Chapter

Innovative *In Vitro* Models for the Study of Lung Diseases

Vittorio Picchio, Vittoria Cammisotto, Francesca Pagano, Roberto Carnevale and Isotta Chimenti

Abstract

Basic and translational research on lung biology and pathology can greatly benefit from the development of 3D *in vitro* models with physiological relevance. Lung organoids and lungs-on-chip allow the creation of different kinds of *in vitro* microenvironments, that can be useful for the elucidation of novel pathogenetic pathways, for example concerning tissue fibrosis in chronic diseases. Moreover, they represent important translational models for the identification of novel therapeutic targets, and for preliminary testing of new drugs. In this chapter, we provide a selected overview of recent studies on innovative 3D *in vitro* models that have enhanced our knowledge on chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF), particularly concerning oxidative stress and pro-fibrotic pathogenetic mechanisms. Despite several limitations, these complex models must be considered as complementary in all respects to in vivo studies on animal models and clinical research.

Keywords: organoids, organ-on-chip, oxidative stress, lung fibrosis, cell spheroids

1. Lung chronic diseases: a brief overview

The primary function of the lungs is the exchange of gas occurring at the level of alveoli, which are arranged as acini in the lung parenchyma. There is strong need to understand the mechanisms of alveolar maintenance and repair because damage to this region underlies many chronic adult lung diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, acute respiratory failure in pneumonia, and acute respiratory distress syndrome. Additionally, insufficient development of alveoli results in various neonatal and childhood diseases including bronchopulmonary dysplasia [1]. Despite the pivotal role of the alveoli in the development of lung diseases, the pathogenesis of these various conditions is still largely unknown, and treatment options for patients remain limited.

There is clear evidence that environmental exposures and genetic predisposition contribute to the pathogenesis of idiopathic pulmonary fibrosis (IPF). IPF is defined as a specific form of progressive chronic fibrotic interstitial pneumonia, that is occurring mainly in older adults, and is limited to the lungs [2]. IPF remains relatively rare, with an estimated incidence of roughly 10 cases per 100,000 person-years. Nonetheless, IPF is a lethal lung disorder with a predicted survival of 3–6 years from the onset of symptoms. Most of the deaths among patients with IPF are due to respiratory failure or complicating comorbidities [3]. The pathogenesis of IPF is

characterized by continuous insults or micro-lesions to the alveolar epithelium, which result in abnormal activation of both epithelial cells and fibroblasts. Finally, there is an alteration in the deposition of collagen, which contributes to the irreversible fibrosis typical of the disease [4]. Various risk factors have been identified in the development of IPF, that can be divided between intrinsic and extrinsic [5]. Intrinsic risk factors include genetics, aging, sex, lung microbiome [6–9], while extrinsic risk factors comprise cigarette smoking, environmental exposures, and air pollution [10, 11]. Moreover, studies of familial clustering of pulmonary fibrosis provided evidence that IPF is associated with genetic susceptibility. Multiple genes can affect alveolar stability, for example, genes encoding surfactant proteins A and C, genes associated with enhanced cell senescence by disruption of telomerase function, with the integrity of the epithelial barrier, and with mutant desmosome proteins [12–15].

Chronic obstructive pulmonary disease (COPD) is another chronic lung pathology, representing a serious and growing global health problem, as it is currently a leading cause of death worldwide [16]. COPD is a disease characterized by irreversible airflow reduction, associated with a decline in lung function and increased inflammatory response [17]. It represents a massive health problem, and it is estimated to affect around 200 million people worldwide, with a projected estimate towards further increase in the near future [18]. COPD is the result of the interaction between genetic susceptibility and environmental factors [19]. A well acknowledged genetic cause is α1-antitrypsin deficiency [20], while among environmental factors cigarette smoking represents the main cause; nonetheless, environmental pollution, occupational exposure to dust and fumes, and exposure to passive smoke can induce an increased risk in non-smokers, as well [21, 22]. Exposure to cigarette smoke, which contains a large number of pro-oxidant molecules [23], causes direct damage to the epithelial cells of the airways, leading to increased inflammation and activation of neutrophils, macrophages and lymphocytes in the airways [24]. There is currently no cure for COPD, but fortunately most symptoms can be treated and controlled mostly pharmacologically, at least delaying its progression and worsening. It represents indeed the most common indication for lung transplantation, that is the only conclusive therapeutic option for severe COPD, particularly in younger patients.

