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Early View

State of the art

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Towards an artificial human lung: modelling organ-like complexity to aid mechanistic understanding

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‘Take home’ message

Rapid advancement in human *ex vivo* modelling is providing new opportunities for delineating novel mechanistic understandings. Combining these with evolving downstream and multiomic analysis techniques could accelerate therapeutic development in the future.

ABSTRACT

Respiratory diseases account for over 5 million deaths yearly and are a huge burden to health-care systems worldwide. Murine models have been of paramount importance to decode human lung biology *in vivo*, but their genetic, anatomical, physiological and immunological differences with humans significantly hamper successful translation of research into clinical practice. Thus, to clearly understand human lung physiology, development, homeostasis and mechanistic dysregulation that may lead to disease, it is essential to develop models that accurately recreate the extraordinary complexity of the human pulmonary architecture and biology. Recent advances in micro-engineering technology and tissue engineering have allowed the development of more sophisticated models intending to bridge the gap between the native lung and its replicates *in vitro*. Alongside advanced culture techniques, remarkable technological growth in downstream analyses has significantly increased the predictive power of human biology-based *in vitro* models by allowing capture and quantification of complex signals. Refined integrated multi-omics readouts could lead to an acceleration of the translational pipeline from *in vitro* experimental settings to drug development and clinical testing in the future. This review highlights the range and complexity of state-of-the-art lung models for different areas of the respiratory system, from nasal to large airways, small airways, and alveoli, with consideration of various aspects of disease states and their potential applications, including pre-clinical drug testing. We explore how development of optimised physiologically relevant *in vitro* human lung models could accelerate the identification of novel therapeutics with increased potential to translate successfully from the bench to the patient's bedside.

Word count: 250 words

Keywords: Lung models, 3D culture, co-culture, lung-on-a-chip, respiratory diseases, lung infections

Introduction

Chronic respiratory diseases are a main cause of rising mortality and morbidity worldwide and represent a major health-care burden [1]. Over three million deaths a year are due to Chronic Obstructive Pulmonary Disease (COPD) alone, which is now the third leading cause of death [2, 3]. Asthma is the most common chronic respiratory disease, affecting over 330 million people worldwide, of which half a million die yearly [4]. Alongside COPD and asthma, other pulmonary diseases associated with chronic inflammation, airway obstruction, airway remodelling and tissue damage, are also a cause of concern and can be caused or exacerbated due to infection [5]. Idiopathic pulmonary fibrosis [6], cystic fibrosis (CF), primary ciliary dyskinesia (PCD) [7] and pneumonia [8] are good examples of these. The burden of lung cancer is also huge; this leads to 1.6 million deaths a year and is the most frequent cause of cancer-related deaths [9]. Screening and treatment programmes for lung cancer represent a significant financial stress on health-care systems around the world. Altogether, the global impact of lung diseases brings to light the urgent need to understand the molecular mechanisms driving disease processes and develop novel therapeutics.

Murine models have been instrumental to understand lung development, homeostasis and disease [10-15]. However, differences in the anatomy, physiology and cell biology between mice and humans hinder the potential of these animal models to recapitulate the human lung behaviour *in vivo* [16]. Thus, translational research from animal models to clinical trials is often unsuccessful [17-21]. This is a major concern in drug development and the biopharmaceutical industry [22].

Standard two-dimensional (2D) monoculture cell models which use human lung-derived immortalized epithelial cell lines are normally the first choice for testing a hypothesis *in vitro* [23, 24]. However, given the complexity of the lung, results derived from studies based on 2D monocultures may not be representative of what occurs *in vivo*. Consequently, a move to more advanced and complex *in vitro* lung replicas, ranging from relatively simple 2D co-cultures [25-27] to more sophisticated three-dimensional (3D) culture models, such as organoids and lung-on-a-chip models [28, 29], which better resemble the native lung, is underway and is significantly improving our current understanding of lung physiology and disease [30-32]. This review highlights the need for physiologically relevant *in vitro* and *ex vivo* models, current advances in the field of human lung modelling, and the strengths and

limitations of each model to facilitate suitable model choice for future lung research. Further development and wider use of more physiologically relevant lung models are key to acquire a better understanding of pathology and thus could accelerate identification of new drug targets, pre-clinical toxicity testing and the translation to successful clinical trials and patient benefits.

The human lung

The human lung is a complex organ comprised of more than forty cell types, which intimately interact to ensure appropriate tissue architecture and functional capability for efficient gas exchange [33, 34]. Thus, proper lung development and homeostasis is critical for survival [35, 36].

The differentiated airway epithelium is highly heterogeneous in cellular composition and function [37]. Comprised of ciliated, secretory, intermediate/undifferentiated and basal cells, it constitutes the first protective barrier localized at the interface between the host and the environment and as such, it is constantly being exposed to potentially harmful agents and airborne pathogens during breathing. Lung epithelial cells serve many important functions, including protection from environmental insults by mucociliary clearance of noxious particles, production of mucus and surfactant by specialised epithelial cells (mainly goblet and type II alveolar epithelial cell (AT2), respectively), initiation of immune responses and tissue regeneration post-injury [38]. Thus, appropriate epithelial cellular responses are key to maintain lung tissue homeostasis and dysfunction of the epithelial barrier has been shown to contribute to the development of many chronic respiratory diseases, such as asthma [39, 40] and COPD [41-43]. The airway epithelium is also frequently the site of metabolism and therapeutic effect of drugs. Therefore, it is an important target of biopharmaceutical research looking at drug development to fight respiratory diseases [44-49].

The alveoli are the principle site of gas exchange [36]. Alveoli are composed of epithelial cells, macrophages, endothelial and mesenchymal cells. Recent single cell transcriptomic studies have identified evidence of significant heterogeneity of these populations [50, 51]. Phenotypic and functional understanding of newly proposed sub-populations remains limited;

however the development of methodologies to incorporate such cell types will be important to better recapitulate the human lung *in vitro* [30].

The alveolar wall is comprised of a single thin layer of squamous type 1 alveolar epithelial (AT1) cells which cover 90-95% of the alveolar surface and facilitates efficient gaseous exchange. Comparatively, AT2 cells produce pulmonary surfactant, a complex layer of lipids and proteins which coats the air-liquid-interface to reduce surface tension and prevent alveolar collapse. Surfactant also has various essential innate immune and immunomodulatory functions which prevent lung infection and facilitate homeostasis to maintain the delicate epithelia and efficient gaseous exchange [52-58]. The distal saccular structures of the alveoli are protected by an active process called mucociliary clearance, which occurs in the bronchi and bronchioles, by which the inhaled air is filtered for removal of bacteria, viruses, and air pollution particles that could be potentially harmful to the organ's integrity and/or function [59]. Alveolar macrophages are located on the luminal epithelial surface of the alveoli and are the first line of cellular defence in this region, capable of phagocytosis and initiating an inflammatory response including secretion of cytokines and chemokines, and recruitment of activated neutrophils [60-62].

