OXFORD FEMS

FEMS Microbiology Reviews, 2024, **48**, fuae007

DOI: 10.1093/femsre/fuae007
Advance access publication date: 26 February 2024
Review Article

In vitro modelling of bacterial pneumonia: a comparative analysis of widely applied complex cell culture models

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Editor: [Claudio Avignone Rossa]

Abstract

Bacterial pneumonia greatly contributes to the disease burden and mortality of lower respiratory tract infections among all age groups and risk profiles. Therefore, laboratory modelling of bacterial pneumonia remains important for elucidating the complex host–pathogen interactions and to determine drug efficacy and toxicity. *In vitro* cell culture enables for the creation of high-throughput, specific disease models in a tightly controlled environment. Advanced human cell culture models specifically, can bridge the research gap between the classical two-dimensional cell models and animal models. This review provides an overview of the current status of the development of complex cellular *in vitro* models to study bacterial pneumonia infections, with a focus on air–liquid interface models, spheroid, organoid, and lung-on-a-chip models. For the wide scale, comparative literature search, we selected six clinically highly relevant bacteria (*Pseudomonas aeruginosa*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*). We reviewed the cell lines that are commonly used, as well as trends and discrepancies in the methodology, ranging from cell infection parameters to assay read-outs. We also highlighted the importance of model validation and data transparency in guiding the research field towards more complex infection models.

Keywords: bacterial pneumonia; air–liquid-interface; spheroid; organoid; lung-on-a-chip; advanced cell culture models

Introduction

As the most lethal communicable disease worldwide, lower respiratory tract infections such as pneumonia continue to rank as one of the primary contributors to global morbidity and mortality (Heron 2015, World Health Organization 2020). The most common classification of pneumonia is dependent on the conditions under which the infection is contracted: community-acquired pneumonia (CAP) refers to an infection contracted outside of a hospital setting, whereas hospital-acquired pneumonia is an infection following a hospital stay of more than 48 h. Ventilator-associated pneumonia (VAP) can develop in patients admitted to the intensive care unit who have been mechanically ventilated (Pahal et al. 2018, Lanks et al. 2019, Lim 2020). Although CAP can be caused by a variety of microbes, the vast majority of the adult population presenting with symptoms of pneumonia has a lung infection of bacterial origin (Torres et al. 2021). The most common cause of bacterial CAP is *Streptococcus pneumoniae*, which is associated with an especially high disease burden in young children, as indicated by the loss of years due to illness, disability, or premature death (Brooks and Mias 2018, Torres et al. 2021). *Streptococcus pneumoniae* is a part of the commensal mucosal flora of the respiratory tract but it can cause invasive diseases such as pneumonia upon further dissemination, often in immunocompromised individuals (Brooks and Mias 2018). Next, *Haemophilus influenzae* remains the second most common pathogen related to CAP (Shoar et al. 2021). In countries where tuberculosis is endemic, *Mycobacterium tuberculosis* remains an important cause of CAP (Wu et al. 2007). Other common causative bacteria of CAP include the atypical pulmonary pathogens *Mycoplasma pneumoniae* and *Legionella pneumophila* (Cunha 2006, Torres et al. 2021). *Staphylococcus aureus*, including the methicillin-resistant *S. aureus* strain, and Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* are more commonly encountered as nosocomial pathogens (Torres et al. 2021). *Pseudomonas aeruginosa*, e.g. is the most common pathogen associated with VAP infections (Tsay et al. 2016).

In vitro modelling of bacterial pneumonia remains indispensable when studying certain aspects of pneumonia pathogenesis and host–pathogen interactions. Cell-based models offer a controlled environment that can simulate the cellular and physiological conditions of the human respiratory tract (Nichols et al. 2014, Barron et al. 2021). *In vitro* modelling also provides a valuable platform to investigate the human host immune responses, pathogen evasion strategies, and the impact of specific virulence factors on disease severity (Nichols et al. 2014, Barron et al. 2021). In addition, *in vitro* models can be of use in assessing the efficacy and toxic side effects of therapeutics in early drug research, without the maintenance of costly preclinical animal facilities (Nichols et al. 2014, Duval et al. 2017). The development and routine implementation of these cell-based *in vitro* models in laboratory research remains an urgent point of action in the global initiative for reducing the use of animal models (Doke and Dhawale 2015, Marshall et al.

Received 2 October 2023; **revised** 29 January 2024; **accepted** 24 February 2024

2022). The European Union, e.g. has issued various guidelines and directives on alternative approaches to *in vivo* research as part of the global '3R strategy' (Marshall et al. 2022). The 2010/63/EU directive protects and restricts the use of animals in scientific research, with an end goal of replacing all animal models with validated alternatives, highlighting again the importance of suitable (bacterial) cell culture models (Directive 2010/63/EU 2010). While mostly used complementary to animal testing today, advanced cell culture models hold significant translational promise. These complex models can bridge the knowledge gap between information unveiled in rodent models, and the true in-human situation (Bosáková et al. 2022). Despite the steady increase of the use of advanced cell culture models within bacterial pneumonia research the past two decades, no comprehensive overview exists to date.

This review aims to present an in-depth summary of the current status of the development of complex cellular *in vitro* models to study bacterial lower respiratory infections. We focus hereby on air–liquid interface (ALI) models, spheroid, organoid, and lung-on-a-chip (LOC) models. These advanced models offer better insights into complex host–pathogen and host–pathogen–drug interactions compared to standard submerged two-dimensional cell monolayers. Although precision-cut lung slices have been documented in literature as an advanced cell culture model, their application in our context is less common, and therefore this *ex vivo* model is not discussed in this review. Additionally, this review provides a thorough overview of the methodologies used by researchers to establish and investigate bacterial infections in these complex cellular models. It can serve as a valuable resource for researchers looking to start their bacterial lung infection cell model. For the literature search, a selection of six highly clinically relevant bacteria (*P. aeruginosa*, *M. pneumoniae*, *H. influenzae*, *M. tuberculosis*, *S. pneumoniae*, and *S. aureus*) was made. This panel of bacteria was responsible for an estimated 3 million deaths related to lower respiratory tract infections in 2019 (Fukunaga et al. 2021, GBD 2019 Antimicrobial Resistance Collaborators 2022). Simultaneously, bacteria were chosen to represent a varied spectrum, both pertaining to the type of pneumonia they cause as to their cell wall type, replication location, and host cell (Table 1). A detailed description of these advanced models and their characteristics falls outside the scope of this review. A thorough examination of currently available *in vitro* human lung models, including advantages and disadvantages, can be found in recent work from Moreira et al. (2022).

Relevant articles describing the use of complex cell culture systems in bacterial pneumonia research were gathered by a comprehensive, systematic literature search of all PubMed-indexed papers of the past 20 years (2003–2023). A total of 157 research articles were found by using the query *('selected bacterial strain'[Title/Abstract]) AND ((lung[Title/Abstract]) OR (pneumonia[Title/Abstract]) OR (airway[Title/Abstract])) AND (cells) AND ((air liquid interface) OR (transwell) OR (spheroid) OR (lung on a chip) OR (organoid) OR ("3-D aggregates") OR (microfluidic-model) OR ("3-D cell culture") OR ("3D cell culture") OR ("3D aggregates") OR (three-dimensional cell culture) OR ("organ culture"))* in the PubMed database (July 2023). This selection was further refined by manually selecting only the relevant research articles, leading to a total of 84 included papers.

Research trends

Within the selected literature, *P. aeruginosa* was the bacterium most commonly investigated in advanced cellular models (Fig. 1A). Fewer advanced cell models, however, have been used to generate *in vitro* infections for the other selected lung pathogens. The use of ALI models applying permeable inserts to grow differentiated cell layers is still far more common in laboratory research than the use of the three-dimensional spheroid and organoid models or the LOC devices. Presumably, the transition from accessible two-dimensional submerged models to air–liquid transwell systems is easier, and therefore more commonplace in research groups, as less new equipment or reagents are needed to set up these ALI models (Silva et al. 2023). Although organoid models have been widely used and accepted for decades, the transition in bacterial infection research has been much slower. In cancer research, e.g. the use of three-dimensional lung cell models is customary, and even in the study of viral infections the use of lung organoid models is more common (Lee and Lee 2020, Bassi et al. 2021, Hofer and Lutolf 2021). One limiting factor that delayed the development of bacterial infection organoid models is the difficulty of delivering the pathogens to the luminal side of the organoid (Blutt and Estes 2022). Recent developments such as the microinjection platform, however, have facilitated the use of organoids in bacterial infectious research (Shpichka et al. 2022). Next, the microfluidic LOC device has also been used in many areas of research but remains underrepresented in the collection of research studies (Singh et al. 2022). The microfluidic chip models are known for their challenging upscaling and validation and are also more time-consuming and costly, which may have hampered their use in bacterial research (Lee and Lee 2020, Bassi et al. 2021). In addition, LOC devices also require the use of a more complex experimental set-up and equipment, as well as specific personnel expertise (Loewa et al. 2023). Importantly, when looking at the selected studies, there seems to be an upward trend in the use of the spheroid, organoid, and LOC devices in the last 4 years within research for bacterial pneumonia, possibly indicating the start of a changing research field (Fig. 1B).

Complex cell culture models of the lung

The lung is a vital organ of the respiratory system and is responsible for essential physiological functions, including the exchange of gases, as well as various immunological mechanisms (Weber et al. 2020, Mettelman et al. 2022). Maintaining a functional ALI barrier, which separates the atmosphere from fluid-containing tissues, allows for the efficient delivery of oxygen to the blood and the removal of carbon dioxide generated by cellular metabolism (Sidhaye and Koval 2017). In addition, the immune defence of the lung plays a crucial role in safeguarding delicate lung tissue against harmful airborne agents (Mettelman et al. 2022). The trachea, located in the most proximal part of the airways, divides into the left and the right main stem bronchi. These bronchi further branch into increasingly thinner bronchioles, eventually connecting to the smallest airways that lead to the most distal part of the airways, the alveoli, i.e. tiny air sacs responsible for gas exchange in the lungs (Nikolić et al. 2018). Airway epithelial cells lining the respiratory tract act as a physical barrier with different subtypes of cells, all contributing to functions such as mucus clearance, pathogen entrapment, and airway integrity (Hewitt and Lloyd 2021). Alveolar epithelial cells in the alveolar sacs facilitate gas diffusion, and immune cells like macrophages, mast cells, T cells, B cells, and natural killer cells safeguard the respiratory system against pathogens and foreign substances (Nikolić et al. 2017, Sidhaye and Koval 2017, Agrawal 2019, Cong and Wei

Table 1. Overview of bacterial pathogens selected for the review literature search. Cell wall, primary replication location, and possible host cells are briefly summarized.