2. Smoke, oxidative stress and fibrosis in lung pathogenesis

Cigarette smoking likely represents the single most significant risk factor for several lung conditions, and it is strongly associated with COPD and IPF, both familial and sporadic. Although observations about environmental risk factors have many biases and limitations [25], increasing knowledge on the underlying causes of lung diseases is evidencing how oxidative stress (OXS) and reactive oxygen species (ROS) play a crucial pathogenetic role (**Figure 1**).

The lungs are indeed highly susceptible to ROS-induced injuries. ROS are commonly thought to be a harmful by-product generated in cellular systems. However, recent studies have suggested that ROS physiological levels regulate important biological functions in cellular processes [2, 26]. Normally, ROS are tightly controlled by enzymes and antioxidant molecules. Nonetheless, excessive ROS accumulation may occur under certain conditions, thus making detoxification by the antioxidant system difficult. The result is indeed a condition called OXS that can affect cell proliferation, differentiation, aging, and death [27]. Cigarette smoke is responsible for significant oxidant burden and decreased antioxidant capacity even in plasma [28, 29]. ROS produced from cigarette smoke, combustion of organic matter and gases, like ozone and nitrogen dioxide, are featured on the lung epithelium [30], and could decrease antioxidant defenses, increasing OXS in the lungs [31].

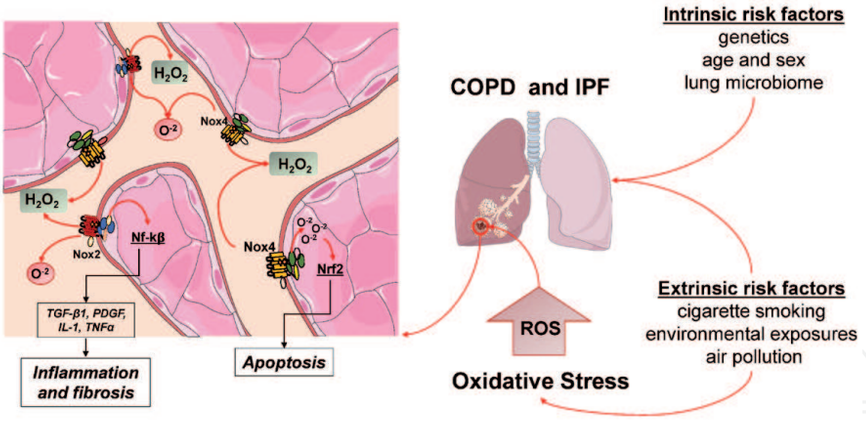

Figure 1.
Oxidative stress in lung pathogenesis. Intrinsic and extrinsic risk factors contribute to the progression of lung damage in the development of idiopathic pulmonary fibrosis (IPF) and obstructive pulmonary disease (COPD). In particular, cigarette smoking, environmental exposures, and air pollution induce an increase of reactive oxygen species (ROS) and oxidative stress condition, which play a crucial pathogenetic role in lung diseases. ROS, such as H_2O_2 and O^{2-}, generated from NOX2 and NOX4, have a central role in the pathogenesis of pulmonary diseases. Indeed, ROS produced by these enzymes are involved in alveolar epithelial cell apoptosis, activation of inflammation, and induction of tissue fibrosis, that are all mechanisms underlying the progression of IPF and COPD. Figure was prepared using images from Servier Medical Art by Servier (https://smart.servier.com), which are licensed under a Creative Commons Attribution 3.0 Unported License.