Although far more attention has been paid to the epithelium, the mesenchyme is also an important element of the lung. The lung mesenchyme encompasses smooth muscle, fibroblast, and endothelial cell populations which support lung structure, producing extracellular matrix (ECM) components and playing an important role in injury and repair [63]. Far from simply providing an inert structure, mesenchymal cells are able to influence the behaviour of nearby cells, through varying the composition of the ECM, and secreting cytokines and growth factors [64-68].

Overall, human lung tissue complexity makes it extremely difficult to fully recapitulate the myriad of possible cell-cell interactions in one *in vitro* model, and some biological and technical questions remain. However, the advancement of novel technologies is beginning to bridge the gap between the native lung and its replicates *in vitro*.

The need for physiologically relevant human lung models

Physiologically relevant human lung models are essential to understanding cellular and molecular features of lung homeostasis and disease pathogenesis. Whilst animal models have been key in helping to delineate human lung development, homeostasis and disease [10-14, 69, 70], due to genetic, anatomical, physiological and immunological differences [16, 71], translation of knowledge acquired from animal models into clinical studies is often unsuccessful [17-20, 72, 73]. Drug candidates that pass pharmacological and toxicological preclinical animal tests but fail clinical testing [22] are the clearest example of the current unmet need of complementary *in vitro* models that could better recapitulate human physiology.

Modelling organ-like complexity: what makes a good model?

Significant advancements have been achieved in the development of a range of different *in vitro* lung models, with varying degrees of structural complexity (FIGURE 1). An ideal lung model should include: (1) all the structural elements (airway and/or alveolar epithelium, distensible and dynamically stretched matrix-embedded fibroblasts and pulmonary endothelium), (2) an air interface, ideally with the possibility of adjustable bi-directional airflow for breathing simulation and (3) a liquid (media) compartment, preferably with an adjustable flow rate representing the bloodstream or interstitial flow through tissue, which may or may not contain leukocytes [16, 74]. However, the characteristics that make a good lung model will be dictated by the research question and determined by its specific application, and so all of the above may not be absolutely necessary for every need. Proper functionality of the model will also depend on the source, integrity, differentiation state and desired confluence of all cell types, all of which are key to achieve a physiologically relevant phenotype [74]. The local microenvironment (extracellular composition and topology, tissue stiffness, oxygen tension) is key for appropriate cellular differentiation and thus should be contemplated. The scale of the model and the ratio between all cell types are also important characteristics for maintaining *in vivo* geometry and structural patterns [75]. Methods for validation, assessment and potential downstream analysis of a particular model should also be considered, such as imaging, in which case optical transparency would be ideal, and immunohistochemical and gene expression profiles analyses, both of which will require the model to enable cell processing [74, 76-78]. In addition, for certain research approaches where scalability is key, the throughput potential of the model may become a limiting factor

and should therefore be contemplated beforehand. All considered, however, there is no such thing as the perfect model, as even the most complex human-relevant *in vitro* replicas of the lung have inherent drawbacks. Depending on their intrinsic features, a particular model may be more suitable than other models to study a specific biological process, disease or infection mechanism. Acknowledgement of the models' advantages and limitations are therefore key to achieve biologically relevant understanding of the native lung.

Cell source matters

Human immortalized cell lines permit the development of well-characterised, homogeneous cell populations which results in high empirical reproducibility [23, 24]. However, their genetic, physiological, morphological and functional differences to original primary cells is a major limitation when extrapolating findings to what actually occurs *in vivo*. Primary cells are, therefore, gold standard for 2D culture systems [79-82]. However, availability (in terms of number of patients and the amount of material that can be obtained from each one) and quality of samples, as well as donor heterogeneity, restrict their widespread utility.
Ex vivo nasal or bronchial samples obtained by brushing or curette biopsy provide primary human airway epithelial cells for *in vitro* culture models, and patient donated cells are in fact the best resort to recapitulate disease processes. Similarly, AT2 and other cell types such as fibroblasts, smooth muscle cells and immune cells, can be isolated from healthy surgically resected lung tissue [83-86].

Human (induced) pluripotent stem cells (hPSC) provide an alternative potentially unlimited source of different cell types, but present with the disadvantage of expensive cell culture media requirements and demanding differentiation protocols that need additional optimization depending on the specific hPSC line in question [87-92]. Nevertheless, these drawbacks are counterbalanced by the fact that hPSC can be used in combination with gene editing technologies such as CRISPR/Cas9 to allow the generation of disease-causing mutations, overcoming the need for screening and sampling patients and healthy subjects to source biologically relevant primary cells [93-95]. However, it is important to ensure that hPSC-derived lung differentiated cells conserve the same phenotype, gene expression profile and functionality as their *in vivo* counterparts, and that no off-target mutations occur if genetically modified *in vitro*.

Limitations of traditional 2D culture systems

Standard 2D monocultures, which involve culturing a single cell type submerged in culture media on a flat surface [23], are widely accepted as first-choice to prove an idea or a hypothesis *in vitro*, mainly because of their simplicity to grow and handle. 2D monocultures are a good option for screening-type experiments that may require running a number of conditions simultaneously, which would turn into a much more labour intensive and potentially much more expensive approach if more complex models were used instead.

2D cultures, however, fail to reproduce the structural complexity of the native lung, where many different cell types with very different functions interact within specific 3D nanostructures to compose functional tissues that collectively define the organ [96-99]. A further major limitation is the lack of polarized cells, with no distinctive apical and basolateral markers which may, for example, impair infection studies [100, 101]. Therefore, despite being practical and simple, more complex models may be required to better understand lung physiology. Recent advancements and development of a range of novel models has led to new opportunities for lung research; these are discussed below.

Air-liquid interface cultures

One system able to better model the native lung epithelia are 3D cell culture insert-based air-liquid interface (ALI) epithelial culture systems. These are capable of producing a pseudostratified epithelium with basal cells, ciliated epithelial cells and mucus producing goblet cells, where culture conditions are adjusted to promote this, such as by supplementation with retinoic acid. Furthermore, they can be produced either with primary cells or cell lines; primary cells can also be transformed using human telomerase reverse transcriptase (hTERT) to generate cell lines that retain their ability to differentiate and ciliate at ALI [102, 103]. Both upper and lower airway culture methods can be exploited to replicate or investigate disease states. For example, ALI cultures derived from primary human bronchial epithelial cells (HBECs) from diseased airways maintain the phenotype of their *in vivo* counterparts and demonstrate a dysregulated barrier [104-106]. ALI cultures can also be used diagnostically for PCD, a rare inherited disease [107-110], where motile cilia proteins are genetically rendered defective and mucociliary clearance is impaired. Using *in vitro* differentiated ALI cultures, primary and secondary ciliary defects can be distinguished using high-speed video microscopy (HSVA) [111-113], transmission electron microscopy (TEM)

[109] and immunofluorescence microscopy [114], supporting functional genomic analysis. Since primary airway epithelial cells can be cryo-preserved after the first expansion step, a bio-resource [111] can facilitate diagnostic development and research, e.g. for asthma, COPD, PCD or CF. Furthermore, ALI culture scale can be reduced to 96-well formats to maximise higher-throughput of rare or limited samples [45].

