Animal models in research examining stem cell therapy for human lung disease: a support in the quest to develop therapies

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Abstract

Acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD) are lung diseases characterized by severe inflammation. No cures are identified for these diseases yet. Stem cell treatment is suggested to be a valid option for these patients, but this option needs to be extensively examined. Animal models are used for drug testing and validating treatments. However, it is extensively discussed which part of the ARDS/COPD pathology is adequately modelled by animal models and whether current modelling is sufficient for making clinical translation possible. This review is aimed to identify whether the use of animal models as step in research towards stem cell therapy for human lung disease (ARDS and COPD) is an utopia, a need, or a support in the quest to develop therapies. It is shown that a generalized and optimized model for both diseases is needed. The absence of comprehensive ARDS and COPD animal models has led research to develop new *in vitro* models in order to examine stem cell treatment and its underlying mechanisms. These *in vitro* models, as lung organoids and lung-on-a-chip models, sound promising but are not optimized yet. To conclude: there is a need for optimizing the *in vivo* and *in vitro* models for ARDS and COPD in order to examine the underlying mechanisms of MSC-treatment for these diseases. Thus, animal models (and *in vitro* models) are a support in the quest to develop therapies and the first step needed now is optimization of these models.

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Introduction

Lung tissue is in close contact with the outside environment and therefore more prone to airborne infections and associated diseases. The external environment contains different harmful pathogens, as bacteria, fungi and viruses, but also detrimental particles. Detrimental particles can be found in substances like toxic smoke or inefficiently burned fuel. The particles are smaller than 10 micrometres and can be as fine as 1 nanometre, which makes it possible for the particles to enter the lung along with inhaled air. Therefore, these particles are one of the main causes of respiratory disease[1]. Respiratory disease is, apart from cardiovascular diseases, the disease-group with the most disability-adjusted life-years, which is a scale that displays the amount of active and productive life lost due to disease[2]. 'Respiratory disease' is a broad term which comprises a lot of different syndromes. Acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), asthma, tuberculosis and acute lower respiratory infections are examples of respiratory diseases which are the most common causes for severe illness globally. More than one billion people are suffering from respiratory diseases, which are either acute or chronic[2].

ARDS and COPD are representatives of both acute and chronic lung diseases respectively. Both diseases are characterized by severe inflammation. Symptoms are not always disease-specific and therefore these respiratory diseases are difficult to diagnose. Measurement of lung function gives an indication of the underlying disease, but symptoms as coughing and an increased work of breathing are also taken into consideration. Identifying diseases based on symptoms contributes to misdiagnosis of ARDS or COPD: not all COPD patients fit the typical disease profile, which makes diagnostics based on symptoms difficult[3]. On top of that, no cure has been identified for these diseases, while the burden and death rates of both are rising: there is a need for a cure that tackles the core problems in pathology instead of focussing on symptom relief.

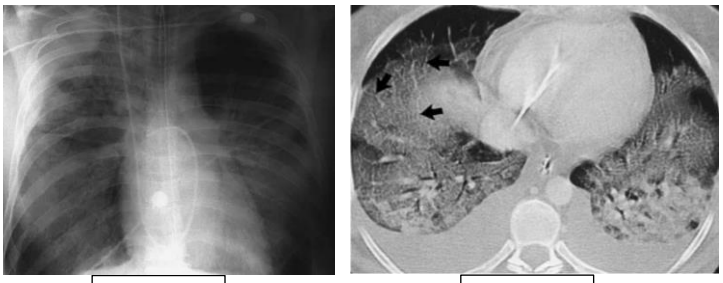
The core problems of ARDS and COPD are disruption of the epithelial barrier and the associated alveoli, as is damage to the blood endothelium because of the inflammatory response in the lungs. In order to tackle the main problems, it is necessary to modulate the immune response so disruption of the epithelium and endothelium is stopped. On top of that it is important to reverse the damage done. Stem cell treatment is suggested to be a valid option for treatment, since these cells are prone to instruction and can (re)construct damaged tissue. However, this option needs to be extensively examined to identify its effect and possible (harmful) side effects on the body.

Stem cell treatment is a hot topic in research nowadays and its effects on disease are investigated in animal models often, since this gives an opportunity to see the effects of this treatment in living organisms. Animal models are widely used in research for examining fundamental mechanisms and physiology, but also for research in relation to pathology. Mostly mouse models are used in research, since they are biologically similar to humans and can be genetically modified in order to express human disease forms. Rat models are used frequently as well. Most importantly, a good animal model has to mirror physiological and pathological characteristics of humans and human disease. However, an animal model cannot mimic human pathology to the full extent. Also, most often healthy animals are acutely induced with disease and are being used to mimic chronic disease, which is a great limitation in the use of animal models. These pros and cons pressure the ongoing use of animal models in research: if there are great restrictions, why use animal models? Therefore, this review is aimed to

identify whether the use of animal models as step in research towards stem cell therapy for human lung disease (ARDS and COPD) is an utopia, a need, or a support in the quest to develop therapies.

Acute respiratory distress syndrome

ARDS is an acute inflammatory lung injury, mainly caused by infection or injury including pneumonia, non-pulmonary sepsis or trauma[4,5]. Lungs of ARDS patients show alveolar opacities that are more prominent in the posterior lung. These opacities are coherent with the presence of pulmonary edema (figure 1).



Panel A Panel B

Figure 1. Lungs of a patients diagnosed with acute respiratory distress syndrome (ARDS). Panel A shows a chest radiograph from a 42-year-old man with ARDS. The lung shows diffuse alveolar opacities, which is consistent with pulmonary edema. Panel B displays a CT-scan of the chest. The alveolar opacities are more dense in the posterior lung. The arrows display thickened interlobular septa, consistent with the presence of pulmonary edema[6].

Once the inflammation in ARDS is induced, a cascade of sequential processes is started which causes the clinical phenotype of ARDS patients. Neutrophils and alveolar macrophages (AMs) are the central inflammatory cells during the acute inflammation in ARDS[7]. AMs are thought to be increased in the alveolar lumen of the lung due to a rapid influx of monocytes from the blood[8]. AMs are sentinel cells: they last in the resting state (M0), unless activated. Once activated by detrimental particles in the outside environment , the AMs become either M1 or M2 macrophages. Both can be found in inflammatory environments, but their functions differ: M1 is a proinflammatory macrophage while M2 macrophages have anti-inflammatory properties. It is proposed that the acute inflammation in ARDS is due to excessive neutrophil extracellular trap (NET) formation, which captures pathogens and kills them. This NET structure causes resting AMs to develop into proinflammatory M1s[9]. The inflammation causes the disruption of the epithelial barrier, the associated alveoli and damage to the blood vessel endothelium (figure 2). Consequently, pulmonary edema is formed which causes respiratory failure and a decreased level of oxygen (oxygenation) in ARDS patients[4,6].