Bacterium	Cell wall	Primary location	Host cell
M. pneumoniae	No cell wall	Extracellular	• Epithelial cells; intercellular survival in the cytoplasm and perinuclear regions possible (Yavlovich et al. 2004, Hu et al. 2022).
P. aeruginosa	Gram-negative		• Epithelial cells; internalization and intracellular survival possible, subpopulations in vacuoles and cytoplasm (Kumar et al. 2022).
H. influenzae		Intracellular	• Epithelial cells; intracellular replication (Duell et al. 2016). • Macrophages; survival possible (Duell et al. 2016).
M. tuberculosis	Acid-fast		• Macrophages; survival and replication, evasion of host immune system (Mahamed et al. 2017).
S. pneumoniae	Gram-positive	Extracellular	• Epithelial cells; intracellular survival possible, possible niche within macrophages (Nyazika et al. 2022).
S. aureus		Facultative intracellular	• Phagocytic and nonphagocytic cells; replication and survival possible (Hommes and Surewaard 2022).

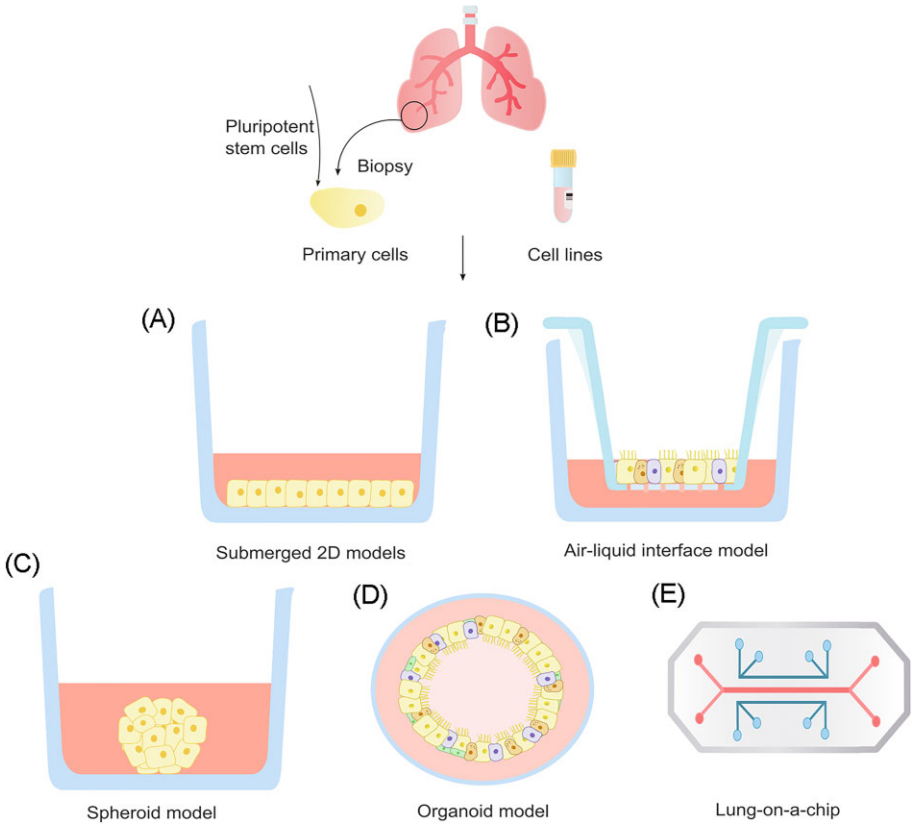

Figure 1. Overview of the most commonly used *in vitro* cellular models in pneumonia research. (A) While still broadly used, the standard two-dimensional submerged model has a lower translational value compared to the more advanced cellular models. (B) ALI models allow for cell differentiation by exposing cell monolayers to air resulting in a pseudostratified epithelium. (C) Spheroid models have a three-dimensional structure but lack self-renewing capacity. (D) Organoids are more complex three-dimensional cell structures with an organ-like architecture, including tissue lumen and self-renewal. (E) LOC devices contain multiple chambers where cells are seeded and exposed to a dynamic microenvironment, including a continuous air and medium flow, and fluid shear stress.

2019, Aegerter et al. 2022, Banafea et al. 2022). The intricate design and organization of the lung makes it challenging to establish a model that fully replicates the complex structure and set of physiological functions. Several advanced models, however, have emerged that mimic the human lung more closely than the traditionally used two-dimensional cell models. Nevertheless, these complex cell culture models face challenges such as high costs, complex protocols, standardization issues, and complex and expensive technologies.

Air–liquid-interface models

In traditional cell culture models, respiratory cells are commonly cultured on two-dimensional surfaces, such as tissue culture flasks or plates, submerged in a liquid medium (Fig. 2A). This approach enables for the study of cellular behaviour, responses, and interactions in a controlled *in vitro* setting (Hermanns et al. 2004, Luyts et al. 2015). A major limitation of submerged cell cultures is the absence of direct and constant contact with atmospheric air. The contact with atmospheric air is crucial for proper cellular dif-

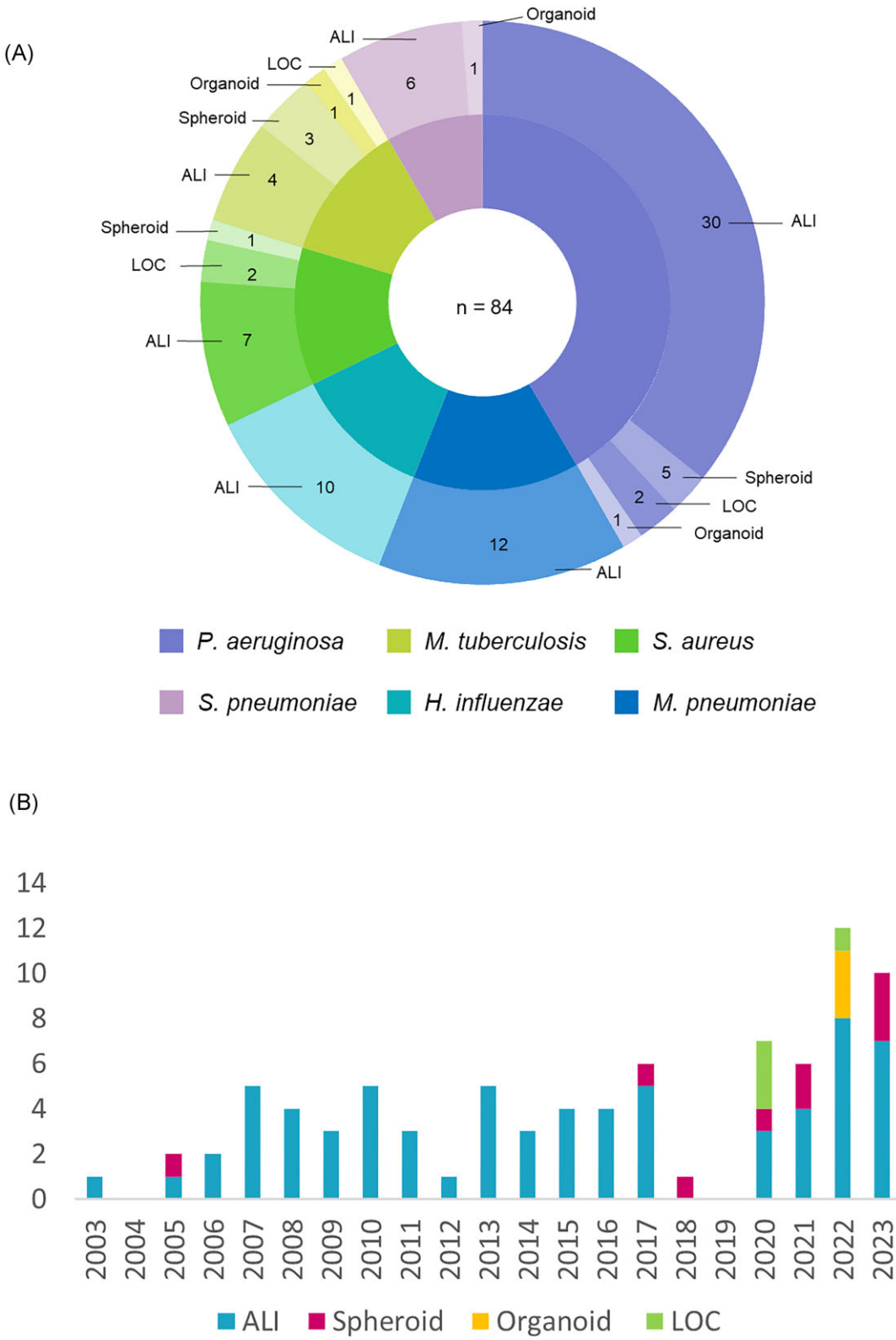

Figure 2. Overview of the 84 selected research articles making use of a complex cellular infection model for bacterial pneumonia as of July 2023. (A) Articles subdivided per bacterium and cell model. (B) Selected articles per publication year, subdivided per advanced cellular model. ALI = air–liquid interface, LOC = lung-on-a-chip.

ferentiation and functional barrier formation, which submerged culture models fail to fully replicate (Gerovac et al. 2014, Hiemstra et al. 2019). As an alternative, ALI models have emerged as valuable tools for studying lung physiology and pathology.

To establish an ALI culture, respiratory epithelial cells are seeded onto permeable transwell inserts, and grown until a confluent monolayer is achieved. Subsequently, the cells are exposed to air to initiate differentiation. This method creates a more physiologically relevant environment compared to traditional submerged cell culture techniques (Fig. 2B) (Hittinger et al. 2016, Chandrasekaran et al. 2019, Cao et al. 2021). ALI models offer several advantages over conventional cell culture methods, including the differentiation of respiratory epithelial cells into functional phenotypes resembling those in the human lung and gaseous exchange with the environment. Culture conditions can be manipulated to recapitulate specific lung cell types. ALI models provide a platform to investigate crucial processes such as mucociliary clearing, important for removing particles and pathogens from the airways (Ross et al. 2007). ALI models are also useful for studying infection dynamics, host defence mechanisms, and barrier function of airway epithelium (Cao et al. 2021).

Three-dimensional models

Advancing towards more complexity, spheroids and organoids have emerged as biomimetic platforms for the investigation of intercellular communications, cell–extracellular matrix interactions, and organ development and functionality (Fig. 2C and D) (Yin et al. 2016, Sharma et al. 2020). Although 'spheroid' and 'organoid' are often used interchangeably, significant differences can be noted. Spheroids are considered simple structures formed through self-assembly without a scaffold or three-dimensional matrix and are often used as tumor models because of the hierarchical composition of the external layers (Barrera-Rodríguez and Fuentes 2015, Hu et al. 2015, Meenach et al. 2016, Zhang et al. 2018, Zanoni et al. 2020, Sato et al. 2021). However, spheroids derived from cell lines (Barrera-Rodríguez and Fuentes 2015, Hu et al. 2015, Meenach et al. 2016) or primary cells from patients (Chimenti et al. 2017, Zhang et al. 2018) often lack self-renewing stem or progenitor cells limiting their ability to sustain a three-dimensional, multicellular structure (Yin et al. 2016). Organoids, in contrast, mimic 'miniaturized organs' with multiple cell types, self-renewal, and differentiation potential (Lancaster and Knoblich 2014). Lung organoids possess the capability to perform specific functions unique to the lung, including surfactant secretion, particle clearance, and defence against microbial infections (Tian et al. 2021). Organoids can be obtained from adult patient stem cells (ASCs), from both healthy tissue (organoids) and malignant tissue (tumoroids) (Heo et al. 2018, Kim et al. 2019, Sachs et al. 2019, Li et al. 2020, Shi et al. 2020), or pluripotent stem cells (PSCs) (Dye et al. 2015, Nikolić et al. 2017, Chen et al. 2018, Meyer-Berg et al. 2020). PSC-derived organoids replicate organogenesis processes (Yin et al. 2016, Takebe and Wells 2019, Schutgens and Clevers 2020), while ASC-derived organoids primarily represent adult tissue repair (Schutgens and Clevers 2020). Various cell culture methods are employed to generate and maintain the structures of spheroids and organoids, tailored to specific research objectives and cell types. For spheroids, methods include the hanging drop method and liquid overlay method, while organoid culture methods encompass the submerged method, ALI method, and bioreactor culture. In this review, we refrain from providing an exhaustive discussion of the methods employed, as comprehensive details regarding these techniques can be found in the work of Gunti et al. (2021).