ALI cultures are preferred over classical submerged exposure techniques for studying the effect and toxicity assessment of exposure to airborne particles, and are as well particularly useful for respiratory microbial-host co-culture and allergen studies [115-123]. Culture integrity (by trans-epithelial resistance and macromolecular permeability) and ciliary function (by HSVA) can be monitored alongside bacterial imaging (by scanning EM and TEM, fluorescence *in situ* hybridisation and crystal violet assay compared to colony forming unit counts), virus particle loading, anti-microbial molecule release (e.g. defensin and cathelicidin), immunological responses (by RT-qPCR and protein immuno-assays) and multi-omics (via RNA-seq and protein mass spectrometry). These pre-clinical *in vitro* models can be exploited to investigate airways diseases, which may elucidate host cellular responses to microbial exacerbation, pathogenesis and treatment responses, to help reduce antibiotic use, exacerbations and hospitalisation of patients. However, because the epithelial cell ALI model is perhaps over simplified and lacks representation of the immune system component, more complex multi-cell models will likely become preferred in the future.

3D spheroid culture systems

An alternative method to ALI cultures are 3D spheroids that can be generated from nasal or bronchial brush biopsies. By preventing epithelial cell attachment to a culture surface during the first 4 hours of culture, already differentiated epithelial sheets form spheroids and can be cultured for several days. The advantage of these *ex vivo* 'cilia out' spheroids is that the cells are already differentiated and include ciliated cells, basal cell, and secretory cells. The disadvantages include the dependence on fresh brush biopsies that need to be processed in a short time window after acquisition, a limited availability as no prior expansion of cells and that spheroids need to be used in a relative short time frame without the ability to establish a bioresource. *Ex vivo* spheroid models have applicability in research and diagnostic. For instance, spheroid culture systems have been used to investigate the effect of environmental

exposures on epithelial inflammation in COPD [125] or to analyse cilia function in PCD diagnostics as a more rapid alternative to ALI cultures [126]. However, the limitation of *ex vivo* spheroid culture methods is that *de novo* ciliogenesis does not tend to occur and the model does not contemplate the immune system. An alternative *in vitro* 'cilia out' spheroid model has been established, using primary nasal or bronchial epithelial cells that can be expanded in 2D culture prior generation of spheroids [127, 128]. These spheroids can be used for CF research, infection studies and other exposures with a variety of downstream analysis methods, including imaging, mediator release and gene expression.

Co-culture models

2D or 3D co-culture systems provide more representative human *in vivo*-like tissue models than single cell systems and allow the analysis of cell-cell communications, investigation of specific lung diseases and development of successful therapeutic treatments [25-27, 129]. The lack of chemically defined culture media suitable for multiple cell types, however, needs to be overcome in the future.

Epithelial-mesenchymal co-culture models

The complex interaction between fibroblasts and epithelial cells plays a key role in the biology of the lung, including development, wound healing and pathological disease processes. Epithelial-mesenchymal crosstalk can be investigated in simple experiments using fibroblast-derived conditioned media on epithelial cells or vice versa. However, this approach recapitulates only unidirectional signalling and is not able to capture the dynamic nature of crosstalk which may occur in response to a range of challenges. In order to overcome this drawback, an *ex-vivo* 3D cell culture insert-based co-culture model of primary alveolar cells and lung fibroblasts that more closely mimics the lung parenchyma was developed to allow more realistic *in vitro* modelling of the complex cellular interactions taking place in the alveolar epithelium where the cells share the same microenvironment [68, 130, 131]. Similarly, a co-culture model incorporating a polarized human bronchial epithelial cell line (16HBE14o-) or differentiated primary HBECs and fibroblasts was designed to study the cell-cell interactions propagating and amplifying the innate immune response to respiratory viral infections [132]. These models replicate the epithelial-mesenchymal trophic unit (EMTU) of the respiratory epithelium, which consists of epithelial cells and underlying

fibroblasts [132, 133] and which, under physiological conditions, drives lung development, tissue repair, and regulation of the inflammatory response.

Macrophage-epithelial co-culture models

Macrophages are the predominant resident immune cell in the lung airway lumen and alveoli, comprising approximately 50% of sputum cells and 90% of bronchoalveolar lavage (BAL) cells [134]. Studying purified macrophages outside their natural environment provides little understanding of the role the alveolar epithelium plays orchestrating the macrophage-driven innate immune response to infection. To study macrophage-epithelial crosstalk, patient-matched primary human alveolar macrophages and fibroblasts can be isolated [135], thus giving rise to the potential for a multi-cell co-culture model of the lung where all component cells are taken from the same tissue sample. Alternatively, since availability of primary cells (especially macrophages) might be limited, macrophage cell lines or macrophages derived from peripheral blood monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), which have phenotypic similarities to alveolar macrophages [136, 137], can be incorporated into co-culture models with primary human alveolar epithelial AT2 cells. Macrophages-airway epithelium co-culture models has also been reported, for example, to study the mechanism of epithelial integrity restoration after epithelial injury upon an external noxious stimuli [138].

Utility of GM-CSF monocyte-derived macrophages model

Despite being relatively easy to access, analyse and culture from human sputum, BAL and lung tissue, human lung macrophages are, however, highly variable and relatively few cells are derived from these procedures. This has limited the ability to manipulate these cells *ex vivo*. An alveolar macrophage cell line has only recently been described [139] and it remains to be seen how well this line fully recapitulates all lung macrophage functions. The use of monocyte-derived macrophages (MDM) differentiated in GM-CSF, which is essential for alveolar macrophage differentiation, is an alternative [140-142].

Whilst it is increasingly recognised that alveolar macrophages arise from the foetal yolk sac and liver and not blood monocytes as previously thought [143], this GM-CSF differentiation has been shown to reveal a lung phenotype that is not evident in blood monocytes [140]. However, lack of consideration of lung disease states that may influence macrophage polarization may become a limitation [144]. Nevertheless, this GM-CSF MDM model has

been used to demonstrate similar responses in alveolar macrophages to influenza infection [145-147] and cigarette smoke [141]. MDM also seem able to present antigen to and activate T cells, MAIT cells and NK cells in co-culture systems [145, 148-150]. In addition, it has been shown that viral infections also compromise the ability of MDM to phagocytose lung relevant bacteria, such as *Streptococcus pneumoniae* [151]. There is also some evidence that some bacterial species such as *Haemophilus influenzae* can survive within airway macrophages [152], and this intracellular bacterial persistence has been recently modelled using the MDM model [153]. In sum, co-culture models which incorporate alveolar macrophages or GM-CSF monocyte-derived macrophages, where appropriate, have been fundamental to comprehend their role in initiation and resolution of inflammation in the lung and thus merit serious consideration in future model developments.