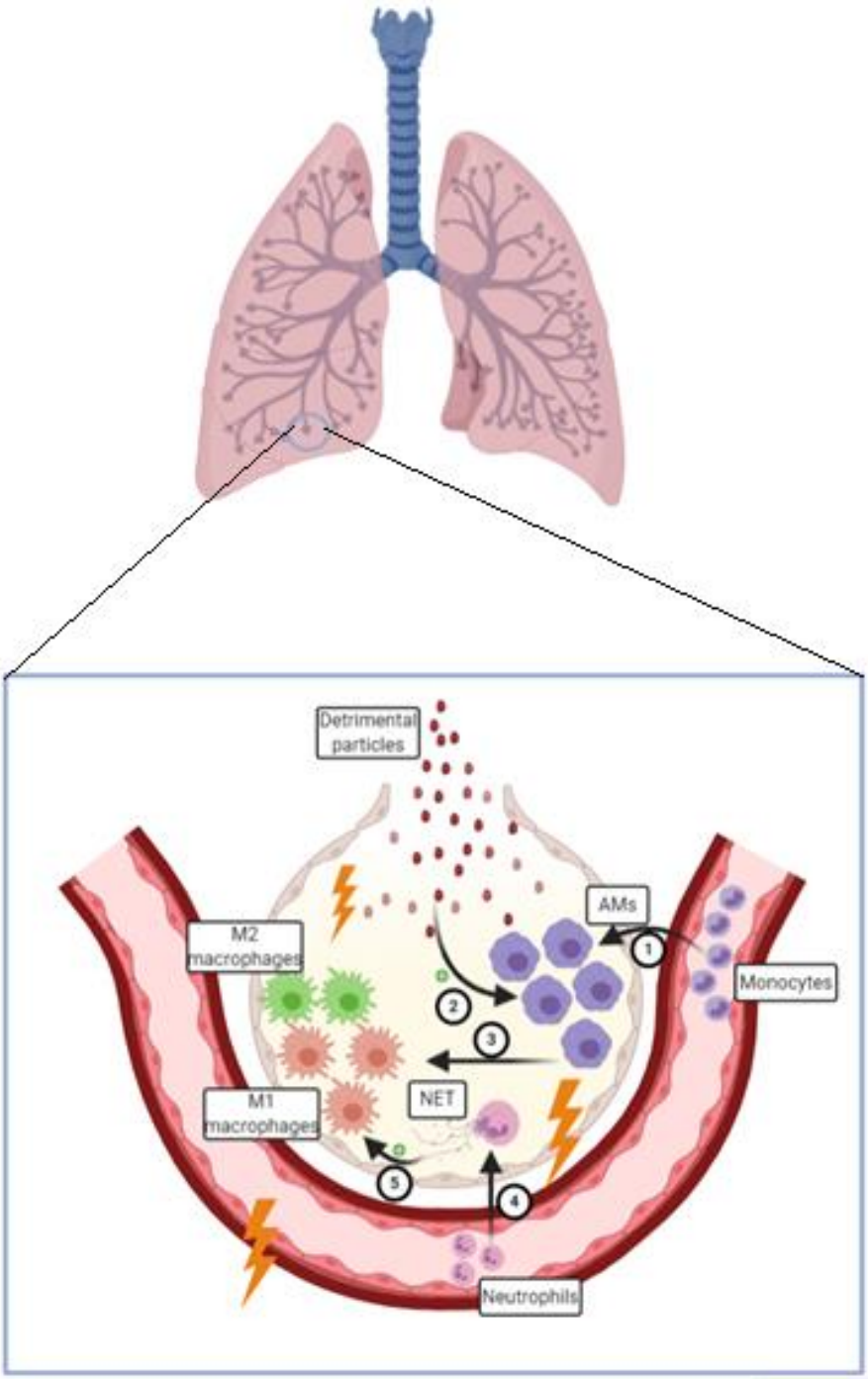


Figure 2. The cellular mechanisms involved in the pathology of ARDS. Monocytes in the blood vessel influx the alveolar lumen and become alveolar macrophages (AMs) (arrow 1). Detrimental particles activate AMs (arrow 2) and cause the AMs to become pro-inflammatory M1 or anti-inflammatory M2 macrophages (arrow 3). Neutrophils from the blood stream infiltrate the alveolar lumen (arrow 4) and start to produce neutrophil extracellular trap (NET) structures, which drives AM development towards M1 macrophages (arrow 5). These inflammatory responses cause the endothelial barrier of the blood vessel, the epithelial barrier of the alveoli and the alveoli itself to disrupt (indicated by orange lightning bolts)[4,6-9]. Generated via Biorender.com.

ARDS in patients is classified as mild, moderate, or severe according to their oxygenation[5]. Identification of ARDS is mostly within 72 hours after recognition of an underlying risk factor. Practically all ARDS patients are identified within 7 days[10]. Classifying patients is performed by measuring the PaO_2/FIO_2 ratio, which is the ratio between arterial oxygen partial pressure (PaO_2) and fractional inspired oxygen (FIO_2)[11,12]. The latter comprises the part of a gas mixture that contains oxygen when inhaled; ambient air contains 21% oxygen, so in that case the FIO_2 is 21%. The ratio displays to what extent hypoxemia, a low oxygen level in the blood, is present: a lower ratio shows a more severe clinical picture. The normal value of the PaO_2/FIO_2 ratio is ~350 mmHg[13]. Mild patients are identified to have a PaO_2/FIO_2 ratio between 200 and 300 mmHg, while moderate patients are between 100 and 200 mmHg and severe patients are below 100 mmHg[11]. It is possible for mild and moderate ARDS patients to progress to a more severe form of ARDS[10]. Next to a low level of blood oxygenation, patients experience an increased work of breathing due to a reduction in lung compliance because of increased lung stiffness[5]. This stiffness is a direct consequence of the acute inflammatory response that occurs in the lung. Macrophages and other immune cells produce cytokines which stimulate the production of extracellular matrix by fibroblasts. The excessive extracellular matrix causes remodelling of the lung and therefore the reduction in lung compliance[6]. Jointly, these symptoms lead to a high ARDS-related hospital mortality of 34.9%, 40.3% and 46.1% for mild, moderate and severe patients respectively, since there is no cure for ARDS[5,11]. Supportive care by placing patients on mechanical ventilation is the only treatment provided so far. However, this treatment is focussed on symptom relief and a treatment should be focussed on resolving the core problems of ARDS[11]. A possibility could be the use of stem cells in order to annul the disruption of the epithelial barrier, the associated alveoli, the damage to the blood vessel endothelium and to normalize the remodelled lung that is occurring because of the inflammatory response. Stem cells could reduce this inflammatory response by decreasing pro-inflammatory mediators and restore lung function by increasing concentrations of necessary growth factors[14].

Chronic obstructive pulmonary disease

COPD is a chronic inflammatory lung disease, mainly caused by exposure to smoke: cigarette smoking, indoor cooking and air pollution are illustrations of this[16]. However, not every individual exposed to (substances of) smoke develops COPD.

