMW 3R’s (114021005) to PJP, the Nuffic Van Gogh Programme (VGP.17/10 to NI), and by the LINK program from the Province of Limburg, the Netherlands.

CONFLICT OF INTEREST

Hans Clevers and Norman Sachs are inventors on patents related to organoid technology. Other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

NI, CC, and PJP designed the experiments with the help of CLI, ON, EM, and GLV. NI, SALI, and CC performed the experiments with the contribution of KK, RBGR, SM, AP, CCh, KC, and CG. SR generated...
the fluorescent Mtb strains. MME and JM provided the lung biopsies. HC and NS developed the lung organoid technology. NI, CC, SALI, and PJP wrote the manuscript.

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**How to cite this article:** Iakobachvili, N., Leon-Icaza, S.A., Knoops, K., Sachs, N., Mazères, S., Simeone, R., et al (2022) Mycobacteria–host interactions in human bronchiolar airway organoids. *Molecular Microbiology*, 117, 682–692. [https://doi.org/10.1111/mmi.14824](https://doi.org/10.1111/mmi.14824)