Epithelial-endothelial co-culture models

Delineating epithelial-endothelial interactions is key to understanding gaseous exchange, immune regulation and metabolic processes in health and disease. However, there are limited studies of co-cultures to mimic the alveolar gas-blood barrier, and those that have been developed often use cell lines or cells from a mixture of species [154]. The endothelial cell tube formation assay was first described in 1988 [155] as a model for studying angiogenesis *in vitro*. It quantifiably measures the ability of sub-confluent densities of endothelial cells to migrate rapidly to form capillary-like structures in response to angiogenic signals. The endothelial cells are plated onto a growth-factor reduced basement membrane for support, and over the next 6-20 hours tubules can form in the presence of angiogenic stimuli. Cells are frequently pre-stained with calcein-AM to allow fluorescent visualisation of the tube formation, but visualisation can also be achieved with a phase-contrast microscope. The assay can be quantified in numerous ways – including total tube length, total branching points (where tubes meet) or total loops (a loop being an area enclosed by tubular structure), much of which can be analysed freely with an ImageJ macro [156]. This assay has particular use in the study of angiogenic promotors and inhibitors. Further advancements to facilitate the modelling of the lung endothelium are required and are discussed below.

Scaffold models

Aside from efforts to replicate the lung epithelium and the alveolar unit focusing on the epithelium and its interactions with proximal cells, there are also efforts to recapitulate the ECM present in the lung interstitium. These have led to the development of several models which more closely resemble this ECM compared to traditional cell culture methods. One approach is to use macromolecular crowding of *in vitro* cultures of lung fibroblasts with the soluble, high molecular weight branched polysaccharide Ficoll® [157-159]. The addition of a high concentration of macromolecules to cell culture media is proposed to more closely resemble the crowded macromolecular environment found *in vivo*, and is associated with an increased rate of collagen fibril production. Similarly, several natural biomaterials such as Matrigel®, Chitosan, gelatine, collagen and alginate, as well as other synthetic materials including polyethylene glycol (PEG), Poly(lactic-co-glycolic acid) (PLGA) and pluronic F-127, mimicking the ECM/scaffold, have successfully been used in 3D bio-printing for construction of lung tissue [16, 160, 161]. An alternative is the long-term culture of lung fibroblasts from human explant lung tissue under defined conditions to promote the self-assembly of a mature 3D ECM [162, 163]. Such an approach can recapitulate the topology and stiffness of the lung cellular microenvironment, and permits functional assessment of tissue stiffness [162].

As one particular example, collagen-alginate can act as a matrix in microspheres that are generated by bioelectrospray methodology. A model incorporating *Mycobacterium tuberculosis* (Mtb)-infected peripheral blood mononuclear cells (PBMC) was developed to study granuloma formation in tuberculosis (TB), a chronic and persistent epidemic that has killed more humans than any other infection [164]. At a histological level, the characteristic process is granuloma formation, an aggregation of activated macrophages, T cells, B cells and fibroblasts [165], which are organised in 3-dimensions with specific microenvironments [166, 167] and where the ECM regulates the host-pathogen interaction [168]. The challenge in each model system is to determine the level of complexity necessary to then investigate the pathophysiological question under investigation [169]. For TB, many variables can be incorporated: cell type, number, activation status and ratio; ECM composition and rigidity; size of granuloma; change in environment over time; and oxygen tension, as human TB lesions are hypoxic [170]. Because of these variables, no single model system can fully reflect clinical disease, but insights from human cellular models are likely to be highly

valuable as Mtb is an obligate human pathogen [171] and animal models have different histological appearances to human disease [172]. This collagen-alginate microspheres-based granuloma model allows for investigation of TB inflammatory processes [173], and it has been recently shown that gene expression patterns reflect those in infected lymph node tissue more accurately than traditional models [174]. Integrative bioinformatics analysis of gene expression datasets can be used to identify new therapeutic targets and, through this modelling, doxycycline has progressed from validation in the 3D microsphere system to a successful clinical trial [175, 176].

A limitation common to all natural and synthetic ECM-mimicking materials is the difficulty to generate compact vascular networks capable of physiologic blood flow and gas exchange [177], in which case decellularization techniques may be more appropriate. Decellularized human lung tissue can also be used as a scaffold for cell seeding, potentially allowing modelling of the contribution of ECM to cellular phenotypes in health and disease [178-180]. Native ECM has the benefit of providing with a pre-formed vascular network and thus, of capturing the complex structure and composition found in tissues [181]. However, the decellularization process itself may modify ECM composition, topology and stiffness, and reproducibility between donors may be challenging [182]. Further development and utility of these scaffold models are rapidly advancing and may further our understanding of pathological processes, particularly in interstitial lung diseases.

Ex vivo lung tissue models

Similarly to scaffold models, *ex vivo* lung tissue models present the advantage of preserving the complex architecture of the lung, including, not only all of the relevant structural cells (e.g. epithelium, endothelium, fibroblasts), but also the tissue-resident immune cells (e.g. macrophages, T cells, NK cells). The obvious caveat to such investigations is donor variability and the limited availability of lung tissue in the specific respiratory disease. However, explant tissue of approximately 2-3 mm^3 in size can be cultured and infected with respiratory pathogens including influenza virus [145, 146, 148, 183] and *Haemophilus influenzae* [150]. Culture supernatants can also be sampled and lung cell responses analysed further by immunohistochemistry and flow cytometry [145, 146].

Human *ex vivo* whole lung perfusion model

Perhaps the pinnacle of lung tissue modelling work is the human *ex vivo* whole lung perfusion model. This uses tissue derived from pneumonectomy [184] which can be challenged with different agents and the different cellular and tissue responses monitored in a number of different ways (reviewed in [185]). Indeed, whole human blood can be added to the perfusate to also allow study of immune cell recruitment to sites of injury. However, the major limitation of this model is the paucity of tissue, which is often only available from major transplant centres. Moreover, the relatively short-term nature of such investigations (<24 h) has limited the majority of work using this model to focus on acute respiratory distress syndrome (ARDS), although this is beginning to change [186].

Precision cut lung slices

One way the short-term nature of the *ex vivo* whole lung perfusion model can be overcome is via the use of precision cut lung slices (PCLS). Here the lung tissue is infused with agarose or other hydrogels and thin sections cut by vibratome (reviewed in [187]). The resulting sections can then be cultured for up to 2 weeks and exposed to agents of interest, such as respiratory viruses and bacteria [188], or air pollutants either through direct exposure to emissions sources [189] or to pre-collected particulate matter [190]. It is noteworthy that one study has shown that murine PCLS exhibit similar patterns of responses to collected particulate matter as whole animal exposures [190]. These cultures have the advantage of accessibility for live cell imaging to track infectious agents, but are not able to recruit immune cells. In addition, the tissue collected needs to retain enough of its architecture to allow infusion to occur, i.e. have a sufficiently large and recognisable bronchus in which to introduce the agarose without a leak. The fact that PCLS are static systems is also a caveat that turns this model inappropriate, for example, for studying cellular processes that will occur *in vivo*. Another disadvantage is that in these models it is not possible to restrict exposure of microorganisms to the compartment of the lung airway/alveolar lumen and thus, these types of exposures in PCLS are less relevant compared to *in vivo* infection in humans. Counterbalancing these drawbacks, to our knowledge, PCLS are the only *in vitro* models in which bronchoconstriction can be measured [191].