During the development of COPD, the epithelium of the airway shows abnormalities. These are due to changes in the transcriptional program of the apical junctional complex in the epithelium, which continues to change as the disease progresses[17]. The apical junctional complex comprises both adherens junctions and tight junctions, which preserve the epithelial barrier function[17]. The molecule E-cadherin is involved in cell-cell interactions and therefore involved in keeping the epithelial barrier function stable. A reduction in E-cadherin protein expression is found in COPD patients[18], which partially shows the mechanism behind the epithelial barrier dysfunction.

Next to disruption of the epithelial barrier, epithelial cells also produce pro-inflammatory factors, as tumour necrosis factor alfa (TNF-α), interleukin(IL)-1β and IL-6 that contribute to the inflammatory response[19]. TNF-α activates NF-κB, which is a protein complex thought to amplify inflammation in COPD. NF-κB activation causes pro-IL-1 to form, and multiprotein-signalling complexes as NLRP3 (inflammasomes) to activate and regulate IL-1β expression

(figure 3). IL-1β is a pro-inflammatory cytokine which contributes to the inflammatory response in the lung, just as IL-6. IL-6 can also be released from AMs and regulatory T-helper 17 (Th17) cells[19]. Endothelial barrier dysfunction and the production of pro-inflammatory cytokines in epithelial cells contributes to inflammation in COPD. However, a lot more contributors to this inflammation have been identified. Neutrophils and macrophages from the innate immune system, but also T-cells and B-lymphocytes from the adaptive immune system are involved[19].

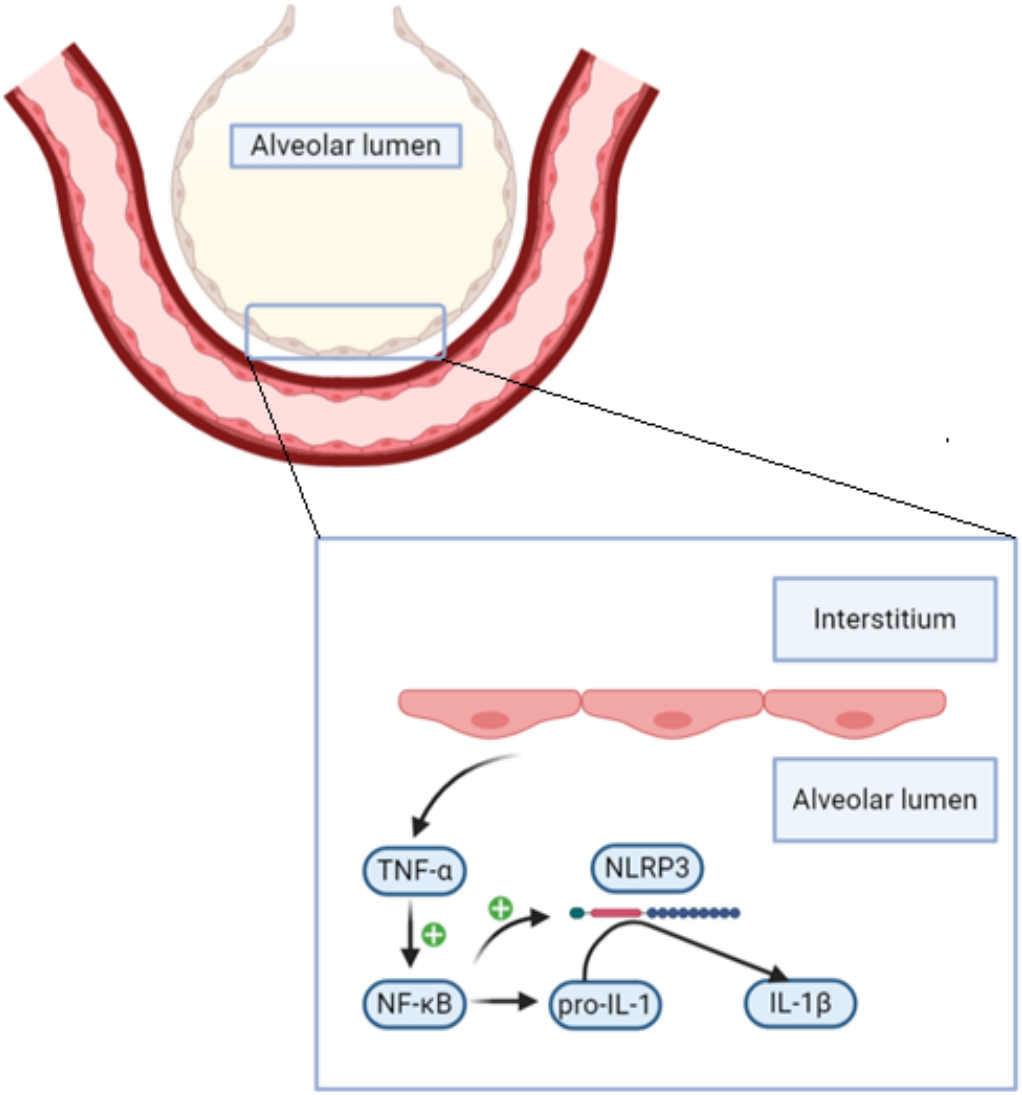


Figure 3. Inflammasome activation in patients with COPD. Epithelial cells of the alveoli produce pro-inflammatory factors as TNF-α and IL-1β. TNF-α activates a protein complex NF-κB, which amplifies inflammation in COPD. NF-κB activation causes pro-IL-1 to form, and multiprotein-signalling complexes as NLRP3 (inflammasomes) will activate and regulate IL-1β expression[19]. Generated via Biorender.com.

Next to inflammation, COPD is characterised by unreversible airflow obstruction. This is due to both small airway remodelling as a result of inflammation (as is seen previously in ARDS) and disruption of the lung parenchyma. The latter involves alveolar attachments to be lost, which increases the size of air spaces in the lungs: emphysema is formed and hyperinflation occurs (figure 4A)[19]. Also, a reduction in pulmonary perfusion can be seen (figure 4B). When a patient displays symptoms related to hyperinflation, dyspnoea, chronic cough and has a history with exposure to risk factors for COPD, a clinical diagnosis will be performed[20]. COPD is mainly diagnosed by examining the FEV_1/FVC ratio of the patient's lung by using spirometry. FEV_1 comprises the forced expiratory volume in 1 second, while FVC comprises the forced vital capacity. The ratio should be <0.7 in order to diagnose a patient with irreversible airflow obstruction and therefore COPD[20]. When diagnosed, patients often get a combination of long-acting muscarinic antagonists (LAMAs) and long-acting β2-adrenergic agonists (LABAs) to relief symptoms[21].