A disrupted function of the redox system can consequentially impact on key cell signaling pathways involved in disease progression. Conversely, several signals can alter the oxidative state of lung cells. For example, the lung is constantly exposed to biomechanical forces, such as fluid shear stress, cyclic stretch, and pressure, due to the blood flowing through the pulmonary vessels, and the distension of the lungs during the breathing cycle. It is indeed known that cells within the lung respond to these changes by activating signal transduction pathways that can also alter their redox state with pathophysiological consequences [32]. Particularly in the vasculature, the two types of biomechanical stimuli, such as frictional force known as shear stress (SS), or wall shear stress (WSS) that acts tangentially to the vessel, could determinate dysregulation of the cellular redox status, that in turn could have effects on intracellular signaling pathways involved in disease progression [33]. For example, exposure of endothelial cells to laminar SS can induce a suppression of ROS levels [34, 35]. Conversely, exposure of endothelial cells to WSS using an irregular flow induces an increase of ROS levels and a reduced bioavailability of the vasodilator molecule NO [36], which is involved in preventing the activation and adhesion of platelets and leukocytes to the wall of the injured vessel [37].

A significant role in the pathogenesis of COPD is precisely the imbalance of ROS production and antioxidant capacity [38]. Changes in the redox balance in the lungs and circulatory system, genetic polymorphisms, and activation of transcription factors, such as the nuclear factor kappa B (NF-κB), lead to the molecular pathogenesis of COPD [39, 40]. Oxidized proteins and lipid products, such as isoprostanes and carbonylated proteins, can be identified in exhaled air, bronchoalveolar lavage fluid, and lung tissue from patients with fibrotic lung diseases and COPD [41, 42]. Furthermore, clinical worsening of COPD is often associated with down-regulation of the antioxidant system, thus a possible therapeutic method for COPD could be the administration of redox-protective antioxidants [38]. Finally, it is possible that maintaining a balance between oxidant and antioxidant species in COPD affected smokers may slow down disease progression [43].

As discussed, smoking, occupational exposures like asbestos or silica, and radiation are the principal sources of OXS with overproduction of ROS, that could lead

and contribute to pulmonary fibrosis [44]. Indeed, OXS is an important molecular mechanism underlying fibrosis in a variety of organs, including lungs. Bleomycin-induced pulmonary fibrosis, the most commonly used experimental animal model, has been shown to be associated with marked increase in the level of ROS, oxidized proteins, DNA and lipids [45]. Following lung injury three main mechanisms (i.e. inflammation, coagulation disturbances, and OXS) are involved and alter the lung interstitial cell compartment and extracellular matrix (ECM) homeostasis, resulting overall in pulmonary fibrosis. ROS can be produced by several cellular types involved in fibrosis including alveolar macrophages [46–48] and lung epithelial cells [49]. In particular, ROS generated from the mitochondria of stressed or damaged epithelial cells are very important; their mitochondrial dysfunction results in the generation and release of ROS, such as H_2O_2 and O^{2-}, further enhancing OXS and cell damage [50]. As previously discussed, NAD(P)H oxidase is the main source of ROS, and isoforms NOX1, NOX2, and NOX4 have a central role in the pathogenesis of pulmonary fibrosis [51] (**Figure 1**). For example, NOX4 is strongly expressed in the hyperplastic alveolar epithelium of IPF patients [52], and ROS produced by NOX4 are involved in alveolar epithelial cell apoptosis. Continuous epithelial apoptosis further supports activation of inflammatory processes and cytokine release, including myofibroblast activating molecules, such as TGF-β1, PDGF, IL-1, and TNFα (**Figure 1**). There is also evidence of direct pathogenetic involvement of these enzymes in IPF, for example for NOX2: in fact, supporting data have shown that mice genetically deficient in NOX2 do not develop IPF after bleomycin or carbon nanotubes exposure [51, 53]. Finally, the interplay between oxidative stress and TGF-β1 signaling is of great importance in promoting fibrosis. In fact, TGF-β1 is the most profibrogenic protein and can directly stimulate NOX-mediated ROS production, while OXS in turn can activate latent TGF-β1, setting up a vicious profibrogenic positive feedback loop [54].