Human embryonic/foetal lung explant culture model

Improving our understanding of the mechanisms underlying human lung development may help understand the early disease origins [192-194]. In addition, further characterisation of the different cell types may help us unlock their potential for lung regeneration and repair [195-198]. To date, it is unclear how to use lung regenerative potential to treat patients with chronic lung diseases [199]. Understanding how embryos build up gas exchange in developing lungs could generate new insights [200-203].

Lung structural complexity is the result of finely-tuned, orchestrated interactions between epithelial and mesenchymal tissues which coordinate the spatial and temporal development, influenced by potential clues from non-structural tissues (e.g. macrophages) which may nonetheless be involved in pathological structural changes. Lung morphogenesis is a dynamic process where multipotent epithelial progenitor cells differentiate and specialise in different restricted cell lineages. This relies on various important processes, including proximal-distal patterning, cell proliferation, migration and differentiation, as well as epithelial-mesenchymal interactions [204]. Lung development begins with the formation of two primary lung buds from the ventral foregut endoderm made by an inner epithelial layer enclosed by mesenchyme with a thin external mesothelial layer [69]. These then expand into the mesenchyme and begin branching morphogenesis. The dual origin of the lung tissue is important because branching derives from the intensive crosstalk between epithelial and mesenchymal cells and the factors they produce [63, 204, 205].

Budding, followed by branching and alveolarization, are phases of lung development and maturation that histologically have been divided into 5 stages: embryonic, pseudoglandular, canalicular, saccular and alveolar [206, 207]. In addition, extra-pulmonary mesenchymal tissues, including vasculature, lymphatic vessels, cartilage, smooth muscle and neurons, undergo development and spatial definition concurrent with the pulmonary airways branching morphogenesis program in response to intra-mesenchymal crosstalks [208-210]. An additional layer of complexity to the process of conducting airways formation involves reactivation of these various developmental programs in response to airway damage, necessary for tissue repair and reestablishment of healthy lung homeostasis [211, 212].

In human embryonic/foetal lung explant models, tissue is collected at the pseudoglandular stage (7 to 10 weeks post-conception) from first-trimester terminations [213, 214], or later at 11.5 to 21 weeks of development when bud tip progenitors have differentiated into alveolar cells [215]. Different culture methods can be used to propagate embryonic/foetal lung explants: from disaggregation of whole foetal lungs to obtain mixed cell populations grown as monolayers, organoids and other 3D cultures [216, 217] to investigate *in vivo* gene and protein expression [218-222], to specific single cell type monocultures to generate lung tissue for personalised disease modelling [223].

While we can study airway branching morphogenesis in foetal explant cultures *ex vivo* for several weeks (FIGURE 2), the main limitations are the restricted availability of explant tissue from all stages of gestational lung development, and the lack of a fully representation of *in vivo* lung development of branching airways, blood vessels and the autonomous nervous system. However, these drawbacks do not prevent the use of these models in regenerative medicine, tissue engineering, pharmaceutical safety and efficacy testing and toxicological screening, and they could provide novel insights for understanding lung diseases in the future [224].

Organoids

3D Organoid systems offer new opportunities for modelling the lung complexity [28]. hPSC-derived organoids are self-organising multicellular structures formed from stem cells which, by recapitulating the developmental signalling pathways required for development of a particular organ, can be differentiated into organoids resembling that organ [225]. Lung organoids can be generated by selective activation and inhibition of key developmental pathways in induced hPSC, and over several months develop into organoids containing tissue-resembling proximal airway epithelium, comprising several distinct but associated cell types, such as basal, ciliated and club cells [226, 227]. These airway-like structures are surrounded by lung mesenchymal cells, as well as cells expressing alveolar epithelial markers, resembling immature alveolar tissue. hPSC-derived lung organoids have been shown to exhibit gene expression signatures more like those present in foetal lung than in adult lung [226], and so provide a particularly useful model for investigating lung development. Lung organoids provide a promising alternative to *in vivo* models for modelling lung differentiation, homeostasis and disease, and the multiple cell types present allow

investigation of cellular crosstalk within the lung [227]. In contrast to hPSC-derived organoids, the adult phenotype can be preserved if organoids are established from primary lung epithelial cells [228, 229]. Limitations of these models include long culture times and the need for previous cell differentiation in 2D cultures if derived from induced hPSC. A further limitation to the organoid model in terms of its culture is the need for Matrigel, which is produced from animal sources. As such, the model does not fully replicate the chemistry/biology of human tissue, while lot-to-lot variability in Matrigel composition may result in variability between experiments. An additional consideration is the directionality of the culture. In organoid culture the lumen or apical side of the epithelium faces inwards, thus preventing replication of exposures of the airborne or apical/luminal surface to inhaled challenges such as aeroallergens or air pollutants. Absence of integration with the immune system and difficulty in visualising cells towards the inside of the organoid are also important drawbacks to be considered. Moreover, hPSC-derived organoids' resemblance to foetal rather than adult lung may have limitations in understanding adult lung disease, in which case primary lung epithelial cell-derived organoids or other 3D culture systems modelling the developed lung become more appropriate.

Mechanical strain/stretch model

Analysis of altered mechanical strain seen in respiratory diseases may be important in understanding pathophysiology of chronic obstructive airways diseases such as asthma, which are characterised by reversible airflow obstruction and bronchial hyper-responsiveness; these are associated with airway inflammation and remodelling. Airway remodelling consists of increased smooth muscle, deposition of ECM proteins such as collagens and proteoglycans, and angiogenesis.

Airway ECM is mainly synthesised by airway fibroblasts, a cell type that responds to mechanical signals by expression of ECM genes [230]. Analysis of airway fibroblasts from asthmatic and control subjects in response to mechanical strain can be achieved using a Flexercell-4000T TensionPlus instrument, which allows mimicking of regular normal breathing and enables microscopic evaluation post-stretching [231]. The advantage of the Flexcell system as a fibroblast stretch model is its wide use in the field of mechano-biology and mechano-transduction [232] and the ease of set up of single cell type cultures (e.g.

fibroblasts). Furthermore, combination with cell culture insert holders and cell culture inserts atop the BioFlex culture plate allows co-culture with at least two or three different cell types such as bronchial epithelial cells, endothelial cells and fibroblasts (FIGURE 3). This has the advantage of becoming a 3D model with the additional functional stretch component. The disadvantage of this model is that there is no continuous media exchange/flow mimicking airway blood supply that would allow continuous biological mediator and biochemical analysis. This can instead be achieved with newer airway-on-a-chip designs [29] using microfluidic-chip-integrated biosensors [233], as discussed in the section below.