Lung-on-a-chip

In recent years, significant advancement has been made in cell-based *in vitro* models and microfabrication technology. Combining physiologically relevant cell culture models with microfluidic chips has led to the development of organ-on-chip (OOC) systems. These bionic devices mimic the microstructure and functions of living organs, offering a more precise simulation of organ physiology and pathology compared to conventional *in vitro* cell culture models (Ingber 2016, Li et al. 2019, Ingber 2022). Numerous organs, such as the liver (Hassan et al. 2020, Moradi et al. 2020), gastrointestinal tract (Marrero et al. 2021, Morelli et al. 2023), and kidney (Chen et al. 2021, Wang et al. 2022), have already been successfully modelled using OOC technology. Despite several reports describing LOC devices (Fig. 1E), this field is still in its early stages, likely due to the complex geometry and diverse cell composition of the lung microenvironment. Typically, a LOC system consists of two channels separated by a porous membrane. The upper channel is seeded with epithelial cells and provides oxygen to form an ALI, the lower channel contains vascular endothelial cells and is continuously perfused with cell culture medium, simulating capillary channels and fluids shears (Huh et al. 2010, Zamprogno et al. 2021). However, a multitude of designs exists, ranging from commercially available options to numerous in-house designs detailed in the literature, some of which may incorporate additional features, such as cyclic stretching of the membrane to mimic respiration, adjusting flow rates of culture media to simulate blood circulation, incorporating sensors to monitor key parameters, and integrating immune cells.

Cell selection for pneumonia models

Pneumonia models mostly mimic either the epithelial structure of the trachea and bronchi, the alveolar epithelium, or in some cases, a combination of both. Additionally, the incorporation of endothelial cells enhances the model, offering a more encompassing representation of host–pathogen interactions. However, there is less focus on endothelium since epithelial cells perceived a primary role in initial infection stages and cultivating cocultures with endothelial cells often involves technical challenges. The pseudostratified epithelium of the respiratory tract is characterized by the tiered arrangement of diverse cell types, each contributing distinct functions essential for maintaining respiratory homeostasis and responding to environmental challenges such as bacterial infections (Figs 3 and 4A) (Hewitt and Lloyd 2021). Basal cells are multipotent stem cells, that participate in tissue repair and regeneration. They can differentiate into various major subpopulations of cells, replenishing the epithelium's cellular pool and ensuring its integrity after injury or inflammation (Evans et al. 2001, Hewitt and Lloyd 2021). Goblet cells secrete mucins, which upon hydration contribute to the production of mucus. This mucus acts as a protective barrier, trapping pathogens and particles, while also facilitating their expulsion from the respiratory system (Birchenough et al. 2015, McCauley and Guasch 2015). Ciliated cells dominate the pseudostratified epithelium, wielding cilia that generate coordinated, directional movements to propel mucus and trapped particles out of the respiratory tract (Chilvers et al. 2003, Park et al. 2008). Club cells, also known as clara cells, produce secretory proteins that protect the respiratory epithelium, regulate inflammation and contribute to the maintenance

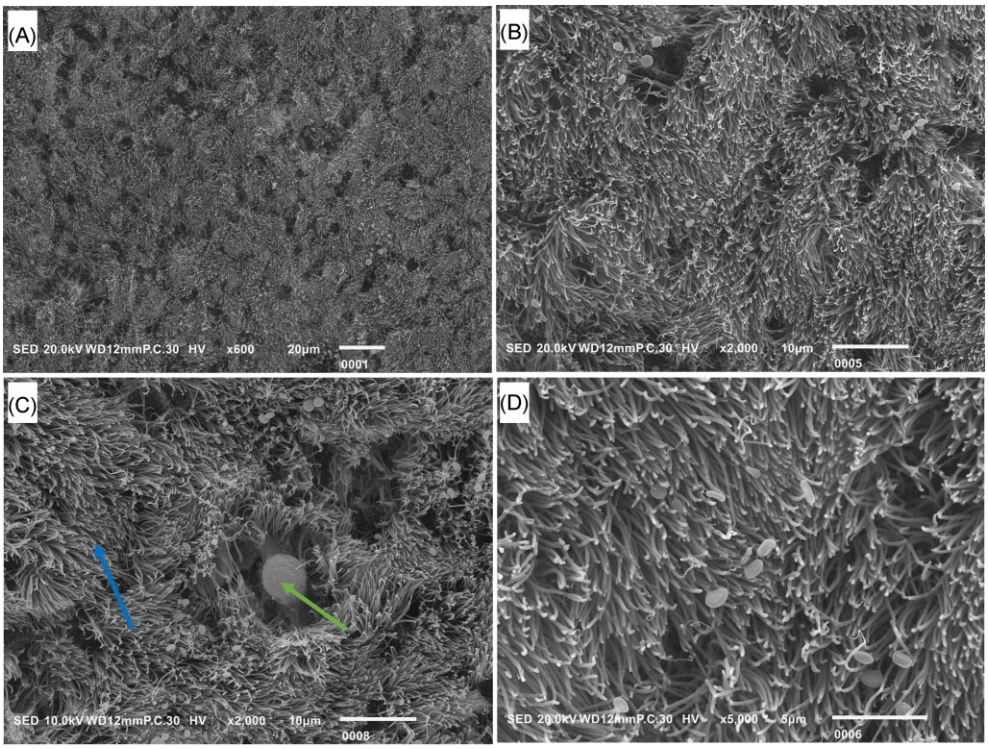

Figure 3. Scanning electron microscopy (SEM) of human, upper respiratory epithelium. Transwell inserts with cells were bought from Epithelix (MucilAir) and cultured at the air–liquid-interface for 2 weeks before imaging. Homogeneous monolayer of bronchial epithelial cells, at different magnifications. (A) SEM at 600x magnification. Cilia are densely packed on the surface, (B) SEM at 2000x magnification. Cilia are densely packed on the surface, (C) SEM at 2000x magnification. Densely packed cilia (left arrow) interspersed with goblet cells (right arrow), and (D) SEM at 5000x magnification. Detailed close-up view of ciliated cells. Images were taken at the Advanced Centre for Advanced Microscopy facility of the University of Antwerp in collaboration with the Laboratory for Microbiology, Parasitology and Hygiene.

of the lung's homeostasis (Rokicki et al. 2016, Zhai et al. 2019, Martinu et al. 2023). More rare epithelial cell types include ionocytes, pulmonary neuroendocrine cells, microfold cells, and tuft cells. Ionocytes play a vital role in regulating electrolyte and fluid balance within the respiratory lining fluid. By controlling ion transport, these cells help establish the optimal conditions for efficient mucociliary clearance and respiratory function (Shah et al. 2022). Ionocytes are rich in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, involved in cystic fibrosis (CF) pathology (Shah et al. 2022). Pulmonary neuroendocrine cells are dispersed throughout the pseudostratified epithelium and are involved in sensory perception and chemosensory detection. They play a role in responding to environmental cues and modulating local immune and vascular responses (Hewitt and Lloyd 2021). Microfold cells are specialized epithelial cells found in the mucosa-lymphoid tissue of the respiratory tract. These cells play a critical role in the immune responses by facilitating the uptake and transport of antigens from the external environment (Man et al. 2004, Sato and Kiyono 2012). Tuft cells are chemosensory cells with immunoregulatory functions (Davis and Wypych 2021). Their role in respiratory disease is less well characterized, but they are implicated in the response to allergens and parasitic infections (Sato 2007, Davis and Wypych 2021). Lower in the lungs we find alveolar epithelial cells, lining the alveolar sacs (Fig. 4A). This epithelium consists of type I alveolar (ATI) cells responsible for gas diffusion, and type II alveolar (ATII) cells secreting surfactant, aiding alveolar stability and immune response (Sidhaye and Koval 2017, Nikolić et al. 2018).

For the *in vitro* cultivation of the respiratory epithelium, primary cells isolated from humans or established cell lines can be used. In the literature review, primary lung-derived cells (ACS's) were most often encountered in the set-up of the advanced cell models. As primary cells contain the subset of basal multipotent stem cells, they can give rise to the formation of a complete, differentiated pseudostratified epithelium. Immortalized cell lines, on the other hand, exhibit lower differentiation potential and usually only mimic the characteristics of a specific cell type or combination of cell types (e.g. ciliated cells or club cells).

Primary cells

Primary cells are most often used in complex cellular models. They retain the characteristics and physiological properties of a true, differentiated tissue, as they are immediately isolated from the organism without undergoing extensive culture or manipulation (Kaur and Dufour 2012). Despite these advantages, there are also some challenges associated with primary cells, e.g. the batch-to-batch and interdonor variability (Bovard et al. 2020). It is noteworthy, however, that this donor variability can also be seen as advantageous by increasing clinical relevancy. Another important disadvantage is that primary cells possess a finite lifespan and can only replicate for specific number of passages before they undergo loss of their original characteristics and functions. Several companies, such as Lonza, MatTek, Cambrex, Cell Biologics, and Epithelix offer primary cells of different regions of the respiratory tract. Moreover, it is also possible to obtain primary cells from tissues or organs in-house. Typically, this is done through enzymatic digestion or mechanical disruption of a specific region of the respiratory tract (Clifford et al. 2019, Golec et al. 2022, Ninaber et al. 2023). For enzymatic isolation, a piece of bronchial