3. Modelling lung diseases in 3D with organoids

As mentioned previously, fibrosis and oxidative stress are linked to a dysregulation of cellular homeostasis and impaired alveolar structure in chronic lung diseases [55]. Despite the paramount importance of animal models in biomedical and clinical research, they often do not fully recapitulate the pathogenesis of human IPF [56]. Moreover, there is increasing social and political pressure on reducing animal experimentation, according to the 3R's principle of replacement, reduction, and refinement. Furthermore, the associated costs of animal purchasing, housing, and handling cannot be ignored, as well [57]. Under this perspective, 3D cultures (such as organoids) and innovative microfluidic devices (such as "organs-on-chip") represent useful platforms to perform significant investigations *in vitro* on multiple topics, including the pathogenesis of COPD, IPF, or other lung diseases. They grant simultaneous multicellular culture and cell–cell interactions that overcome the limitations of standard monolayer cell cultures, allowing a step forward towards reproducing the complexity of tissues. Moreover, specific protocols and setups make it possible to simulate many more elaborated pathogenetic features, such as ontogenetic-like mechanisms, tunable biomechanical cues, altered gas/liquid interfaces, as well as immune cells recruitment and activation (**Figure 2**). Physiologically relevant *in vitro* systems are also suitable to discover and test new drugs and therapeutics, supporting the clinical translation of novel protocols in a "personalized medicine" perspective [1].

Three-dimensional culture systems offer multiple advantages for *in vitro* phenotype control in order to obtain physiologically relevant settings [58]. The simplest 3D culture system is represented by spheroids, which can be obtained

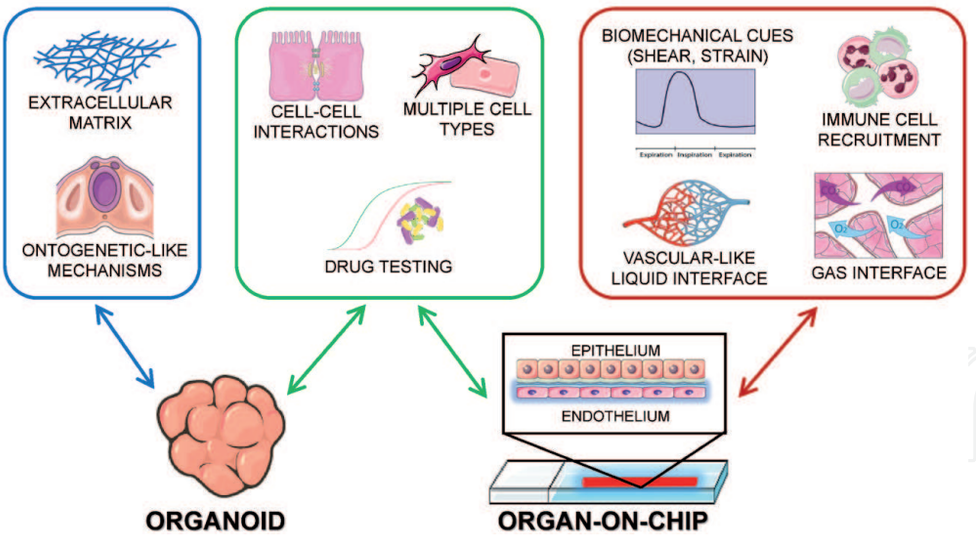

Figure 2.
In vitro models of lung pathology. Both organoids and lungs-on-chip allow the creation of 3D systems where complex cell–cell interactions and multi-cellular cultures are possible. Moreover, drug discovery and testing in these settings can provide important preliminary results in vitro. Several features, though, are better reproduced in specific 3D systems, for example the inclusion of an extracellular matrix, or the modelling of ontogenetic-like mechanisms better fit in organoid cultures. Conversely, biomechanical cues, gas and liquid interfaces, and immune cells response are more finely tunable with organs-on-chip technology. Figure was prepared using images from Servier Medical Art by Servier (https://smart.servier.com), which are licensed under a Creative Commons Attribution 3.0 Unported License.