Lung-on-a-chip

Microphysiological systems (MPS) are miniaturised models that combine microfluidics, engineering and cell biology to recreate certain aspects of organ physiology *in vitro* and are a promising alternative to conventional models. Cells (primary, stem-cell derived or cell lines) are cultured within biopolymer or tissue-derived matrix in microfluidic devices with flow. The systems can have integrated sensors to maintain cultures and monitor responses [234]. Lung-on-a-chip technological complexity highlights this model as the closest replica of the native lung, but it also accounts for the limitation of expertise required for its development and application. The absence of non-standardized protocols is also a drawback. Moreover, ECM-coated and biocompatible polymeric membranes may not reflect native tissue interface, as it may, for instance, present with different structure and transport properties. However, second generation of lung-on-a-chip models are now beginning to address this limitation [238].

Lung-on-a-chip MPS have been developed to recapitulate different compartments of the airway including the alveolar compartment [29] and conducting and small airways [235-239]. In these models it is important to accurately recapitulate the airway epithelial barrier which is central to the maintenance of tissue homeostasis in the lung and acts as a physical, chemical and immunological barrier [240]. Typically epithelial cells are differentiated on a nanoporous membrane on-chip under flow before challenge with environmental agents (e.g. cigarette smoke, viruses, dsRNA, LPS) [235-238] or inflammatory mediators (e.g. IL-13) [235, 236] to model certain aspects of lung disease and determine the effect of drug treatments on cellular responses [235, 236, 238]. Commercial airway MPS offerings include differentiating

epithelial cultures on-chip (Emulate, Synvivo, Kirkstall) or introduction of statically differentiated cultures into MPS in combination with other organ systems (Draper [241], CNBio [242], TissUse [243]). Monitoring of epithelial barrier function on-chip has been demonstrated by Guenat's group in an alveolar model [244] and by Ingber's group in an airway model [239]. Researchers at the University of Southampton have developed an "airway barrier-on-chip" MPS that can monitor epithelial barrier integrity in real-time using electrical impedance spectroscopy (EIS) [245]. The system uses microfluidics to recapitulate interstitial flow and includes time-dependent sampling of cellular secretions [246, 247] (FIGURE 4). Cellular crosstalk on-chip has also been demonstrated [248]. Monitoring cell Trans-Epithelial Resistance (TER) in real time with integrated micro-electrodes provides data on the formation and disruption of the airway epithelial barrier. The system includes droplet microfluidics for collection of cellular secretions to provide time-dependent measurements of epithelial responses during differentiation and environmental exposure. Delivery of challenges and compounds in a physiologically relevant manner to the apical surface is accomplished using surface acoustic wave technology to generate aerosols. Combining microfluidics and cell biology provides a MPS that more closely recapitulates the physiological environment of the lung for understanding human (patho)physiological mechanisms, providing more predictive pre-clinical models for drug discovery and developing personalised medicine strategies.

Lung microbiome: the unappreciated hallmark of *in vitro* lung models?

The impact of homeostatic disruption to the lung microbiome in respiratory diseases is becoming increasingly understood [249-253]. Modelling these interactions in the future will be key to understand disease pathological processes. Most of our understanding about host-microbiome interactions comes from germ-free (GM) and antibiotic-treated (AT) mouse models [254, 255]. Analysis of antibiotic resistance in lung diseases and drug development is strongly dependent on the consideration of native 3D matrices within the lung (e.g. mucus and biofilms) that can significantly affect drug permeability and allow the formation of self-protected bacterial clusters [256]. Significant progress has been achieved on the development of a variety of *in vitro* models replicating several aspects/compartments of the human lung, but technical impediments or merely the lack of consideration of its microbiome in the

experimental setting is a major limitation to all of these. The incorporation of not only commensal bacteria, but also fungi and viruses [257], as components of 3D pulmonary models, would better reflect lung homeostasis *in vivo*, significantly enhancing their physiological relevance as *in vitro* replicas of the human lung. These biometric systems may serve to validate and expand the scope of lung microbiome studies reliant on sampling techniques for culture-independent analysis [258], with the ultimate goal of developing novel therapeutics and improve translational lung research that impacts clinical outcomes.

Future perspectives and clinical impact

Pre-clinical modelling of the human lung is paramount for delineating new mechanistic insights of respiratory diseases and identifying novel therapeutic targets. Murine models are useful for understanding the whole *in vivo* system and are still widely accepted as a pre-clinical 'must'. However, there is a clear disconnect between demonstration in murine models and their translation to the patient. Differences between the murine and human lung in genetics, anatomy, physiology, and metabolic and immunological processes are widely understood, and thus there are clear limitations in using mouse models to recapitulate the human lung. These issues, alongside ethical and scientific dilemmas around the Three Rs - Reduction, Refinement and Replacement - highlight the need for further utility and development of physiologically relevant human *ex vivo* models [259].

Within the last decade, unprecedented technological advances have enabled the replication of organ-like complexity *in vitro* using human cells/tissue, overcoming some of the major limitations of animal testing (TABLE 1). However, even at the highest level of complexity and sophistication, current prototypes represent only partial pictures of much complex processes *in vivo*, and it is unlikely that a single model will completely replicate all the native lung functions. The choice of a lung model should, therefore, be determined to fit-for-purpose, for which all cell types and biological functions relevant to address a specific question should be represented.

The ability to investigate disease pathology in human lung samples or organotypic models offers unparalleled opportunity for understanding the molecular mechanisms underpinning lung diseases versus health. However, the breadth of investigations is often limited by the

model size, and read-out complexity. Advances in omics technologies over the past 3 decades have opened opportunities for investigations of complex biological systems, across human tissues and advanced culture models, and are readily applicable for investigations of human lung physiology. Recent studies demonstrate the usefulness of high throughput transcriptomics approaches, allowing delineation of transcriptional programming of specific cell populations, identifying key regulatory switches and hubs in biological processes amenable to therapeutic intervention, and interrogating complex interactions between different cell populations [50, 51, 260-263]. The last decade has seen successful application of omics analyses to describe human lung development [264], for discovery of pathways dysregulated in pulmonary fibrosis [265], COPD [266, 267], cancer [260, 263] and for investigation of cell types specific responses to SARS-CoV-2 across ages and patient groups [268, 269]. Moreover, recent studies provide a proof-of-concept of how the power of gene co-expression analyses can be used for identification of novel therapeutic targets [262] and treatment drugs [261] for TB.

Most excitingly, analysis of transcriptional programmes and networks offers the opportunity to improve advanced culture models of lung physiology and disease. Open access data sharing initiatives, such as the Molecular Cell Atlas of the Human Lung [50, 51], allow *in silico* interrogation and cross-comparison of specific population- or disease-related gene signatures, curated in laboratories across the world. *In silico* validation and testing of transcriptome features against such gold standards will facilitate improvement of advanced cell culture models, to reproduce lung disease or its specific aspects faithfully. Importantly, standardisation of models with open access to detailed methodology will advance the field enormously allowing for cross model comparisons, collaboration and acceleration of discovery and translational activities. In sum, development of reliable *in vitro* and *ex vivo* models of the human lung combined with powerful downstream analyses could not only decrease reliance on animal models, but also accelerate the identification of drug targets, facilitate the development of novel therapeutics and improve clinical trial outcomes, to ultimately benefit patients with lung diseases and infections.