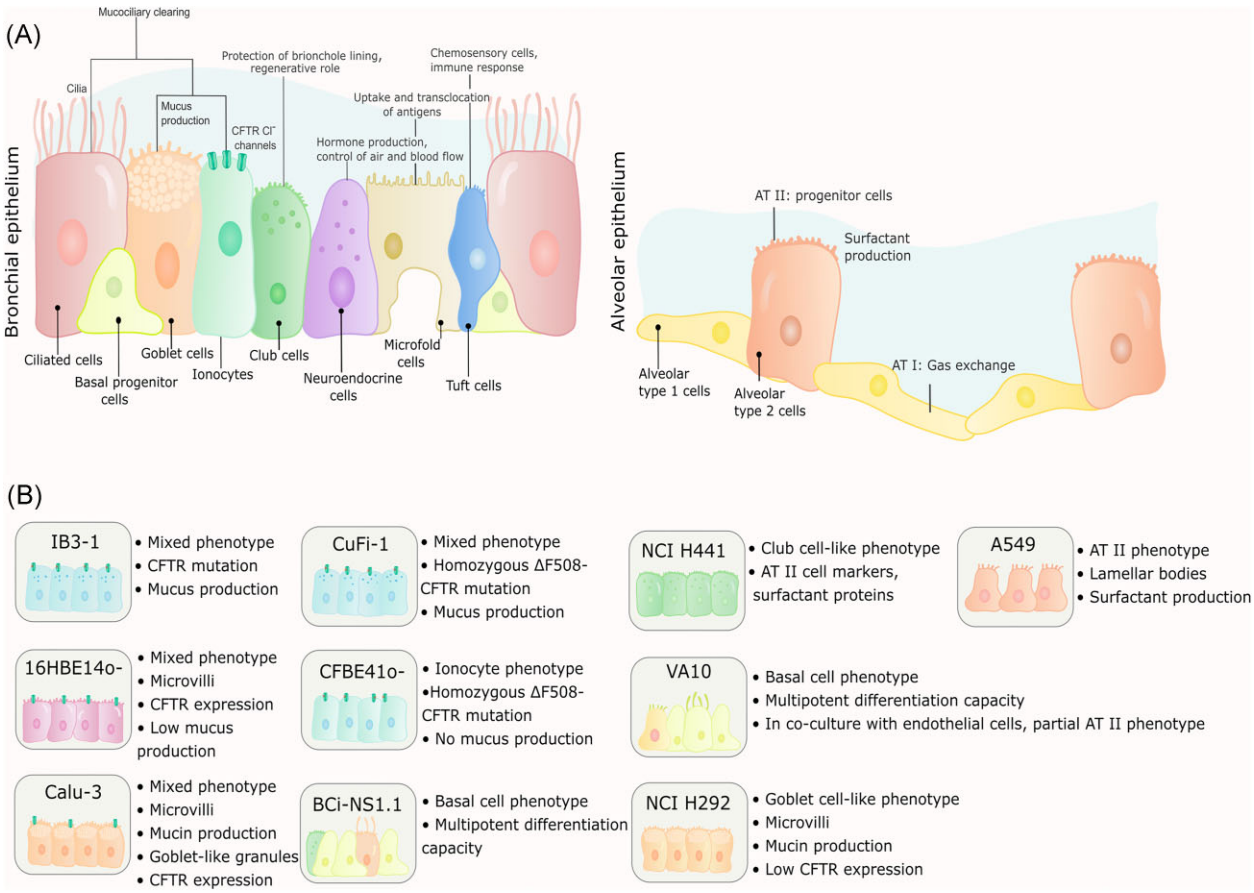

Figure 4. (A) Main cells of the differentiated bronchial and alveolar epithelium. The respiratory epithelium is characterized by a multitude of cell subsets, including ciliated cells and serous goblet cells, club cells and basal progenitor cells. More rare cell types include the ionocytes, microfold cells, neuroendocrine cells, and tuft cells. The alveolar epithelium mainly consists of two cell types, the alveolar type I (ATI) and type II cells (ATII). (B) Cell lines used to establish complex bacterial infection models and some key characteristics are depicted. CFTR = cystic fibrosis transmembrane conductance regulator.

ring tissue is excised from lung tissue and exposed to enzymatic treatment to detach and dissociate the cell layer (Ninaber et al. 2023). Mechanical disruption relies on a cytology brush, which is inserted into the respiratory tract of a dissected lung and vigorously stroked along the wall of the bronchi. Subsequently, the cells are washed and distributed into a cell culture flask for expansion (Golec et al. 2022). Upon sufficient expansion the isolated cells can be cryopreserved for later usage (Clifford et al. 2019). The majority of articles reporting on the use of primary cells describe self-isolating cells from a donor. In these articles, mechanical disruption and enzymatic digestion are equally represented. Although both methods can yield satisfactory cell communities, it has been reported that brush biopsies can lead to a lower count of basal cells, which is especially important when creating cell structures with a self-renewing property (Gowers et al. 2018). Primary cells used in pneumonia research can originate from the whole respiratory tract, including the nasal, sinusal, tracheal, and bronchial regions. As isolating cells from structures located deep in the lungs is challenging, alveolar primary cells are rarely used (Gonzalez and Dobbs 2013). However, due to some recent successes in isolating these cells, an increase in the use of primary alveolar cells can be expected in the near future (Katsura et al. 2020). Respiratory and alveolar cells can be isolated from both healthy and disease-affected lungs, such as COPD or smoking-related conditions. The use of primary cells from patients with respiratory disease, may have added value in the study of bacterial infections in more specific conditions.

Continuous cell lines

Apart from primary cells, established cell lines can also be used. These continuous cell lines offer major advantages due to their ease of cultivation, reproducibility, and relatively unlimited supply. When properly maintained, they can retain a stable phenotype throughout many subcultures. However, this phenotype may differ from the original tissue, which can compromise their ability to fully replicate the *in vivo* physiological state. Using continuous cell lines often involves a trade-off between convenience and suitability, as these cells typically retain features more associated with the original tumor, including uncontrolled proliferative growth and a dedifferentiated phenotype (Geraghty et al. 2014).

Cell lines with a tracheobronchial character are commonly used in complex bacterial pneumonia cell models (Fig. 4B). Calu-3, e.g. is a well-characterized cell line originating from adenocarcinoma submucosal glands, which are sources of airway surface liquid, mucins, and other immune-active substances in the lung. As a result, Calu-3 cells are serous cells that have a goblet-like phenotype, containing mucin-secreting granules and microvilli (Dubin et al. 2004, Joo et al. 2004, da Paula et al. 2005, Ghanem et al. 2021). The CFTR protein is also abundantly present in Calu-3 cell layers (da Paula et al. 2005, Wiese-Rischke et al. 2021). Calu-

3 cells are mostly cultured using an ALI model to mimic the tracheobronchial epithelium in (i) host–pathogen interaction studies, (ii) biofilm research, and (iii) antibacterial drug studies (Starner et al. 2006, Hasan et al. 2018, Juntke et al. 2021, Qi et al. 2023). Calu-3 cells can form a polarized cell layer with tight junctions (Ghanem et al. 2021). Another cell line with bronchial character is the 16HBE14o- airway epithelial cell line, immortalized with simian virus 40 (SV40) (Cozens et al. 1994). This cell type exhibits multiple features of a differentiated epithelium, such as strong cell–cell junctions, microvilli, and expression of the CFTR protein. Hence, this cell line is mostly used in CF research and studies investigating the pulmonary barrier function (Haws et al. 1992, Cozens et al. 1994, Forbes et al. 2003, Oliynyk et al. 2010). However, mucus production by these cells is low (Sonntag et al. 2022). The use of the 16HBE41o- cell line is also reported for ALI culture models in the study of host–pathogen interaction (Sajjan et al. 2008, John et al. 2010, Junkins et al. 2014). Importantly, Qi et al. (2023) note that it is challenging to culture the 16HBE14o- cell line at ALI conditions for longer periods, in contrast to the Calu-3 cell line, a factor, i.e. worth considering when aiming for long-time infection models(111). Another less common bronchial cell line is BCi-NS1.1, a basal cell immortalized-nonsmoker 1 cell line, used by Prescott et al. (2023) to investigate *S. aureus* infection in ALI cultured models (Prescott et al. 2023). These cells retain many original characteristics of primary cells and give rise to cells with goblet and club cell character, as well as mucus-producing and ciliated cells (Walters et al. 2013, Prescott et al. 2023). Remarkable is also the use of Caco-2 cells by Nair et al. (2016), as these cells originate from human colorectal adenocarcinoma, and are typically used as a ALI model of the intestinal barrier (Nair et al. 2016). In coculture with the lymphoblast-like cell type RajiB, Caco-2 cells are known to differentiate into microfold epithelial cells (Nair et al. 2016, Selo et al. 2021). A disease-specific bronchial cell line is CFBE41o-. This cell line is homozygous for the ΔF508 CFTR mutation and SV40 immortalized (Ehrhardt et al. 2006). The CFBE41o- cell line has been used in ALI infection models for *P. aeruginosa*, a pathogen common in CF patients (John et al. 2010, Juntke et al. 2021, Kuek et al. 2022). Unfortunately, unlike certain pulmonary cell lines such as Calu-3, the CFBE41o- cell line cannot produce and release mucus (Grainger et al. 2006, Haghi et al. 2010, Mura et al. 2011). As mucus production is an important characteristic of normal airway behaviour and bacterial infection, the lack or absence of mucus production must be taken into account when choosing commercial cell lines for airway research (Ehrhardt et al. 2006). Importantly, the addition of human tracheobronchial mucus to CFBE41o- cell structures can compensate for the lack of intrinsic mucus production (Juntke et al. 2021). Alternatively, other CF-specific cell lines used to model a *P. aeruginosa* infection are the IB3-1 and CuFi-1 airway epithelial cell lines, also derived from CF patients (Zeitlin et al. 1991, Dechecchi et al. 2011, Sheikh et al. 2020).

Aside from bronchial cell lines, complex *in vitro* cellular models can also be established using alveolar-like cells, such as the A549 human lung adenocarcinoma cell line. This cell line has characteristic features of ATII cells, such as lamellar bodies and surfactant proteins, and has been a mainstay of respiratory research for over four decades (Lieber et al. 1976). However, more recent studies working with an early passage of A549 cells have led to conflicting results regarding their ability to exhibit these features (Swain et al. 2010, Corbière et al. 2011). For example, they lack the ability to form tight monolayers of polarized cells, due to their compromised tight junctions (Foster et al. 1998, Elbert et al. 1999). The use of A549 cells in ALI models is common, with applications ranging from host–pathogen interaction studies to the investigation of epithelial barrier translocation (Bermudez et al. 2002, Hurley et al. 2006, Tamang et al. 2012, Ahmed et al. 2023). Three-dimensional A549 aggregate models for a *P. aeruginosa* infection are described by Carterson et al. (2005) and Wang et al. (2023). LOC application was described by Zhang et al. (2013), who used A549 cells to determine bacterial adhesion to single-host human lung epithelial A549 cells (Zhang et al. 2013).