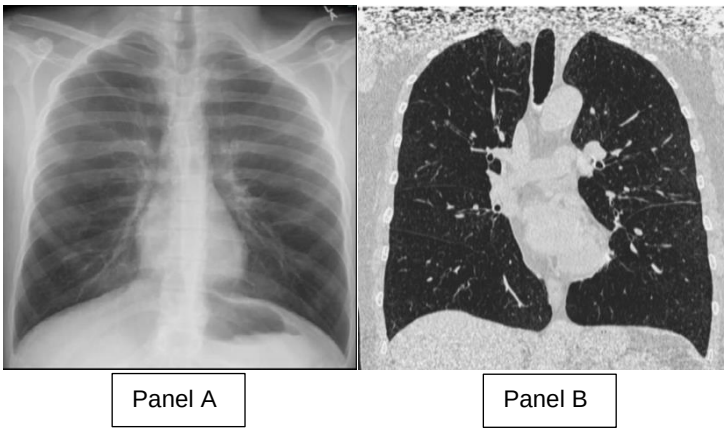


Figure 4. Lungs of a patient diagnosed with chronic obstructive pulmonary disease (COPD). Panel A shows a chest radiograph from a patient diagnosed with COPD. The lung shows hyperinflation, identified as larger lungs in comparison to normal lungs as a result of trapped air. Panel B displays a CT scan of the lungs from a patient diagnosed with COPD. A reduction of the pulmonary perfusion is shown[22].

So far, there is no cure for COPD other than medicaments focussed on symptom relief. Knowing that the prevalence of COPD was identified at 118 million worldwide in 2019[23], it is necessary to find a treatment that tackles the core problems of COPD instead of tackling symptoms that are results of the problem. The core problems comprise disruption of the lung epithelial barrier, production of pro-inflammatory factors by epithelial cells and unreversible airway obstruction due to small airway remodelling and lung emphysema. Stem cell treatment is a valid option in order to normalize lung tissue and lung function and could focus on solving the core problems. Stem cells could reduce inflammation by decreasing the pro-inflammatory cytokine levels, improving pulmonary perfusion and improving parenchymal repair mechanisms by increasing levels of associated growth factors[14].

Stem cells

Since there are no cures present for either ARDS or COPD, there is a need for innovative treatments that tackle endothelial and epithelial dysfunction, disruption, inflammation and remodelling of the lung. The last years, mesenchymal stem cell (MSC) based treatment has raised the attention in research and showed promising results in clinical trials in both ARDS and COPD[24–27]. However, a window of opportunity in which the tissues in ARDS and COPD are most prone to stem cell treatment is difficult to determine and has not been determined to date. The degree of disease has a correlation with the dosage of stem cells needed, but further research determining the time frame when ARDS/COPD patients are most susceptible to stem cell treatment is not determined, which is an important factor that should be examined[28].

Stem cells are characterised by the ability of self-renewal, forming clonal progeny and differentiating into different specific cell types, which makes them suitable and deployable for damage control in the human body[29]. The range of specific cell types a stem cell can become varies between different types of stem cells. They could be totipotent, pluripotent or multipotent, however most stem cells belong to one of the latter two forms. Pluripotency comprises the ability of the stem cell to become most cells/tissues of an organism. When a cell is multipotent, it can become a smaller number of options of cells/tissues. Whether a stem cell is pluripotent or multipotent, is determined by the genetic profile of the cell[29].

Next to potency of stem cells, these cells are classified in source of origin: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells. ESCs are found in the inner cell mass of the blastocyst and are pluripotent. Because of the ethical debate surrounding isolating and obtaining ESCs and rejection of ESCs after implantation, these are almost completely displaced in stem cell research. iPSCs are on the other hand emerging in stem cell research. iPSCs comprise the reprogramming of somatic cells by means of nuclear transfer to induce stem cells. Expression of transcription factors Oct3/4, Sox2 and Nanog is the basis for inducing pluripotency in somatic cells. Somatic cells can be found in every department of the body[30]. Thirdly, adult stem cells can be found in specific tissue compartments and are important in maintaining the stability of these tissues, as skin, blood, fat and bone marrow, but also in tissues as lung tissue[29,31]. The bone marrow is well-known for the origin of two different types of stem cells: hematopoietic stem cells and MSCs[29,32].

Mesenchymal stem cells

All stromal tissue, which is the non-parenchymal part of tissue, contains vasculature embedded in extracellular matrix that is maintained by mesenchymal cells such as fibroblasts and mesenchymal stem cells (MSCs). The only exceptions are neural tissues and cartilage. The MSCs are proliferative cells, while small subsets may even self-renew and thus represent genuine stem cells. All other MSCs share the capacity to differentiate in the tri-lineage: bone, cartilage and fat. The cell surface characteristics of MSCs differ, depending on where they are situated in the human body. Almost all (>95%) MSCs express at least cluster of differentiation (CD) markers CD73, CD90 and CD105[34]. Additional CD markers are known, but depend on the origination tissue of MSCs[34]. Unfortunately, the currently used CD markers are by far specific enough to designate MSCs even if combinations are used. When MSCs are eventually differentiated, they start producing markers that correspond to the specific cell type they become.

MSCs are often isolated from adipose tissue or bone marrow to be used in *in vitro* experiments[35] and regeneration of organs. When MSCs are isolated, their plasticity and properties still depend on cell-cell contact and the biophysical microenvironment[33,36]. Cell-cell contact is of importance in determining the plasticity of MSCs. It has been known that culturing MSCs on a stiff matrix causes spreading of the cells and subsequently, MSCs are more prone to differentiate into bone-forming osteoblasts than towards adipose tissue cells: differentiation into fat tissue is slowed down[33]. Contradictory, culturing on a soft hydrogel withholds cells from spreading and MSCs will be more prone to differentiate into adipose tissue cells than osteoblasts. There is thus a direct effect of cell-cell contact on MSCs plasticity, which may be an important factor that contributes to the effect of MSC-treatment in ARDS/COPD patients. When MSCs are administrated and are present in the lung, the behaviour of MSCs needs to be determined. As a result of inflammation, ARDS and COPD patients show (small) airway remodelling and thus an increase in lung stiffness. Because of the relatively stiff matrix, spreading of MSCs could occur and have an impact on MSC plasticity and its degree of differentiation.

The biophysical microenvironment has, next to cell-cell contact, an influence on determining MSCs plasticity *in vitro*. Changes in the biophysical environment of MSCs cause them to behave differently[36]. Using soft (0.5 kilopascal (kPa)) and stiff (40 kPa) hydrogels, MSCs were cultured and cell area was measured. The soft hydrogel showed a lower spread area than the stiff matrix, as mentioned before. Hereafter, MSCs cultured on soft hydrogels were transferred to stiff matrixes and vice versa. After switching, the MSCs that ended on stiff hydrogels showed a significant increase in cell spread area, while MSCs that ended on soft matrixes showed a decrease. Thus, specification towards a cell type is reversible when switching the biophysical environment, which shows the importance of the microenvironment for MSC differentiation *in vitro*[36]. Unfortunately, these experiments have been performed on 2D substrates *in vitro* and therefore do not mimic the 3D environment of cells *in vivo*, which limits the knowledge of MSC behaviour in patients or animal models. I*n vivo*, when MSCs are administrated to ARDS/COPD patients and these cells are present in the biophysical environment of the lung, the behaviour of MSCs may be different. Because of the stiffness, MSCs might differentiate into e.g. alveolar epithelial cells. However, when fibrosis is reduced, the stiffness of the substrate will decrease. MSCs may then differentiate into other cell types. The biophysical environment thus has an influence on MSC plasticity.