Competing interests

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MVH, CMS, JB, CB, FC, ERD, LSND, PE, HMH, CJ, MGJ, ML, JSL, HM, MP, KJS, ES, LT and AW declare that they have no conflicts of interest.

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Authors' contributions

MVH and TMAW conceived the project. MVH designed and managed the review. MVH, CMS, JB, CB, FC, ERD, LSND, PE, HMH, CJ, MJ, ML, JSL, HM, MP, KJS, ES, LT and AW wrote the review. MVH, CMS, AW and TMAW edited the review. TMAW supervised the project. All authors approved the final version to be published.

TABLE 1

MODEL	Characteristics/Strengths	Limitations	Reproducibility
Monolayers (2D)	Simple to grow and handle Cost-effective Confluence can be achieved rapidly Easy to monitor cells and extracellular flux	Static system No cellular heterogeneity No cellular polarization Lack of native structural complexity Absence of air-liquid interface	+++
Air-liquid interface (ALI)	Pseudostratified, differentiated epithelium with mucociliary barrier Allow co-culturing Allow reduced scale cultures	Over-simplified 3D model Full differentiation will take 1-2 months culture Labour-intensive Absence of immune system	++
Spheroids	Simple to handle Self-assembling 3D cultures that reflect native lung structure Rapid alternative to ALI cultures	Absence of *de novo* ciliogenesis (*ex vivo*) Dependence on fresh brush biopsies Limited availability Short-term viability Absence of immune	+++

		system	
Organoids	Patient and disease relevant Self-organising multicellular structures *In vivo*-like structural complexity	Organoids derived from PSCs require previous cell differentiation in 2D cultures. It does not allow for apical exposure Difficult visualization of inner cells Resemblance to foetal rather than adult lung (immature cells) (if derived from hPSC) Absence of immune system Long culture times	+
Whole perfused lung	Patient and disease relevant Preserved native architecture: structural and tissue-resident immune cells	Donor variability Limited availability Short-term viability (<24h)	-
Precision cut lung slices (PCLS)	Patient and disease relevant Preserves tissue-specific features (e.g. cellular polarity and architecture) Long-term viability Allows measurement of bronchoconstriction Allows live cell imaging	Donor variability Limited availability Static system Requires preservation of native architecture for agarose-perfusion Exposure of microorganisms is not restricted to the lung airway/alveolar lumen Absence of immune cells	-
Tissue explant	Patient and disease relevant It does not require full preservation of structure	Donor variability Limited availability	-
Embryonic/foetal lung explant	Patient and disease relevant Allows long-term culturing Applicability in tissue engineering and regenerative medicine	Donor variability Restricted availability from all stages of gestational lung development Lack of representation of blood vessels and the autonomous nervous	-

		system	
GM-CSF monocyte-derived macrophages (MDM)	Not restricted to yield and consistency of lung macrophage extraction methods Alternative to the alveolar macrophage cell line which has not been fully characterized	Lack of consideration of lung disease states that may influence macrophage polarization	++
Natural and synthetic ECM-mimicking materials	Recapitulate the lung's native topology and stiffness Permits functional assessment of tissue stiffness	Difficult to generate compact vascular networks capable of physiologic blood flow and gas exchange	+++
Decellularization	Patient and disease relevant Suitable microenvironment for cell growth and differentiation Decellularized scaffolds can be incorporated into bioinks for 3D bioprinting of the lung	Donor variability Restricted by limited advances on recellularization techniques (seeding, cell survival and maturation/differentiation) It may modify ECM composition, topology and stiffness	-
Mechanical strain/stretch	Allows mimicking of breathing patterns Applicability in mechano-biology and mechano-transduction Permits co-culturing	Absence of continuous media exchange/flow mimicking airway blood supply	++
Lung-on-a-chip	*In vivo*-like structural complexity Allows multi-co-culturing High spatiotemporal precision of physiologically relevant biochemical and mechanical cues Allow analysis of dynamic processes	Technologically complex Experts required ECM-coated and biocompatible polymeric membranes may not reflect native tissue interface (e.g. different structure and transport properties) Non-standardized protocols	+

TABLE 1. State-of-the-art *in vitro* models of the human lung. The main characteristics/strengths and limitations are listed for each model for comparison. The degree

of reproducibility of the models is merely an estimation based on the ease with which they can be developed and tested (+, ++, +++), also influenced by whether a cell line or primary cells are used. Lack of reproducibility (-) refer to the ones that are patient specific and thus imply donor variability.

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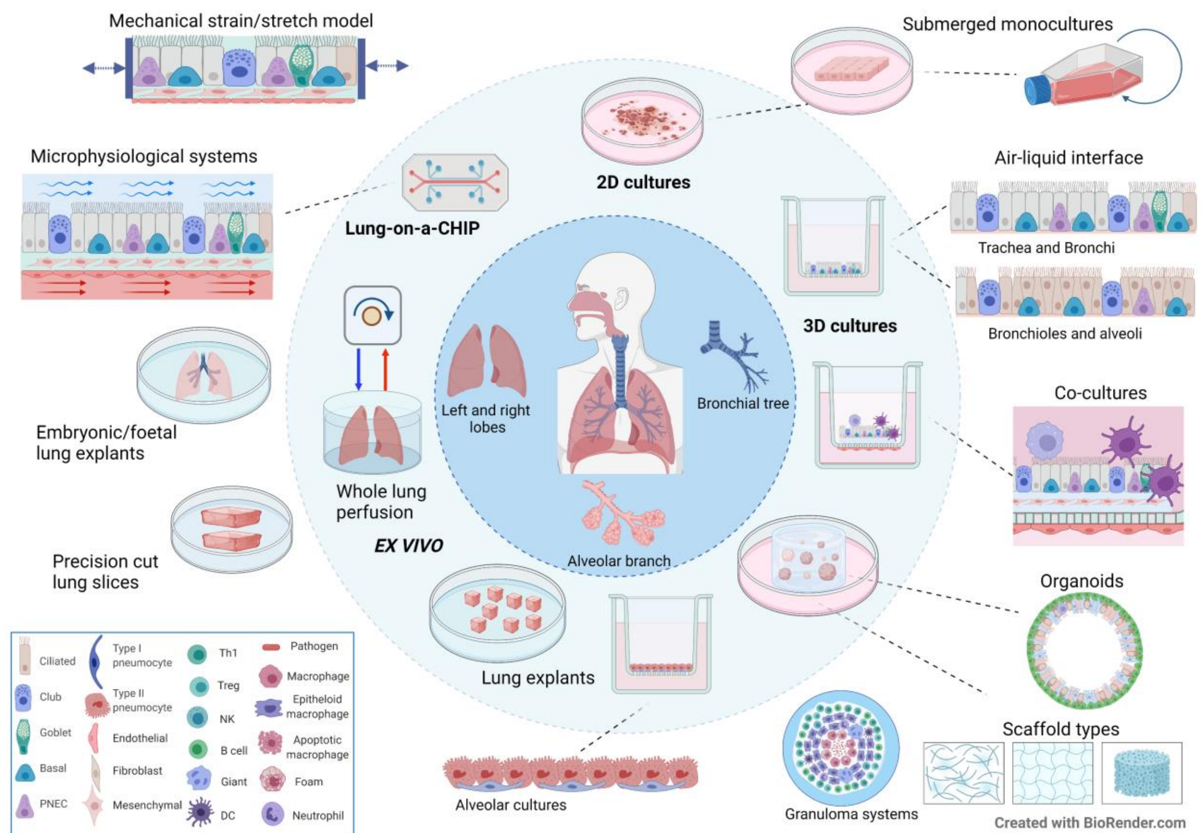