Lastly, various cell types exhibit a mixed bronchial and alveolar character (Fig. 4B). The NCI-H441 cells, e.g. are derived from the pericardial fluid of patients with papillary lung adenocarcinoma and exhibit characteristic features of club cells, while also producing ATII-related surfactant proteins (Vuong et al. 2002, Hermanns et al. 2004, Salomon et al. 2014). Deinhardt-Emmer's group used this NCI-H441 cell line in LOC models, cocultured with endothelial cells and macrophages (Deinhardt-Emmer et al. 2020, Schicke et al. 2020). Garnett et al. (2013) describe the use of ALI cultures with NCI-H441 cells to investigate the relationship between elevated blood glucose concentration and *S. aureus* growth and the effect of treatment with the antidiabetic drug metformin (Garnett et al. 2013). Lastly, its use in ALI cultures is also described by Carey et al. (2022) in research on antibacterial innate immunity (Carey et al. 2022). NCI-H292 is an epithelial cell line derived from a pulmonary mucoepidermoid carcinoma that constitutively expresses the epidermal growth factor receptor. Upon stimulation by its ligands, these cells exhibit an increase in mucus secretion. Their mucus-producing capacity makes them valuable for studying respiratory diseases associated with excessive mucus production (Takeyama et al. 1999, 2000). In contrast to the NCI-H441 cell line, NCI-H292 cells have difficulty forming a tight epithelial barrier (Salomon et al. 2014, George et al. 2015). This cell line has been used in ALI culture infection models for *P. aeruginosa* by Yonker et al. (2017) and Pazos et al. (2015). Next, VA10 cells, a human bronchial epithelial cell line, established through retroviral transfection of constructs containing E6/E7 oncogenes from the human papilloma virus-16 (Halldorsson et al. 2007). VA10 exhibit both basal- and stem cell phenotypes, expressing basal cell markers such as cytokeratins 5/6 and 14, along with the basal-associated transcription factor p63 (Halldorsson et al. 2007). The stem cell characteristics of VA10 are manifested by its ability to form bronchioalveolar-like structures in a three-dimensional culture (Franzdóttir et al. 2010). Furthermore, VA10 demonstrates the generation of active tight junctions in ALI culture, as evidenced by high transepithelial electrical resistance (Halldorsson et al. 2007). In coculture with endothelial cells, differentiation into a partial AT II phenotype has also been reported (Halldorsson et al. 2007). The murine lung epithelial cell line can also express surfactant proteins (Wikenheiser et al. 1993, Pazos et al. 2015).

Beyond epithelial cells

Incorporating secondary cell types, whether primary cells or cell lines, alongside epithelial cells, helps approximating the human-like situation in complex cell culture models. Endothelial cells can be incorporated to emulate the presence of pulmonary arteries, essential for the gas exchange in the lungs (Comhair et al. 2012). Plebani et al. (2022) and Thacker et al. (2020), e.g. extend their LOC model by incorporating primary human lung microvascular endothelial cells for a *P. aeruginosa* and *M. tuberculosis* infection, respectively (Thacker et al. 2020, Plebani et al. 2022). In transwell ALI models, endothelial cells can be seeded in the lower, basal chamber to establish a cell bilayer, as seen in an infection model used by Bermudez et al. (2002) to study *M. tuberculo-*

sis translocation (Bermudez et al. 2002). Apart from endothelial cells, immune cells can be incorporated. The murine macrophage cell line J774A.1 was used in an *M. pneumoniae* cell coculture ALI model by Shi et al. (2023), whereas human THP-1 monocytes were used in both ALI and spheroid model infected with *M. tuberculosis* (Reuschl et al. 2017, Mukundan et al. 2021). Conversely, Kotze et al. (2021) employ a spheroid model comprising primary alveolar macrophages and T cells, resembling early 'innate' and 'adaptive' stages of the tuberculosis granuloma (Kotze et al. 2021). Most cell models incorporating macrophages are *M. tuberculosis* models, as macrophages are their natural host cell (Mahamed et al. 2017). Apart from macrophages, mast cells (HMC-1 cells) have also been used to incorporate immune functions into respiratory epithelial models (ALI) as described by Junkins et al. (2014). LOC models with a coculture of epithelial cells, endothelial cells, and macrophages have been developed for *S. aureus* by Schicke et al. (2020) and Deinhardt-Emmer et al. (2020).

In general, cell cocultures are only sparsely used in bacterial pneumonia research, and most models still focus on monocultures of epithelial cells. However, other cell types including immunity-related cells and fibroblasts play a crucial role in bacterial lung injury processes (Greeley 2017). Moreover, cross-talk with endothelial cells is also important for epithelial cell differentiation (Burkhanova et al. 2022). While omitting these cells from *in vitro* models simplifies laboratory set-up and validation, some key cell–cell or cell–bacteria interactions can be missed due to a lack of human tissue representation.

Initiation of bacterial infection

After choosing the cells and cultivating the cell model, bacteria are added in the mix to simulate a lung infection. When comparing the protocols described in our literature search, a high variety can be observed in key parameters, including infection dose and infection duration. Even studies using the same cell model and bacterium (e.g. transwell models for acute *P. aeruginosa* infections), often use very different infection protocols. Here, we highlight some of the main parameters to look out for when establishing a bacterial infection. Table 2 gives a detailed overview of each study and its infection set-up parameters. Two critical parameters for the creation of an infection model are the duration of bacterial exposure, as well as the multiplicity of infection (MOI), defined as the ratio of the number of bacteria added to the number of cells seeded for the *in vitro* model. Here, we make a distinction between the primary bacterial exposure time, and any subsequent incubation periods following one or multiple washing steps.

Infection duration

Most research applying advanced cellular models for bacterial pneumonia simulates acute infections, studying host–pathogen interactions within 24 h of bacterial exposure. Short timeframes are very suitable for studying the bacterial adhesion and internalization processes, as well as the first host responses to bacterial invasion, including the early immune responses such as chemokine and cytokine production, and mucociliary clearing mechanisms. Adhesion of *P. aeruginosa* and *H. influenzae* to airway epithelial cells, e.g. can be quantified within 1 h after bacterial infection (Raffel et al. 2013, Badaoui et al. 2020, Kuek et al. 2022). Similarly, attachment of *M. pneumoniae* to ciliated epithelial cells can be visualized microscopically as fast as 2 min after initial exposure (Krunkosky et al. 2007). In an ALI *M. tuberculosis* infection model, bacterial-cell association and internalization were reported 30 min after infection, especially with nonvillous cells (Nair et al. 2016). Although *P. aeruginosa* is typically considered to be an extracellular pathogen, internalization can also be measured in the first hours following infection (Carterson et al. 2005, Bucior et al. 2010, Higgins et al. 2016). Interestingly, Carterson et al. (2005) observed that adhesion and invasion of airway epithelial cells occur slower in 3D-aggregate cell structures than in 2D-cell monolayers, due to increased tight junctions and more defined cell polarity (Carterson et al. 2005). Timepoints for experimental readouts should, therefore be chosen carefully and validated, as they can be dependent upon the chosen cell model. As soon as bacterial contact occurs, the early immune cascade of respiratory cells is set in motion. In both the ALI models and the 3D-cellular models, the release of proinflammatory cytokines and chemokines typically produced by epithelial cells such as IL-6, IL-1β, TNF-α, and the monocyte chemoattractant protein 1 can be quantified in the cell supernatant the first hours following infection (Carterson et al. 2005, Dechecchi et al. 2011, Bissonnette et al. 2020, Deinhardt-Emmer et al. 2020, Qi et al. 2023). Short ($\leq$ 24 h) bacterial exposure studies can also be used to study the initiation of mucociliary clearing mechanisms, e.g. by quantification of the MUC5 genes characteristically expressed by goblet epithelial cells (Hao et al. 2014). When investigating the integrity of the cellular layers, a decrease in tight junctions, cilia function, and viability can also occur hours after infection, even at low bacterial inoculum (Qi et al. 2023).

Although bacterial pneumonia typically manifests as an acute infection, chronic or recurrent infections are also highly clinically relevant. For example, *S. aureus* and *P. aeruginosa* often persistently colonize the lungs of CF patients in biofilm-type infections, and *H. influenzae* is known to cause recurrent, pervasive infections in COPD patients (Sato 2007, Kaur and Dufour 2012, Davis and Wypych 2021). *Mycobacterium tuberculosis* can also be present latently in lung tissue before causing an active, long-lasting infection (Bovard et al. 2020). Unravelling the dynamics of a chronic bacterial infection requires the use of long-term infection models. However, establishing an actively propagating long-lasting bacterial infection while also maintaining cellular structural integrity and viability is known to complicate the development of stable chronic infection models *in vitro* (Shi et al. 2019). This difficulty is also reflected by our literature search, where chronic models were only encountered sporadically. Models were deemed chronic when (i) the author specifically mentioned the development of a chronic infection model or, (ii) the model was used to study specific chronic infection lesions, such as the organoid and spheroid granuloma models for *M. tuberculosis* (Gowers et al. 2018, Clifford et al. 2019, Golec et al. 2022, Ninaber et al. 2023). For *P. aeruginosa*, one chronic ALI model was encountered where cells were repeatedly infected with bacteria over 16 days. To prevent high levels of cell death and bacterial overgrowth, the cell layers were washed daily, tobramycin was added to the cell–bacteria culture, and infectious doses of *P. aeruginosa* were kept low (MOI of 0.0025) (Endres et al. 2022). Several ALI models for long-term *M. pneumoniae* infections have been described as well (Green et al. 2009, Thaikoottathil et al. 2009, Gross et al. 2010, Prince et al. 2018). Prince et al. (2018), e.g. employ a model where *M. pneumoniae* remained closely associated with epithelial cells up to 28 days after infection, despite multiple mucus-washing steps (Prince et al. 2018). MOI for chronic *M. pneumoniae* models were also generally on the lower end (Cunha 2006, Wu et al. 2007, Heron 2015, Brooks and Mias 2018, Pahal et al. 2018, Lanks et al. 2019, Lim 2020, World Health Organization 2020, Shoar et al. 2021, Torres et al. 2021) compared to the acute models (Evans et al. 2001, Chilvers et al. 2003, Hermanns et al. 2004, Man et al. 2004, Cunha 2006, Ross et al. 2007, Sato 2007, Park et al. 2008, Directive 2010/63/EU 2010, Huh et al. 2010, Kaur and Dufour 2012, Sato and Kiyono 2012, Gonza-

Table 2. Methodology used to establish a bacterial infection in different cell culture models. General classification based on different bacterial species (*P. aeruginosa*, *M. tuberculosis*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and *M. pneumoniae*) and categorized according to the specific cell culture model [air–liquid interface (ALI), spheroid, organoid, and lung-on-a-chip (LOC)]. Further subdivision is delineated by infection type (acute = interaction within 24 h of bacterial exposure; chronic = author-specified or when studying specific chronic conditions; and biofilm = treatment with mature biofilm), and more detail between primary infection time, followed by subsequent incubation periods postwashing. Critical to each model's characterization is the MOI = the number of bacteria added to the number of cells seeded for the *in vitro* model. * = MOI estimate, calculated based on information disclosed in the article.