In addition to plasticity of MSCs, these cells have a specific property: they can modulate the immune response. It has been shown that transplanted MSCs in spinal cord injury show an upregulation of their gene expression which are related to the immune response, cytokine production and phagocytosis[37]. Therefore, it can be concluded that MSCs are able to adapt immune cell-like properties.

Next to the adaptation of MSCs to immune-cells, MSCs are also able to induce an anti-inflammatory reaction by means of inducing other cells. MSCs were shown to induce mature dendritic cells (DCs) towards regulatory DCs via paracrine pathways and thereby causing reduction of disease[38]. This induction is due to secretion of hepatocyte growth factor (HGF) by MSCs. HGF causes differentiation by stimulation of the Akt pathway, a pathway associated with cell survival and cell growth[38]. The ability of MSCs to convert immune cells by using paracrine pathways shows another route for modulating the immune response.

Besides, MSCs can also exert influence on the fate of macrophages. When macrophages are isolated and cultured with MSCs, CD206 is highly expressed on the cell surface of macrophages[39]. CD206 is a marker for M2 macrophages. This shows that MSCs educate macrophages and thus modulate the immune response by inducing anti-inflammatory macrophages. Regarding ARDS, this education of macrophages is also seen. Macrophages cocultured with MSCs become educated macrophages. When *in vitro* educated macrophages are injected in LPS-treated mice, a reduction of lung inflammation and pulmonary edema is seen. LPS-treated mice function as an animal model for ARDS and reflects the inflammatory response in human ARDS[39]. The macrophages also caused a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines in bronchial alveolar lavage samples of the mice. In addition, serum samples of moderate-to-severe ARDS patients were obtained and educated macrophages were incubated with one of the serum samples. The serum decreased the expression of CD68 and CD206, which are markers for M2 macrophages. However, when MSCs were cultured (directly or indirectly) with these educated macrophages, the expression of M2 macrophage-specific CD markers was reestablished[39]. So, direct cell-cell contact and indirect contact of the secretions of the MSCs with macrophages influence the expression of M2 macrophage CD markers in ARDS.

Next to the insight that MSCs modulate the expression profile of macrophages in ARDS serum, it is shown that MSCs induce regulatory dendritic cells (DCregs) by activation of the Notch signalling pathway in order to reduce acute lung injury (figure 5)[40]. LPS-induction caused an increase in DCs, and the pathology of ARDS worsened in time: an increase in edema and inflammatory cell infiltration were characteristics shown. This cascade of reactions, including the maturation of dendritic cells, was shown to be inhibited by administration of MSCs 4 hours after LPS-induction[40]. DCs that were maturated, differentiated into DCregs by MSCs via the Notch signalling pathway, important in regulating differentiation by means of direct cell-cell contact. Jointly, these results show that MSCs induce DCregs to reduce acute lung injury, like ARDS, via the Notch pathway[40]. The underlying mechanisms and functions of MSCs in a mouse model for ARDS are thus elucidated and elaborated on in recent research.

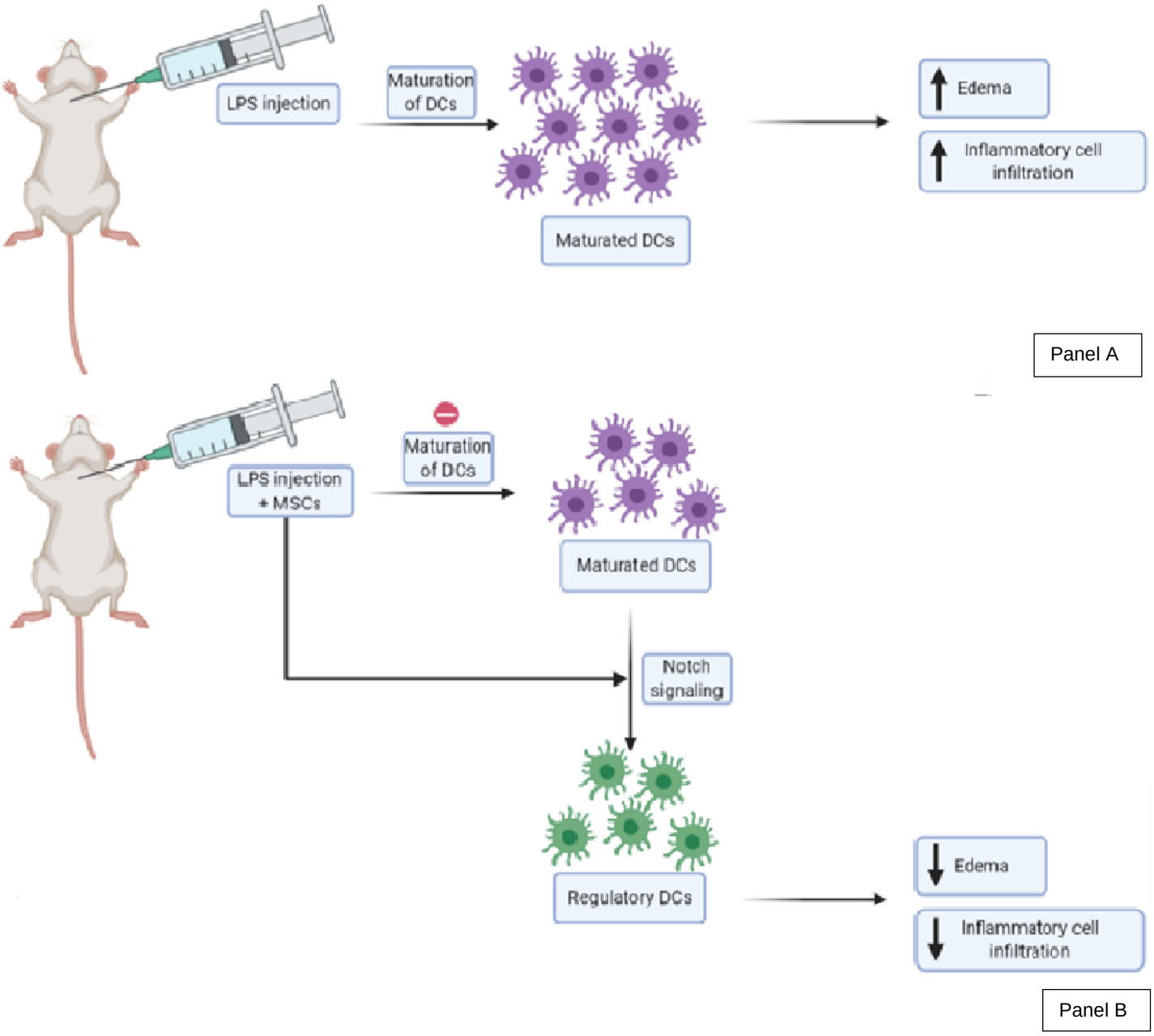


Figure 5. MSCs induce regulatory dendritic cells (DCregs) by activation of the Notch signalling pathway in order to reduce acute lung injury. In panel A, LPS-induction causes an increase in dendritic cells (DCs), and subsequently an increase in edema and inflammatory cell infiltration. In panel B, MSC-administration causes an inhibition of the maturation of DCs and matured DCs become DCregs via Notch signalling. Subsequently, edema and inflammatory cell infiltration are decreased[40]. Generated via Biorender.com.