FIGURE 1. Illustration of in vitro and ex vivo human lung models (Graphical Abstract). Ex vivo nasal or bronchial samples, obtained by brushing or biopsy, in addition to human pluripotent stem cells and immortalised epithelial cell lines, provide human airway epithelial cells for in vitro 2D culture models as submerged monocultures. Air liquid interface cultures are 3D epithelial culture systems where basal cells are able to differentiate in a pseudostratified epithelium resembling the area of the lung they originate from. The model can be enriched in co-culture systems where the epithelium differentiate in the presence of pathogens and immune cells such as macrophages. Further complexity is achieved by addition of fibroblasts and mesenchymal cells to mimic epithelial-mesenchymal crosstalk. To recapitulate the ECM present in the lung interstitium, natural or synthetic macromolecules can be added to the cultures to promote the self-assembly of a mature 3D ECM. Similarly, 3D cultures made out of cells encapsulated in macromolecules-enriched media support the growth of organoids. To investigate pathophysiological questions and in particular inflammatory processes, one example are TB granulomas, which can be developed using collagen-alginate microspheres generated by bioelectrospray methodology incorporating Mycobacterium tuberculosis-infected PBMCs. Amongst other cell types, alveolar cells can be isolated from healthy surgically resected lung tissue that together with lung fibroblasts support 3D cultures mimicking the lung parenchyma. Availability of lung tissue may be exploited in ex vivo lung tissue models using small lung sections preserving its complex native architecture. Whole lung or tissue derived from

pneumonectomies allow development of human ex vivo whole lung perfusion models or provide with precision cut lung slices, both preserving native structural features. Human embryonic/foetal lung explant models support lung development studies. Foetal tissue is collected between 7 and 21 week post conception and the mixed cell population is cultured as monolayers, organoids and other 3D cultures. Miniaturised models such as Lung-on-a-chip combine microfluidics, engineering and cell biology to recreate certain aspects of organ physiology in vitro and are a promising alternative to conventional models. Specific devices allow mimicking normal breathing patterns and enable microscopic tissue evaluation post-stretching. Other micro-physiological systems permit relevant cells of the respiratory tree to be cultured within a biopolymer or tissue-derived matrix within microfluidic devices incorporating flow. These systems can have integrated sensors to maintain cultures and monitor responses. Created with BioRender.com

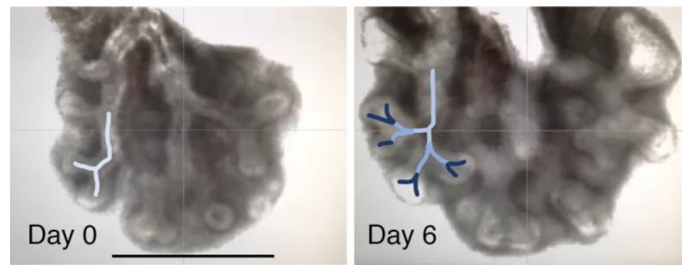

FIGURE 2. Human embryonic/foetal lung explant culture model. Snapshots of time-lapse microscopy of human embryonic/foetal lung explants cultured for 8 days, showing day 0 and 6. Light and dark blue scale lines show primitive branching with 2 generations of branching at day 6 (black bar = 10 mm). (Adapted from: Haitchi HM et al., JACI 2009:124(3):590-7).

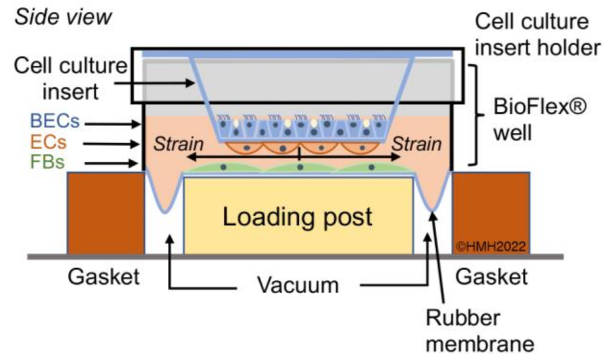

FIGURE 3. Mechanical strain/stretch model. Schematic of a cell culture insert holder with a cell culture insert supported atop the rubber membrane in the well of a BioFlex® culture plate. Bronchial epithelial cells (BECs) on top of cell culture insert membrane and endothelial cells (ECs) underneath of cell culture insert membrane are co-cultured with fibroblasts (FBs) in the BioFlex well. The FBs are stained by applying vacuum to the bottom of the rubber membrane (adapted from: https://www.flexcellint.com/product/transwell-holder).

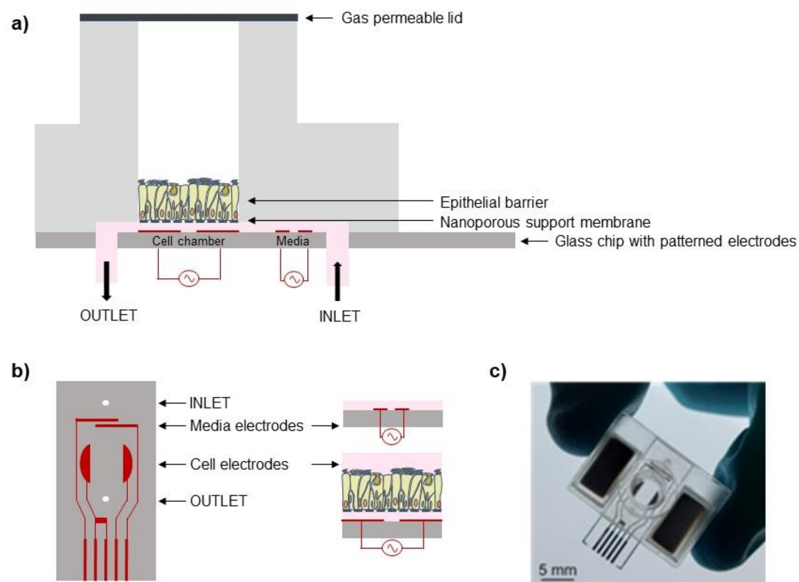

FIGURE 4. On-chip airway barrier. (a) Cross-section of the airway barrier-on-chip, showing the cell and media electrodes. (b) Arrangement of the two sets of electrodes, together with the position of the fluid inlet and outlet holes. (c) Photograph of a microfluidic chip.