Complex cellular infection models

Bacterial species	Cell culture model	Infection type	Primary infection time	Postwashing period	MOI	References
P. aeruginosa	ALI	Acute	≤ 6 h	No washing step	0.1	Higgins et al. (2016)
					0.6*	Badaoui et al. (2020)
					1	Kassim et al. (2007)
					5	Kassim et al. (2007)
					≤ 7.5*	Yonker et al. (2017)
					10	Carterson et al. (2005), Dechecchi et al. (2011)
					15*	Pazos et al. (2015)
					20	Bucior et al. (2010), Halldorsson et al. (2010)
					50	Dechecchi et al. (2011)
					1–1000	Zhang et al. (2013)
					Not specified	Zhao et al. (2011), Carey et al. (2022), Carey et al. (2023), Qi et al. (2023)
				≤ 24 h	0.5*	Noutsios et al. (2017)
					Not specified	John et al. (2010), Farberman et al. (2011), Kuek et al. (2022)
				> 24 h	30	Lin et al. (2022)
					Not specified	Farberman et al. (2011), Collin et al. (2021)
			6 h ≤ 24 h	No washing step	0.001	Laucirica et al. (2022)
					1	Kassim et al. (2007)
					2	Ramirez-Moral et al. (2021)
					5	Kassim et al. (2007)
					60*	Garnett et al. (2013)
					0.0018*–67*	Junkins et al. (2014)
					Not specified	Woodworth et al. (2008), Brunnström et al. (2015), Qi et al. (2023)
		Biofilm	1 h	4 h	Treatment with biofilm	Juntke et al. (2021)
				24 h		
			Up to 72 h	No washing step		Thorn et al. (2021)
		Chronic	16 days	No washing step	Repeated infection at MOI 0.0025	Endres et al. (2022)
	LOC	Acute	≤ 6 h	No washing step	1–1000	Zhang et al. (2013)
				≤ 24 h	0.000125*	Plebani et al. (2022)
	Spheroid	Acute	≤ 6 h	No washing step	10	Carterson et al. (2005)
					25	Rodríguez-Sevilla et al. (2018)
					30	Crabbé et al. (2017)
					Not specified	Ma et al. (2023)
			≤ 6 h	24 h	30	Crabbé et al. (2017)
			6 h ≤ 24 h	Washing every 24 h, up to 5 days	0.001	Wang et al. (2023)
	Organoid	Acute	6 h ≤ 24 h	No washing step	Not specified	Tang et al. (2022)

Table 2. Continued

Complex cellular infection models						
Bacterial species	**Cell culture model**	**Infection type**	**Primary infection time**	**Postwashing period**	**MOI**	**References**
M. tuberculosis	ALI	Acute	≤ 6 h	No washing step	5	Nair et al. (2016)
					10	Bermudez et al. (2002)
					25	Nair et al. (2016)
			6 h ≤ 24 h	No washing step	3	Lastrucci et al. (2015)
					10	Bermudez et al. (2002), Ma et al. (2023)
			> 24 h	No washing step	10	Bermudez et al. (2002)
		Chronic (granuloma lesions)	> 24 h	Washing step after 5 days to establish ALI, incubation up to 7 days	Addition of *Mtb*-infected monocytes	Braian et al. (2015)
	LOC	Acute	≤ 6 h	No washing step	Not specified	Thacker et al. (2020)
	Spheroid	Chronic (granuloma lesions)	≤ 6 h	Up to 5 days	1	Kotze et al. (2021)
			6 h ≤ 24 h	Washing every 24 h, up to 5 days	0.05	Berry et al. (2020)
				Up to 4 days	0.1–10	Mukundan et al. (2021)
	Organoid		≤ 6 h	2–21 days	Not specified	Iakobachvili et al. (2022)
S. aureus	ALI	Acute	≤ 6 h	No washing step	0.0005*	McMichael et al. (2005)
					10	Tzani-Tzanopoulou et al. (2022)
					40*	Kiedrowski et al. (2016)
					200*	Kiedrowski et al. (2016)
				≤ 24 h	7–14*	Prescott et al. (2023)
					666*	Mitchell et al. (2011)
				> 24 h	666*	Mitchell et al. (2011)
			6 h ≤ 24 h	No washing step	40*	Kiedrowski et al. (2016)
					Not specified	Garnett et al. (2013), Ahmed et al. (2023)
			> 24 h	No washing step	40*	Kiedrowski et al. (2016)
					200*	Kiedrowski et al. (2016)
	Spheroid		> 24 h	No washing step	Not specified	Liao et al. (2023)
	LOC		≤ 6 h	≤ 24 h	5	Deinhardt-Emmer et al. (2020), Schicke et al. (2020)
S. pneumoniae	ALI	Acute	≤ 6 h	No washing step	30	Nguyen et al. (2015)
					Not specified	Fliegauf et al. (2013), D'Mello et al. (2023)
			6 h ≤ 24 h	No washing step	10	de Groot et al. (2022), Sempere et al. (2022)
					20	Karwelat et al. (2020)
					100	de Groot et al. (2022)
					Not specified	Moreland and Bailey (2006)
			> 24 h	No washing step	20	Karwelat et al. (2020)
	Organoid		≤ 6 h	No washing step	10	Sempere et al. (2022)

Table 2. Continued

Complex cellular infection models						
Bacterial species	**Cell culture model**	**Infection type**	**Primary infection time**	**Postwashing period**	**MOI**	**References**
H. influenzae	ALI	Acute	≤ 6 h	No washing step	100	Balder et al. (2009)
					Not specified	Schrumpf et al. (2017)
				≤ 24 h	50	Raffel et al. (2013)
			6 h ≤ 24 h	No washing step	20	Poh et al. (2020)
					500–5000	Humlicek et al. (2007)
					Not specified	Sajjan et al. (2008)
				Daily medium change, up to 10 d	10	Ren and Daines (2011)
				Daily medium change, up to 72 h	100	Walker et al. (2017)
		Chronic	Up to 5 days		20	Starner et al. (2006)
				No washing step		
			Up to 10 days		Not specified	Ren et al. (2012)
M. pneumoniae	ALI	Acute	≤ 6 h	No washing step	10	de Groot et al. (2022)
					20	Kraft et al. (2008)
					50	Krunkosky et al. (2007), Kraft et al. (2008)
					100	de Groot et al. (2022)
					Not specified	Prince et al. (2014)
			6 h ≤ 24 h	No washing step	10	de Groot et al. (2022)
					25	Hao et al. (2014)
					50	Krunkosky et al. (2007), Hao et al. (2014)
					100	de Groot et al. (2022)
			> 24 h	No washing step	10	Chu et al. (2007), Kraft et al. (2008), Chu et al. (2010)
					20	Kraft et al. (2008)
					50	Krunkosky et al. (2007), Kraft et al. (2008)
					Not specified	Shi et al. (2023)
		Chronic	≤ 6 h	Washing every 3 days, up to 28 p.i.	Not specified	Thaikoottathil et al. (2009)
			Up to 14 days	No washing step	1	Green et al. (2009), Gross et al. (2010)
			Up to 7 days	No washing step	10	Green et al. (2009), Gross et al. (2010)

lez and Dobbs 2013, Gerovac et al. 2014, Lancaster and Knoblich 2014, Nichols et al. 2014, Barrera-Rodríguez and Fuentes 2015, Birchenough et al. 2015, Doke and Dhawale 2015, Dye et al. 2015, Hu et al. 2015, Luyts et al. 2015, McCauley and Guasch 2015, Hittinger et al. 2016, Ingber 2016, Meenach et al. 2016, Rokicki et al. 2016, Tsay et al. 2016, Yin et al. 2016, Chimenti et al. 2017, Duval et al. 2017, Nikolić et al. 2017, 2018, Sidhaye and Koval 2017, Chen et al. 2018, 2021, Gowers et al. 2018, Heo et al. 2018, Zhang et al. 2018, Agrawal 2019, Chandrasekaran et al. 2019, Clifford et al. 2019, Cong and Wei 2019, Hiemstra et al. 2019, Kim et al. 2019, Li et al. 2019, Sachs et al. 2019, Takebe and Wells 2019, Zhai et al. 2019, Bovard et al. 2020, Hassan et al. 2020, Lee and Lee 2020, Li et al. 2020, Meyer-Berg et al. 2020, Moradi et al. 2020, Schutgens and Clevers 2020, Sharma et al. 2020, Shi et al. 2020, Weber et al. 2020, Zanoni et al. 2020, Barron et al. 2021, Bassi et al. 2021, Cao et al. 2021, Davis and Wypych 2021, Fukunaga et al. 2021, Gunti et al. 2021, Hewitt and Lloyd 2021, Hofer and Lutolf 2021, Marrero et al. 2021, Sato et al. 2021, Tian et al. 2021, Zamprogno et al. 2021, Aegerter et al. 2022, Banafea et al. 2022, Blutt and Estes 2022, Bosáková et al. 2022, GBD 2019 Antimicrobial Resistance Collaborators 2022, Golec et al. 2022, Ingber 2022, Marshall et al. 2022, Mettelman et al. 2022, Moreira et al. 2022, Shah et al. 2022, Shpichka et al. 2022, Singh et al. 2022, Wang et al. 2022, Loewa et al. 2023, Martinu et al. 2023, Morelli et al. 2023, Ninaber et al. 2023, Silva et al. 2023). For *H. influenzae*, Ren et al. (2012) optimized a chronic ALI infection model where bacteria survived up to 10 days intracellularly without significant damage to the epithelial cell layers (Ren et al. 2012).

When studying chronic infections, the incorporation of biofilm models can be desirable. Biofilm formation is often a hallmark of chronic, difficult-to-treat lung infections. These bacterial, matrix-encapsulated structures are more tolerant to antimicrobial therapy and the host immune responses (Ascenzioni et al. 2021). Starner et al. (2006) created an *H. influenzae* biofilm infection ALI model in which the development of large biofilm and matrix structures could be observed over 5 days without loss of epithelial integrity (Starner et al. 2006). Again, the most noticeable difference with the acute models was the choice of a lower MOI. The complex structural and chemical microenvironment of biofilms makes them challenging to mimic *in vitro*. Modelling biofilm formation in cell structures adds another layer of complexity to the *in vitro* biofilm models (Vyas et al. 2022). Two *P. aeruginosa* ALI models were encountered where cells were treated with mature bacterial biofilm (Juntke et al. 2021, Thorn et al. 2021). Both models mainly focus on the effect of the biofilm on host cell viability and barrier integrity. Thorn et al. (2021) additionally studied tobramycin penetration in the cell–biofilm model (Juntke et al. 2021, Thorn et al. 2021). Although these models allow the study of biofilm–cell interactions, they lack the ability to study the initiation process of biofilm formation in a cellular environment. As many questions on the influence of specific airway host cells, genetic predisposition, and the lung microenvironment on the formation and function of biofilms remain unanswered, complex biofilm models could help in our understanding and managing of biofilm-related infections (Hall-Stoodley and McCoy 2022).