Research is also aimed at examining the fundamental mechanisms of MSCs in reducing COPD. It has been known already that MSCs repair cigarette smoke-induced emphysema by increasing type 2 alveolar epithelial cell and vascular endothelial cell proliferation in the lung one week after MSC administration in rats exposed to cigarette smoke. Administration of MSCs also decreased apoptosis of those cells, increased the number of small pulmonary vessels, decreased vascular remodelling and decreased hypertension[41]. However, the underlying working mechanisms of MSCs in COPD were not known. Nowadays, different pathways by which MSCs induce these repairs by activating macrophages are specified increasingly. As is seen in ARDS, MSCs do reduce inflammation, but also emphysema which is a characteristic for COPD[42]. A cigarette smoke (CS)-induced rat model was used to examine the mechanisms behind reduction of inflammation in COPD. Since it is already known that the upregulation of enzyme COX-2 in macrophages is responsible for production of prostaglandins (PGs) associated with this inflammation, it was examined if MSCs could cause a change in this upregulation in the COPD model. Results showed that MSCs reduce CS-induced COX-2 upregulation and PGE2 production in the cells of the bronchial epithelium and alveolar spaces of the lung[42]. Macrophages are known to produce different cytokines as both COX-2 and PGE2. Further investigation showed that cigarette smoke causes an increase in $CD68^+$ macrophages and also an increase in COX-2 expression in these cells. Intratracheally infusion of MSCs after seven weeks of exposure to cigarette smoke in rats lead to a decrease in $CD68^+$ macrophages and COX-2 expression in the macrophages as seen in *in vitro* examination of bronchial epithelium and alveolar spaces in the lung[42]. Next to the decrease of COX-2, MSCs also reduce PGE2 and IL-6 production and promote anti-inflammatory IL-10 production in macrophages. The underlying mechanism comprises the p38 MAPK and ERK pathway, which are both activated in CS-stimulated macrophages, but inhibited by exposure to MSCs. Jointly, these results show that MSCs reduce airway inflammation by downregulation of COX-2 and PGE2 synthesis via inhibition of the p38 MAPK and ERK pathways in alveolar macrophages[42]. So far, the underlying mechanisms of MSCs show a reduction of COPD in a mouse model for this disease.

In vivo testing of MSCs: animal models

MSC-treatment shows promising results in animal models for ARDS and COPD. However, it is extensively discussed which part of the ARDS/COPD pathology is adequately modelled by animal models and whether this is sufficient in making clinical translation possible. Different disadvantages and benefits can be identified concerning whether ARDS/COPD pathology is accurately mimicked and whether the use of these animal models in validating MSC-treatment for these diseases is adequate. The frequently raised question is whether animal models are relevant in this research.

Focussing on lung-related diseases as ARDS and COPD, no cure or treatment is available, which causes the need for an immediate solution from the patient's and the clinician's point of view. Regarding this, using animal models for identifying underlying mechanisms and validating stem cell treatment is time-extensive, and may thus impede the pace of research. On the other hand, adequate animal models make it possible to test the safety of MSC-treatment in living organisms. Next to this, animal models often have a shorter life span than humans. This makes it possible to examine the effect of MSC-treatment on the lifespan. Additionally, the use of human patients in validating a treatment is unethical in most cases, so animal models are sufficient because the treatment can still be examined in a living system. However, the Dutch state secretary for Economic Affairs submitted a letter to the National Committee on Animal Experimentation Policy in which he proposes the Netherlands to be the world leader in animal experimentation free innovations by 2025[43], which lowers the possible use of animal models and lowers chances for optimizing them. Jointly, these pros and cons have their influence on the use of animal models on modern research. The relevance of animal models is still doubted upon, since the discussion regarding the adequacy of these animal models makes using them for clinical translation difficult in itself: it is unknown which parts of ARDS/COPD pathology are adequately mimicked. Research nowadays is determined to find the most suitable animal model for mimicking ARDS/COPD pathology because only then it is possible to thoroughly examine the mechanisms and functions of treatment for diseases like ARDS an COPD.

Different rodent models are used in research to mimic ARDS pathology and to perform research on (table 1). LPS-induced mice are often used as model for sepsis-induced ARDS, but the ARDS pathology is not accurately modelled. When LPS is induced via tracheal instillation or inhalation, the alveolar epithelium is damaged and with local administration the inflammatory response is mimicked[44]. However, after LPS-induction, a quick recovery phase is started, which does not accurately mimic the human pathology of ARDS[45]. Therefore, research is devoted to improving existing animal models. An animal model for ARDS should display damage of the epithelial barrier, the associated alveoli, damage to the blood vessel endothelium and the remodelled lung all as a result of the inflammatory response. In rodents, parts of the pathology are mimicked in an LPS+oleic acid model[45]. Various dose combinations of LPS and oleic acid were administered to rats to be able to identify the most representative pathology of ARDS. LPS was given intratracheal, while oleic acid was administered intravenously. Epithelial and endothelial injuries were displayed after administration of LPS and oleic acid, but the recovery phase was noted 30 hours after injury, which is not an adequate representative of ARDS. To lengthen the course of LPS-induced lung injuries to display an improved pathology, LPS-induced mice pre-administered with corn oil were examined[46]. Addition of corn oil caused lung endothelial injury and lung inflammation to extend in time (17-20 days), mimicking sepsis-induced ARDS pathology in human disease in which

lung repair takes 14-28 days[46]. Since ARDS does not always have to be induced by sepsis, it is needed to generalize animal models for ARDS which mimic the complete pathology instead of different components. Unfortunately, a general model for ARDS is not identified yet, which makes research into MSC-treatment difficult.

Table 1. Overview rodent models used in research to mimic ARDS pathology. The different animal models used in research nowadays are listed, as are the ways of administrating substances to induce ARDS-related pathology, the pathology mimicked and the disadvantages of each model.