from embryonic-like stem cells or several resident lung cell types, particularly those with a facultative stemness potential (e.g. pneumocytes, Clara cells [59]). Lung cell spheroids, despite their simplicity, can provide useful preliminary models even for the study of complex pathological issues. Alveoli-like structures obtained from distal airway stem cells [60] or Oct-4+ progenitor cells [61] have been used for the study of viral infections (e.g. H1N1 influenza virus, or SARS-CoV), the pathogenesis of tissue damage, and subsequent mechanisms of tissue repair. As another example, lung spheroids from stromal primitive cells [62] have significantly contributed to the elucidation of novel pathogenetic mechanisms during organ reconditioning procedures, in particular during ex vivo lung perfusion (EVLP) protocols before lung transplantation. In fact, OXS strongly contributes to tissue damage during EVLP. It has been shown that inhibition of NOX2 activity during thermic stress and starvation (mimicking EVLP conditions) can reduce ROS production, thus being protective for lung epithelial cells [63, 64]. Finally, specific interference of cigarette smoke with Wnt/β-catenin signaling has been described in human fibroblasts, impairing their capacity to support spheroid growth of lung epithelial cells, which can be considered in this case as a stemness assay linked to the activation of a repair mechanism [65].

The more complex example of organotypic 3D cultures is represented by organoids. Lung organoids are self-assembling structures of lung cell types that replicate cell–cell interaction, cell-ECM interaction, and organ structure and function at the microscale, as similar as possible to in vivo histological architecture. They can be used as models of both physiological and pathological settings. Strikoudis et al. have modelled pulmonary fibrosis in lung organoids to study Hermansky-Pudlak syndrome (HSP) [66]. IPF and HSP both are characterized by lung fibrosis, and are now considered as similar clinical entities, albeit with distinct etiology. Lung organoids were generated from embryonic stem cells (ESCs) with specific mutations that strongly predispose to HSP. The resulting organoids displayed a fibrotic phenotype, with an enhanced number of mesenchymal cells, and increased deposition of fibronectin and collagen. Interestingly, HSP organoids share a strong signature with lung

samples from IPF patients, including the overexpression of interlukin-11 (IL-11), a key driver of the fibrotic process that is stimulated also from OXS [67]. This finding validates HSP lung organoids as a tool to study IPF and other lung diseases characterized by fibrosis [66]. Similarly, Wilkinson et al. have developed an organoid from induced pluripotent stem cell (iPSC)-derived fibroblasts functionalized with hydrogel beads, that acts as a 3D alveolar template within a rotating bioreactor [68]. Interestingly, they discovered that organoid formation was not possible in their conditions without the inclusion of fetal lung fibroblasts. Treatment of cultures with exogenous TGF-β1 consistently increased contraction, expression of Collagen 1 and α-SMA, and the emergence of fibroblastic foci within the treated organoid. This system showed features of tissue scarring similar to IPF, thus confirming the feasibility of organoid culture systems to model lung fibrosis. Moreover, these lung organoids can recapitulate even a more complex and representative lung microenvironment when cultured with endothelial and epithelial cells [68]. As an example, using lung organoids from patients with IPF, Surolia et al. described a 3D model to predict the invasive response of IPF fibroblasts to antifibrotic drugs therapy. They observed that inhibition of vimentin intermediate filaments assembly can reduce the invasiveness of lung fibroblasts derived from the majority of the IPF patients tested, uncovering a possible novel therapeutic target for pulmonary fibrosis [69].

Overall, these 3D self-assembled systems recapitulate numerous pathogenetic features of diseases, but nonetheless still show several limitations in their application as models, such as lack of vascular network, immune cells, and other supporting cells (**Figure 2**). These features need to be implemented to reach higher levels of physiological relevance for lung disease modelling [69].