Infectious dose

Apart from the timepoints chosen for readouts and the duration of the infection, the infectious dose is critical for creating a functional infection model. A broad range of MOIs can be found within the study set-up of the selected articles. The highest discrepancy in infectious dose is seen in the *P. aeruginosa* models, with MOIs ranging from 0.0001 to 1000 for acute, single-treatment studies. Although a certain amount of cell death can be acceptable and even warranted depending on the desired investigation parameter, care must be taken to select a suitable bacterial inoculum. Excessive cell death can interfere with various experimental readouts and can lead to misinterpretation of the results. Various authors report on the impairment of cellular integrity after bacterial infection, specifically for *P. aeruginosa*. Induction of cytotoxicity is a major virulence strategy for *P. aeruginosa* and significantly complicates the validation of an optimal MOI when creating a stable infection model (Wood et al. 2023). Qi et al. (2023), e.g. noted decreased cilia function and loss of tight junctions 6 h after *P. aeruginosa* exposure in an ALI model, even at low bacterial doses (MOI 0.05) (Qi et al. 2023). At a high MOI of 50, Kassim et al. (2007) recorded extensive, unwanted cell death within 24 h after *P. aeruginosa* infection in an ALI model (Kassim et al. 2007). In addition, Green et al. (2009) also make note of significant cell death within hours after infection with *P. aeruginosa* at ALI, while this same extensive cell death was not seen for *M. pneumoniae* (Green et al. 2009). For *S. aureus*, a broad MOI range for acute infections was noted as well (0.005–200), while the MOI range for the other selected bacteria is more narrow (10–100 for *H. influenzae*, *M. pneumoniae*, and *S. pneumoniae*, and 0.05–25 for *M. tuberculosis*) for acute studies. As stated earlier, most established chronic models are carried out at an MOI lower than those seen for the acute models. Important to note is that for a significant number of studies, exact information on the bacterial inoculum is missing, complicating the replication of the infection model by other research groups. Besides the careful selection of the MOI, a washing step could also be considered. Washing the infected cell layer or structure removes most nonadherent or noninternalized bacteria, which can reduce the cytotoxic effects of the initial bacterial load. For example, Prince et al. (2017) showed that ∼2% of *Mycoplasma* bacteria remain cell-associated after 4 h of infection upon apical washing of their ALI model (Prince et al. 2018).

Analysing the pneumonia model

After the addition of bacteria to the cell model, infection parameters can be tracked and measured. Analysis can be done via the means of nonendpoint readouts (i.e. in real-time) or endpoint readouts at the end of the infection process. Both parameters pertaining to the cell or the bacterium can be investigated.

Nonendpoint readouts

Nonendpoint assays are a valuable tool for studying bacterial diseases, as they enable the acquisition of experimental read-outs during the course of an infection, while also maintaining a stable cellular environment. These noninvasive assays can be used to assess bacterial and cellular movement, morphology, and activity dynamically at any given timepoint during infection (Fig. 5).

Multiple cell parameters can be examined, such as the evaluation of transepithelial/transendothelial electrical resistance (TEER) serving as a commonly used technique to assess the integrity of a cellular barrier (Strengert and Knaus 2011, Srinivasan et al. 2015). The ionic conductive strength can be measured in live cells using a pair of electrodes. As bacteria can compromise the tight junctions of cell barriers, TEER can be an effective assay for measuring the severity and progression of a bacterial infection in cell culture models (Strengert and Knaus 2011). TEER is aimed at analysing cell monolayers, making it less suitable for spheroid or organoid models (Srinivasan et al. 2015). It is, however, com-

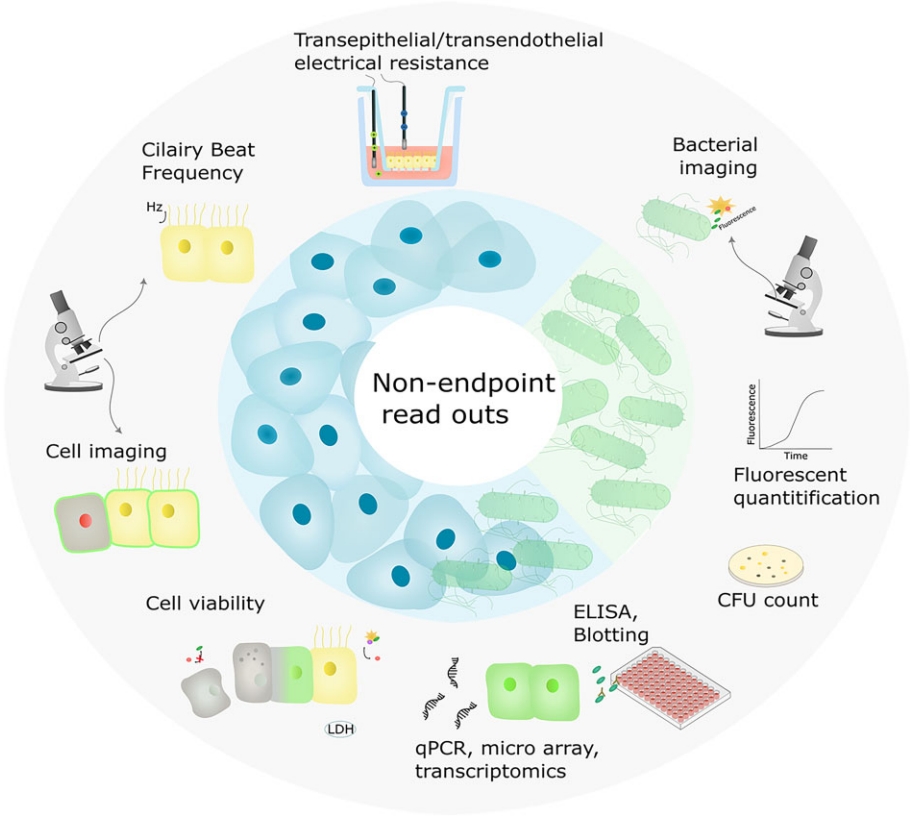

Figure 5. Concise overview of nonendpoint assays. When developing an advanced infection cell model, implementing nonendpoint read-out experiments can help to obtain reliable, dynamic information of a singular cell infection condition throughout the duration of the infection, avoiding the use of multiple dependent replicates. Nonendpoint readouts can focus on both cellular (left-hand side) or bacterial (right-hand side) visualization, quantification, and activity.

monly used in the study of ALI cultures and LOC models, which can have built-in electrodes for TEER monitoring (Henry et al. 2017). Alternatively, epithelial integrity can be measured at different time intervals by analysing the migration of fluorescently labelled molecules, such as FITC-dextran, FITC-inulin, or sodium fluorescein (Dowling and Wilson 1998, Mallants et al. 2008, Sajjan et al. 2008, Slater et al. 2016, Barron et al. 2021, Thorn et al. 2021). Ciliary beat frequency (CBF) is another cell parameter that can be measured in real time, using high-speed video microscopy (Nawroth et al. 2023). CBF is indicative for the cell's mucociliary clearing capacity and can be impaired by the presence of bacteria and their toxins, such as pneumolysin from *S. pneumoniae* or pyocyanin from *P. aeruginosa* (Ren et al. 2012, Nawroth et al. 2023). CBF can be applied in ALI models, as well as spheroids, organoids, and LOC models (Srinivasan et al. 2015, Booij et al. 2019). General cell viability can be measured by various nonendpoint assays, such as continuous bioluminescent monitoring using luciferase and a reducible prosubstrate, or the lactate dehydrogenase (LDH) assay, which can be performed on cell culture supernatant. Recently, a modified LDH viability protocol was proposed by Van den Bossche et al. (2020). Pathogens such as *P. aeruginosa* and *S. pneumoniae* are known to interfere with the LDH activity assay, which can lead to an underestimation of cytotoxicity (Van den Bossche et al. 2020). Next, tracking cell activity in real-time can be performed by analysing cell supernatant or mucus washes while maintaining the cells in culture afterwards (Dowling and Wilson 1998, Mallants et al. 2008, Sajjan et al. 2008, Slater et al. 2016, Barron et al. 2021, Thorn et al. 2021). Microarrays and RT-PCR are commonly used to measure gene expression, while ELISA or blotting techniques are often applied to quantify cell proteins, such as inflammatory mediators or mucins (Dowling and Wilson 1998, Mallants et al. 2008, Sajjan et al. 2008, Slater et al. 2016, Barron et al. 2021, Thorn et al. 2021). Additionally, single-cell or bulk RNA analysis and mass spectrometry can be used to analyse the secretome. Another nonendpoint technique is real-time cell imaging, e.g. by fluorescence microscopy. While dynamic imaging is often preferable to nonlive imaging as it can deliver valuable, real-time information on cellular behaviour, phototoxic effects due to e.g. photobleaching over time can be a challenge for this technique (Ettinger and Wittmann 2014). In general, imaging of organoids and spheroids is more complex and time-consuming, as it requires a z stack of multiple xy images using automated microscopes (Ren et al. 2012, Nawroth et al. 2023). Cells can be stained by a range of dyes; a combination of membrane permeable and nonpermeable stains can be useful to track cell death over time. Iakobachvili et al. (2022) used TOPRO-3 iodide, a nucleic acid stain, and CellMask Green, a membrane stain, to perform time-lapse imaging of cell death in organoids infected with *Mtb* (Iakobachvili et al. 2022). Cells that constitutively express fluorescent markers can also be useful in live cell microscopy (Ren et al. 2012, Nawroth et al. 2023).

Beyond relevant cell parameters, bacterial movement and replication can also be tracked in real-time. Similarly to cells, bacteria expressing fluorescent proteins can be employed to visualize movement, multiplication and viability in real-time. Thacker et al. (2020) performed time-lapse microscopy for a LOC model infected with a *Mtb*-mCherry strain to visualize multiplication and internalization (Thacker et al. 2020). Besides imaging, fluorescently labelled bacteria can also be used for live bacterial quantification.

Plebani et al. (2022), e.g. used GFP *P. aeruginosa* bacteria to construct a real-time bacterial growth curve in ALI culture and chip models (Plebani et al. 2022). CFU plating of supernatant can be useful in ALI or LOC models to track bacterial migration from one compartment to another, but should not be used to estimate a total bacterial count, as bacterial adhesion and internalization will lead to underestimation of bacterial presence (Iakobachvili et al. 2022, Tang et al. 2022, Ma et al. 2023). Most bacterial quantification techniques, however, require an endpoint step such as cell lysis (Kotze et al. 2021, Thorn et al. 2021, Prescott et al. 2023). Release of bacterial material, including RNA and exotoxins, could be studied by analysing cell supernatant by similar techniques as described above. However, most studies still focus on the intracellular bacterial environment to study changes in physiology and gene expression, which also requires an endpoint intervention (Kiedrowski et al. 2016).

Endpoint readouts

Experiments with endpoint read-outs encompass all assays that require the termination of the cell culture model, preventing any subsequent incubation steps and read-outs at later time points. These assays mostly involve a cell lysis step to obtain intracellular material, including cell metabolites, RNA, and internalized bacteria. In addition, microscopy experiments requiring fixation steps are also classified as endpoint readings.