Animal models	Administration options	Pathology ARDS	Disadvantage
LPS[44,45]	Tracheal instillation/ inhalation, local	Damage alveolar epithelium, inflammatory response/fibrosis	Quick recovery phase
LPS+oleic acid[45]	LPS: intratracheal Oleic acid: intravenous	Epithelial and endothelial injuries	Recovery phase 30 hours after injury
LPS+pre-administration corn oil[46]	Intraperitoneal	Extended (17-20 days) endothelial injury and lung inflammation/fibrosis	Focussed on sepsis-induced ARDS only

Research devoted to the mechanisms of MSC-treatment for ARDS is in full swing despite the fact that a generalized, adequate animal model is lacking. The animal model for ARDS used to determine the action of MSC-treatment is still mainly the LPS-induced rodent model: other animal models have not been used extensively for making a bridge towards clinical translation of MSC-treatment. In an LPS-induced rat model, a therapy consisting of administration of MSCs in combination with preactivated and shape-changed platelets was given as potential treatment for ARDS-related lung injury. The MSCs and platelets were both autologous. The combination therapy decreased inflammatory cytokines, fibrosis, and apoptosis, while it increased anti-inflammatory cytokines significantly in relation to only MSC or only platelet therapy. The anti-inflammatory effects of MSC-treatment are due to induction of M2 macrophages by MSCs secreting soluble factors as PGE2 and IL-13[47]. This is shown in LPS-induced mice intravenously administrated with (human umbilical cord) MSCs or concentrated conditional medium. Since human umbilical cord MSCs are able to modulate the immune response and/or evade host rejection by having cell surface hyaluronan on the glycocalyx[48], these MSCs are not rejected in rodents. This shows a promising feature of using animal models: because umbilical cord MSCs can evade the host response, the outcome of these MSCs (acceptation in the body) is the same when administered to the mouse as it is to a human being. It was found that these MSCs improve survival, attenuate lung inflammation and regulate the immune response. Markers of M1 macrophages, TNF-α, IL-1β, and IL-6, were significantly downregulated as a result of MSC-treatment. Markers associated with M2 macrophages, IL-10, CCL17 and CCL22, were upregulated. So, macrophages are induced to become M2 macrophages in LPS-induced mice. Also, *in vitro* investigation of MSCs showed that MSCs secrete soluble factors that are responsible for anti-inflammatory effects. One of the secreted factors by MSCs is PGE2, which is responsible for an increase in the survival rate of LPS-induced mice and attenuation of lung-related inflammation[49]. Lung inflammation was also attenuated when administration of MSCs overexpressing TGF-β1 was performed in LPS-induced mice. An inhibition of lung fibrosis, an improvement of lung permeability and pulmonary histopathology, and a significant modulation of the differentiation of T cells into

Th17 cells and Tregs was seen, while inhibiting the Th17/Tregs ratio[50]. Together these results show that a lot of mechanisms and cells are involved during MSC-treatment of LPS-induced animals as a model for ARDS. Jointly, the additionalities to MSC treatment (combinational treatment with platelets, overexpression of TGF-beta1) but also just administering MSCs intravenously showed a decrease in lung fibrosis, inflammatory cytokines, M1 macrophages and an increase in survival rate, anti-inflammatory cytokines, M2 macrophages, Th17 cells and Tregs without disrupting the Th17/Tregs ratio. All these results in decreasing the pathology of ARDS were obtained by using LPS-induced rodent models. Despite the fact that a general animal model for ARDS showing all the pathology characteristics is missing, it can be stated that the use of an animal model that displays parts of pathology can be useful in determining a treatment strategy and its underlying mechanisms for the pathology parts present. MSC-treatment for targeting the inflammation and fibrosis parts of ARDS pathology sounds promising when examined in an animal model, but the question remains whether this is representative enough for translation in humans or whether it is just an additional step in MSC treatment research which slows down the research process.

Focussing on COPD, different rodent models are used in research to mimic (parts of) COPD pathology (table 2). The main rodent model used for mimicking COPD pathology is cigarette smoke-induced. Despite the fact that cigarette smoke is the most frequent cause of COPD, other causes of COPD are left behind in this model. Next to this, there is no standardized method for the use of cigarette smoke for animal models in research. Commercial cigarettes, research cigarettes, the constituents of smoke, the dose of smoke and administration via nose or whole body are important factors that could influence results[51]. Despite the lack of standardization, this rodent model shows pulmonary inflammation, emphysema and airway remodelling, which are nearly all essential problems that need to be induced in animal models for COPD. A good animal model for COPD should display a disruption of the lung epithelial barrier, production of pro-inflammatory factors by epithelial cells (consequently: inflammation) and small airway remodelling. LPS-induced animal models are sometimes used to induce COPD, but this is a short term model which displays only a few features of disease, mostly pulmonary inflammation[51]. Another animal model which displays a few characteristics of COPD concerns mice exposed to elastase, an enzyme released by neutrophils which causes disruption of alveolar tissue and causes emphysema[51]. Elastase is administered orally[52] or nasally[53], sometimes in combination with other substances as LPS[51,53]. Lately, a mouse model is identified that induces a lot of the characteristics linked to COPD. Mice exposed to cigarette smoke via the nose in combination with airway LPS inhalation showed chronic airway inflammation, but also an impairment of the lung function, an induction of emphysema and right ventricular dysfunction[54]. Since this model most closely resembles the full spectrum of the COPD pathology, it should therefore be used increasingly in research regarding COPD pathology and potential treatment identification..

Table 2. Overview rodent models used in research to mimic COPD pathology. The different animal models used in research nowadays are listed, as are the ways of administrating substances to induce ARDS-related pathology, the pathology mimicked and the disadvantages of each model.

Animal models	Administration options	Pathology COPD	Disadvantage
Cigarette smoke-induced[51]	Nasally or whole body	Pulmonary inflammation, emphysema, airway remodelling	Focussed on cigarette smoke-induced COPD only No standard method for the use of cigarette smoke
LPS[51]	Via instillation or chorionic exposure	Pulmonary inflammation	Short term model, not a comprehensive pathology
Elastase[51,53]	Orally or nasally	Disruption alveolar tissue, emphysema	Not a comprehensive pathology
Cigarette smoke + LPS[54]	Nasally (smoke) + inhalation (LPS)	Chronic airway inflammation, impairment lung function, emphysema, right ventricular dysfunction	Airway remodelling/fibrosis is not present

Research is becoming more devoted to determining the underlying mechanisms of potential MSC-treatment for COPD. Different animal models are used to investigate the function of MSC-treatment, since a general, adequate and complete COPD model is still missing. Despite this, research is already devoted to identifying an optimal dose of MSCs for treatment. A mouse model for emphysema was induced via an intravenous injection of porcine pancreatic elastase. With a dose of $5x10^4$ MSCs, therapeutic effects were seen in reducing emphysema[55]. To examine the effect of MSC-treatment on the full course of COPD, an acute study (4 days) and chronic study (20 weeks) were performed on intranasally LPS-induced mice. It was seen that MSC-treatment decreased pulmonary inflammation, but also decreased systemic inflammation as was seen because of the decreasing number of immune cells in acute COPD. However, chronic emphysema and chronic pulmonary inflammation were not affected by MSC-treatment[56,]. The mechanisms by which acute COPD is reduced is an important part of research. In order to identify the role of administrating MSCs on acute lung inflammation and pulmonary function, rats were exposed to cigarette smoke alone and intratracheally infused with MSCs. A reduction in the expression of pro-inflammatory cytokines as IL-1β, IL-6 and TNF-α was seen. In addition, an increased expression of TGF-β1 was seen in the lungs and anti-inflammatory effects of MSCs were confirmed by examining bronchoalveolar lavage fluid: administration of MSCs lead to a decrease in inflammatory cells. The infusion of MSCs also caused a reduction in airflow obstruction. This research showed that MSCs can reduce inflammation of lung tissue by reducing pro-inflammatory cytokines and inflammatory cells via TGF-β1 signaling[57]. The use of cigarette smoke-induced mice provided insight into the mechanisms of lung inflammation reduction and airflow obstruction and is therefore sufficient in gaining insight into MSC-treatment for parts of the COPD pathology. However, an important part of research has been neglected in animal models so far. Insight is gained into the soluble factors MSCs secrete or modulate, but not where the cells will be situated when transplanted. There is little space for MSCs to engraft in the lung: a niche is needed. The relevance of a gene system in MSC engraftment and lung repair was studied in LPS-induced rates exposed to cigarette smoke. The gene system comprises the pulmonary surfactant associated protein A (SPA) suicide gene system. The use of rAAV-SPA-TK (adeno-