4. Organs-on-chip for the study of lung diseases

In the last decade, the integration of advanced bioengineering approaches (e.g. 3D multicellular cultures) with microfluidic and microfabricated substrates has led to the development of devices called "organs-on-chip" [70]. These bioengineered tools allow fine control and tuning of the microenvironment architecture, media composition, and cell–cell interactions. The combination of lung cells and micro/nanoengineering devices gave rise to new *in vitro* models for the study of therapeutic approaches in pulmonary diseases. In fact, lungs-on-chip can recapitulate typical features of the parenchymal structure, and primary physiological or pathological conditions of the human lung microenvironment, such as liquid and gas interfaces [71] (**Figure 2**). In 2010, Hu et al. for the first time created a lung-on-chip using a soft lithography technique. Soft lithography offers the advantage to control the molecular structure of surfaces, the pattern of complex molecules relevant to biology, and to fabricate channel structures appropriate for microfluidics [72]. They produced a biomimetic microdevice that recapitulates the crucial alveolar-capillary interface of the human lung. This device is a 2.5D system since it contains monolayers of epithelial and endothelial cells that mimic the alveolar-capillary barrier, and permits investigation under dynamic conditions, with biomechanical cues in the form of SS due to perfusion, and strain similar to breathing [71]. However, ECM components are lacking in this model, and this significantly limits the relevance of this device, in particular concerning the study of pulmonary fibrosis. To address these limitations, other groups have designed arrays of 3D microtissue that are suspended over multiple flexible poly-dimethylsiloxane (PMDS) micropillars [73–75]. In particular, Sellgren et al. produced an advanced model by co-culturing interstitial fibroblasts with epithelial and endothelial cells [75]. They demonstrated the feasibility of including a stromal layer within lung-on-chip devices. Similarly, Asmani et al.

have developed a human lung device to model key biomechanical events occurring during lung fibrogenesis, which include progressive stiffening and contraction of alveolar tissue. They used this system for predicting the efficacy of anti-fibrotic drugs for IPF patients, demonstrating that preventative treatments with these drugs can reduce tissue contractility, and counteract tissue stiffening and decline in tissue compliance [73]. Overall, these new approaches will give a better understanding of the complex pathogenesis of IPF.

As discussed above, COPD is a syndrome defined by progressive and chronic airflow limitation, due to the fact that lungs become inflamed, damaged, and narrowed. The main cause is smoking, but others exist such as long-term exposure to harmful fumes or dust, and rare genetic conditions [43]. As for IPF, the animal models of COPD present some limitations. For example, modelling cigarette smoke exposure fails to recapitulate some major airway phenotypes of COPD, such as hyperplasia of basal and mucin-producing cells, and mucus plugging of the airways [76]. Before the advent of lung-on-chip technology, the best-established *in vitro* model to study COPD disease and to address cigarette smoke-induced damage on human airway epithelial cells was the air-liquid-interface (ALI) culture system [77]. The defining feature of ALI cultures is that the basal surface of the cell is in contact with a liquid culture medium, whereas the apical surface is exposed to air [78]. These systems mimic the conditions found in the human airway, and drive differentiation towards different phenotypes [79]. One major limitation of conventional ALI models is that these static culture systems make dynamic processes, such as nutrient exchange and immune cell migration [80], difficult to study.