In contrast to live fluorescent microscopy, fluorescent imaging of static, fixed cells is still regularly performed (Woodworth et al. 2008, Halldorsson et al. 2010, Juntke et al. 2021, Thorn et al. 2021). Immunohistochemistry is a common method to fluorescently visualize various antigens in cell layers or structures (Halldorsson et al. 2010, Junkins et al. 2014, Collin et al. 2021, Carey et al. 2022, Lin et al. 2022, Qi et al. 2023). For example, β-tubulin which is involved in the motility of ciliated cells, e-cadherin, which plays a role in cell adhesion and tight junction formation, and mucin proteins were detected using static immunofluorescent microscopy in a *P. aeruginosa* infection model (Collin et al. 2021). Although photobleaching and phototoxicity is not an issue for endpoint fluorescent microscopy, cell artifacts are more commonly encountered due to the required fixation steps (Yoshida et al. 2023). Apart from light microscopy, electron microscopy can also be used to visualize cell structures. Electron microscopy requires a prior fixation of the cell layers, but can deliver highly detailed photographs. Scanning electron microscopy (SEM) can be used to study cell health at various timepoints after infection, e.g. by looking at cellular detachment and cellular morphology changes such as rounding and blebbing (Kiedrowski et al. 2016, Juntke et al. 2021). Microvilli, cilia, and mucus can also be seen using electron microscopy (Kiedrowski et al. 2016, Juntke et al. 2021). Cell lysates can also be used to study a wide variety of metabolite transcription and/or production using qPCR, western blot, or ELISA assays, similarly as described for the nonendpoint read-outs (Noutsios et al. 2017, Collin et al. 2021, Lin et al. 2022). Surface proteins can be detected by cell sorter analysis, which also requires detachment of the cell layers in case of ALI models or dissociation into single cells of spheroid and organoid structures (John et al. 2010, Iakobachvili et al. 2022).

Next, to study bacterial localization qualitatively or quantitatively, similar endpoint microscopy techniques can be used as described above. For example, electron microscopy allows the visualization of interactions of bacteria with different cell structures (Nair et al. 2016, Deinhardt-Emmer et al. 2020). Next, Badaoui et al. (2020) used immunostaining of *P. aeruginosa* to quantify bacterial cell attachment in an ALI model (Badaoui et al. 2020). However, dead or physiologically compromised bacteria can interfere with these microscopy-based read-outs. Hence, traditional enumeration methods are still commonly used to quantify the cell association of live bacteria (Kim et al. 2019). (i) The cell adhesion assay can be applied to differentiate between external adherent and nonadherent bacteria. To remove non cell-associated bacteria, cell structures are first thoroughly washed with buffer. Subsequently, a mixture of washing buffer and detergent, such as Triton X-100 or saponin, is added to remove adherent bacteria, which can then be plated on agar for CFU determination (Balder et al. 2009, Bucior et al. 2010, Kuek et al. 2022). (ii) The cell invasion assay can be used to quantify the number of internalized bacteria after a certain time point. External bacteria are first killed by the addition of an aminoglycoside antibiotic such as gentamycin, which has poor cell permeability. Next, cell layers or structures are lysed, homogenized, and plated to quantify the internal bacteria (Bucior et al. 2010, Higgins et al. 2016). Recently, however, questions have been raised about the accuracy of this gentamycin-based invasion assay (Kim et al. 2019). As some degree of cell diffusion can still be noted for aminoglycosides, this assay likely leads to an underestimation of internal bacteria. Kim et al. (2019) proposed the use of lysostaphin, a nonmembrane permeable endopeptidase, to kill all external *S. aureus* bacteria (Kim et al. 2019). Lysostaphin has also been used for this purpose in complex *S. aureus* cell infection models (Mitchell et al. 2011, Deinhardt-Emmer et al. 2020, Schicke et al. 2020). Alternatively, flow cytometry can be used to study the attachment of live (fluorescent) bacteria to cells. This technique can also identify weaker bacterial–cell interactions, in contrast to the traditional plating method (Hytönen et al. 2006). Several studies also reported a general viable count without distinguishing between internal or external, adherent, or nonadherent bacteria (McMichael et al. 2005, Garnett et al. 2013, Prescott et al. 2023).

Validation of advanced *in vitro* models

When working towards a routine implementation of complex cellular infection models in laboratory research, the public sharing of validated protocols is indispensable. This knowledge transfer between research groups will facilitate and speed up the transformation of the bacterial (pneumonia) research field. However, in many crucial details on either the construction of the cell model itself or the establishing of a bacterial infection are missing in publications. Moreover, there does not seem to be a consensus on key performance indicators and cut-off values for the parameters necessary for the validation of such complex cell models. First, before adding bacteria, the maturity of the cellular structure should be assessed. Often, cells are grown for a set amount of time or until a desired outer morphology has been reached. Proliferation of cells, however, is dependent on cell-type and lineage and the medium of choice. For cells grown at an ALI, TEER can be an effective measurement to estimate cell barrier integrity. Striving towards a fixed TEER value or range before bacterial infection can increase experiment reproducibility. Within the chosen studies, however, a broad scale of target TEER values are mentioned, ranging from $> 200\ \Omega \times cm^2$ to $1888\ \Omega \times cm^2$. Woodworth et al. (2008), e.g. reported that they obtained a more robust *P. aeruginosa* infection when inoculating cell layers with a TEER higher than $500\ \Omega \times cm^2$ (Woodworth et al. 2008). As TEER measurement is challenging for three-dimensional cell models, other parameters such as analyte secretion could be followed-up using biosensors to estimate the development of the cell model (Hofer and Lutolf 2021). How-

ever, this is not yet commonly applied and complicates the set-up of such cell models even more (Hofer and Lutolf 2021). As three-dimensional cell models often rely on the incorporation of a matrix such as collagen or a hydrogel, the choice of biological material should also be taken into account during the initial validation step (Zhao et al. 2022). In general, due to a higher inherent heterogeneity and complexity, the validation of three-dimensional cell models is more challenging, and standardized protocols for growing such models are sparse (Kim et al. 2020). Additionally, working with primary cells or stem cells, especially when derived from patients with a varying genetic background, also leads to higher experimentational variability, complicating interlaboratory validation of cells models (Kim et al. 2020). Using cells from and depositing new cells to widely available biobanks can help to establish more robust infection models (Kim et al. 2020).

Finally, a bacterial infection protocol should also be carefully optimized before carrying out the desired experiments. Factors such as MOI, time of infection, incorporation of washing steps, and choice of bacterial strain can all greatly influence the course of the infection. Cell viability should be tracked throughout the infection period, especially for pilot studies where the bacterial inoculum is validated. Ideally, an acceptable amount of cell death should be outlined and communicated in the study protocol. Apart from cell viability, cellular barrier integrity can also be used as a parameter to optimize bacterial load or the timepoint of infection. Crabbé et al. (2017), e.g. mention tracking of cellular detachment in order to choose an optimal readout timepoint. Overall, few studies report on the optimization process that was followed to establish the complex cell infection model. Obtaining a validated, robust infection model is increasingly challenging for complex cellular models with multiple variables, but transparency on the validation process and data sharing can help pave the way towards a less fragmented research field. In the future, interlaboratory ring trials can be carried out to verify any publicly shared infection protocols.

Towards a replacement of animal models?

The development and implementation of physiologically relevant *in vitro* models is an indispensable step when reducing the amount of animal testing in research (Barron et al. 2021). Currently, however, the use of animal models for bacterial pneumonia is still ubiquitous in both preclinical and clinical research. While useful to study host–pathogens interactions, invertebrate models (e.g. *Drosophila melanogaster* and *Caenorhabditis elegans*) and zebrafish models bare little similarities to the human host due to the lack of lungs or airway tissues (Waack et al. 2020). In pneumonia research, mammalian models are often chosen for their phylogenetic relation to humans. Mice, specifically, have been used more than any other animal species in pneumonia-related studies (Mizgerd and Skerrett 2008, Williams and Roman 2016, Waack et al. 2020). Mice are often favoured for their cost-effectiveness, ease to handle and breed, and their suitability for genetic manipulation (Williams and Roman 2016). Nevertheless, the use of mouse models in bacterial pneumonia research comes with several caveats. First, many human respiratory pathogens are non-pathogenic for mice, unless specific laboratory conditions such as high bacterial inoculi are applied (Dietert et al. 2017, Mark and Grant 2020). General disease pathogenesis and progression, including tissue lesions and immune cell infiltration, can present differently in mice than in humans (Dietert et al. 2017). Moreover, the lung anatomy of mice is significantly different from that of humans (Gkatzis et al. 2018). Thus, although mice experiments continue to provide us with valuable new information, experimental outcomes can be difficult to translate to the human host (Gkatzis et al. 2018, Mark and Grant 2020). Complex human cell-derived cell culture models can overcome some of these murine model limitations. From all advanced cell culture models, organoids most closely mimic the *in vivo* situation due to their 3D-architecture based on human stem cells (Kim et al. 2020). Physiological responses in organoids can be similar to those seen in human organs, giving these models high translational value (Kim et al. 2020, Silva-Pedrosa et al. 2023).

Currently, however, advanced cell culture is mostly used complementary to animal testing in infectious disease research, as even the most complex models lack key functions such as interorgan communication (Kim et al. 2020). The immune response, specifically, is key when accurately modelling an infection. Most advanced bacterial pneumonia models used today are not cocultured with immune cells, and the complex immune cascades involving other organs are always absent. In addition, advanced cell culture models require a complex workflow and often struggle with attaining good reproducibility (Biju et al. 2023). The cell culture medium often lacks the complexity of the human microenvironment (Galbraith et al. 2018). Next, for mice infection models of pneumonia, validated protocols are readily available. As this review has shown, however, optimizing bacterial lung infections in complex cell models is still in its early stages, and few validated methods are published. Nevertheless, the research field is ever changing and evolving and more complex multiorgan systems such as body-on-a-chip models are gaining increasing attention (Sung et al. 2019, Li and Tuan 2022). Such technological breakthroughs and advancements will likely close the gap between *in vitro* cell models and animal testing in the future even more. However, optimizing bacterial lung infections in these advanced cellular models has proven to be time consuming and complex, and the current state-of-the-art does not yet fully support replacement of mammal pneumonia models.

Concluding remarks

The use of advanced cell culture models is rapidly increasing. These complex models ranging from ALI monolayers to organ-like cell aggregates are valued for their high lab-to-human translational value. Characteristics such as cell differentiation, microfluidics, and three-dimensional cell networks offer a better imitation of the true in-human condition. Within bacterial pneumonia research ALI models are regularly used, while organoid, spheroid, and LOC models are surfacing more slowly. The available literature on establishing bacterial infections in such advanced cell models is fragmented, which can be challenging for researchers looking to establish these models in their laboratory. In this review, studies describing the use of advanced cell models for bacterial pulmonary infections have been compiled and reviewed, with a focus on the methodology aspect. Many researchers opt for the use of primary cells, enabling high degrees of cell differentiation and patient-specific models. Coculture of different cell types, however, is lacking and immune cells are often still omitted from these infection models. Evaluation of the current infection protocols has exposed a large variation within experimental parameters, as well as an urgent need for model optimization and validation. Most studies focus on short term bacterial–cell interactions, while chronic infections are only rarely mimicked in advanced cellular models. It has become clear that, while advanced cell culture models are widely applied, adding bacteria into the mix creates an added layer of complexity, leading to a still under-

developed and underused powerful research tool within bacterial pneumonia.

Acknowledgements

The authors would like to acknowledge Bronwen Martin for the thorough proofreading of the manuscript. We thank Sofie Thys and Isabel Pintelon of the Laboratory of Cell Biology and Histology (University of Antwerp) for help provided with the scanning electron microscopy pictures.

Conflict of interest: None declared.

Funding

This work was supported by the Research Foundation Flanders (FWO Vlaanderen) (S008519N, G066619N, and G055517N).

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Received 2 October 2023; **revised** 29 January 2024; **accepted** 24 February 2024