In this regard, innovative approaches, such as microfluidic lungs-on-chip, have been developed in the last years and helped filling this gap. In 2016, Benam et al. developed the human lung "small airway-on-a-chip", a microfluidic device that supports and drives full differentiation of a columnar, pseudostratified, mucociliary bronchiolar epithelium, composed of cells isolated from healthy individuals or people with COPD, underlined by a functional microvascular endothelium [81]. They demonstrated that COPD small airway chips recapitulate important features of the disease, such as selective cytokine hypersecretion and neutrophil recruitment from the vascular flow in response to epithelial activation by pathogen-like stimuli. Moreover, exposure of the healthy epithelium to interleukin-13 (IL-13) reconstituted the asthmatic phenotype that involves goblet cells hyperplasia, cytokine hypersecretion, and decreased ciliary function [82]. The same group improved this system by developing a "Breathing-Smoking Human Lung-on-Chip", a novel device that consists of four components: a small airway on-chip, a smoke generating robot, a micro-respirator, and a control software that mimics human smoking and breathing. This smoking airway-on-a-chip system effectively recapitulated several key smoke-triggered molecular changes that are known to occur in lung epithelial cells, including increased OXS [83]. When human airway chips fabricated using cells from healthy donors were exposed to whole cigarette smoke, the authors observed a significant increase in the expression of the anti-oxidant gene heme oxygenase 1 (HMOX1), and increased phosphorylation of the transcription factor nuclear factor-like 2 (Nrf2). The latter induces expression of cytoprotective genes, including HMOX1, protecting cells from OXS and chemical toxicity. Furthermore, they identified new smoke-induced dysfunction, such as reduced ciliary beating, a novel biomarker of COPD disease, and studied the epithelial responses to smoke generated by electronic cigarettes [84]. However, the main limitation of this system is the absence of cellular stromal components.

As mentioned before, COPD represents a group of lung diseases that also include refractory severe asthma. In this regard, Nesmith et al. have designed a human airway musculature-on-a-chip with bronchiolar smooth muscle cells on

an elastomeric thin film. To recapitulate asthmatic inflammation *in vitro*, they exposed this biomimetic tissue to IL-13, which resulted in hypercontractility and altered relaxation. Interestingly, the authors were able to show reverse asthmatic hypercontraction of smooth muscle cells using a muscarinic antagonist and a β-agonist, which are used clinically to relax constricted airway [85]. Similarly, Villenave et al. developed a model of severe asthma-on-chip containing a fully differentiated mucociliary bronchiolar epithelium underlined by a microvascular endothelium with fluid flow [86]. They infected the engineered tissue with human Rhinovirus (HRV), a leading cause of asthma exacerbation in children and adults; this led to a pro-inflammatory response characterized by ciliated cells death, goblet cells hyperplasia, release of cytokines, recruitment from the fluid flow and extravasation of human neutrophils across the endothelium. Infection of IL-13-treated Airway Chips with HRV to mimic the molecular response observed in severe asthma patients, induced upregulation of adhesion molecules (E- and P-Selectin, ICAM-1) in endothelial cells, and increase of neutrophil recruitment when compared with IL-13 or HRV stimulation alone [87]. The same group implemented this device to study the integrity of epithelial monolayers-on-chip, measuring trans-epithelial electrical resistance (TERT). They designed a new microfluidic device within a human lung airway chip that contains embedded electrodes, and demonstrated its utility for the assessment of airway barrier function, formation, and disruption in response to relevant external stimuli [88]. These studies suggest that Airway Chips may provide unique opportunities to explore lung pathogenesis, including responses to drug treatments for the evaluation of safety and efficacy of new drugs. Moreover, the possibility of studying the involvement and activation of immune cells certainly brings added value to these systems, allowing the study of physiologically relevant issues within an integrated model.

5. Conclusions

Basic and translational research on lung biology and pathology can greatly benefit from the development of 3D *in vitro* models that can maintain cell phenotypes and functions in a physiologically relevant way. Lung organoids and lungs-on-chip allow the creation of different kinds of *in vitro* microenvironments (**Figure 2**), that can be useful for the study of specific diseases, and for the elucidation of novel pathogenetic pathways. They represent in fact important translational models for the study of clinically relevant issues, for the identification of novel therapeutic targets, and for preliminary testing of new drugs. The main challenge in future developments is represented by the standardization of integrated protocols for the simultaneous inclusion of extracellular matrix, stromal components, immune cells, and biomechanical cues within 3D *in vitro* models. This step forward would provide a clinically relevant system for lung research, which would include all the actors involved in endogenous responses occurring *in vivo*. Nonetheless, despite several limitations still existing, the complexity of these models has been rapidly increasing in the past decade, and they must be considered as complementary in all respects to *in vivo* studies carried on in animal models.

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Conflict of interest

The authors declare no conflict of interest.

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