associated virus-SPA-thymidine kinase) causes apoptosis of type 2 alveolar epithelial cells and clears the associated niche in the lung. Therefore, this vector could be used in COPD treatment to provide MSCs with an appropriate niche. However, a great limitation is the increase in collagen deposition in the lung caused by MSCs[58]. Jointly, all the findings show relevance and importance. It can be stated that the use of different animal models that display parts of pathology can be useful in determining an optimal dose for MSC-treatment and its underlying mechanisms for the pathology parts present. However, a general COPD model which shows the complete pathology would be helpful in MSC-treatment research. MSC-treatment for targeting the systemic and pulmonary inflammation and fibrosis parts of COPD pathology sounds promising when examined in animal models, but also here the questions remain whether this is representative enough for translation in patients or whether it is just an additional step in MSC-treatment research which slows down the research process.

All in all, it can be seen that different options for animal models for ARDS and COPD are available, but adequate and comprehensive models are limited since they only display a part of the clinical pathology of these diseases. Despite the lack of an optimized, extensive model for ARDS/COPD, research regarding MSC-treatment and its underlying mechanisms is rising. However, the question remains whether animal models are a good representative for human disease: clinical translation may be difficult.

MSCs: *in vitro* testing

The absence of comprehensive ARDS and COPD animal models has led research to develop new *in vitro* models in order to examine MSC-treatment and its underlying mechanisms. This development is fairly new and still in progress[59]. The limitation of former *in vitro* models is that the 3D-microenvironment could not be mimicked in culture. However, the developments in this field are rising: the use of a 3D system of an organ tackles the former problem of *in vitro* models. Next to this, also organs-on-a-chip are examined increasingly. Both organoids and organs-on-a-chip show features that may enable replacing animal models in research regarding MSC-treatment for ARDS and COPD.

In vivo research in ARDS and COPD patients and animal models for these diseases has shown that lung epithelium and associated alveoli get damaged. An effective repair of this tissue is required. It was already seen *in vivo* that MSCs secrete factors that induce anti-inflammatory macrophages, but also *in vitro* research shows this[60]: a miniaturized version of the lung was made, a lung organoid, to mimic the 3D structure of lung tissue. MSCs were added and increased formation of the organoid, mostly by secreting factors as TSP1, which is one of the substances that causes the differentiation of lung progenitor cells to alveolar cells. Further, it was shown that MSCs are stimulating the differentiation of epithelial cells into alveolar cells. Also, MSCs are involved in irreversible lung progenitor cell differentiation. Correlated is a decrease of self-renewal capacity. In this research, a lung organoid was used to provide more insight into the function of MSC-secreted factors and their action on lung progenitor cells to transform into alveolar cells[60]. Here, a ‘healthy’ lung organoid was used. Furthermore, lung organoids could be used to model disease and herein the effects of MSCs can be examined. Also, when a MSC-treatment is identified, organoids may function for drug-screening and could advance regenerative medicine as organoids made from tissue of the patient might be better engrafted that single MSCs[61].

Next to organoids, an *in vitro* lung-on-a-chip model and its potential role in lung disease-related research, mostly regarding COPD, has been examined. The origin of COPD is often smoking. A lung-on-a-chip model comprises a 3D environment of a breathing human lung on microscale. In this research, a small airway-on-a-chip containing human bronchiolar epithelium (from normal or COPD-patient lungs) is connected to an instrument which mimics human smoking behaviour. In this manner it can be seen whether there is an effect of smoke on human lung responses *in vitro*, without the difficulty of finding an adequate, representative animal model. Also, effects can be seen on different levels: genetic, molecular, cellular and tissue level[62]. Next to being able to mimic the dynamic conditions of COPD and investigating effects multi-levelly, mechanistic links between COPD and other diseases can be explored in a lung-on-a-chip model. An air chamber, porous membrane, liquid chamber and basement component were bundled on top of each other to mimic the structure of human terminal bronchi. Epithelial and endothelial cells were used in the chip. Exposure to cigarette smoke extract triggered inflammation and induced air-blood barrier malfunction. Also, E-cadherin expression was significantly decreased, which initiated epithelial-mesenchymal transition. On top of that, cell division was promoted when exposed to cigarette smoke extract, which shows a link with tumour-like transformation[63]. This *in vitro* model sounds promising, but the difficulty remains how this will be translated clinically: this may be even harder for *in vitro models* compared to *in vivo* animal models. Lung-on-a-chip models can be used for drug development and sensitivity and thus also for examining MSC-treatment, but since *in vitro* models are upcoming, this is not optimized and an accurate model for disease is still lacking.

Both organoids and lung-on-a-chip models may eventually be used for examining lung diseases and potential MSC-treatments. Since these *in vitro* models are still developing, the results on the long-term are pending. It is for example not known what MSC-treatment does in lung organoids or lung-on-a-chip models for ARDS or COPD. Adequate *in vitro* models for ARDS are not even there yet. However, an advantage of using *in vitro* models is the possibility to work with human cells, but also cells of patients, which brings research closer to clinical translation. Another advantage is that specific parts of tissue repair can be replicated. The next step however is difficult: how to translate this to clinical ARDS/COPD. So, there is still a long way to go for lung organoids and lung-on-a-chip models to be considered as adequate *in vitro* models for ARDS and COPD and eventually MSC-treatment.

Discussion

Respiratory diseases are a huge burden for society: it is one of the most common causes for severe illness globally and over one billion people suffer from one kind of respiratory disease. Focussing on ARDS and COPD, acute and chronic syndromes of respiratory disease respectively, no cure is identified. Both diseases show inflammation and disruption of the lung epithelium and blood-endothelial barriers as main pathology. These core problems should be tackled in a future treatment, which starts by accurately and comprehensively mimicking these problems in *in vivo* and *in vitro* models.

Currently, animal models used for mimicking ARDS and COPD pathology are only displaying parts of the whole pathology. A good animal model has to mirror all pathological characteristics of ARDS or COPD in order to eventually be able to test the functions and mechanisms of the desired treatment. However, that is where the main problem kicks in. The animal models used nowadays mainly exhibit partial symptoms and pathology of those diseases and should therefore be optimized. An animal model for ARDS should display damage of the epithelial barrier, the associated alveoli, damage to the blood vessel endothelium and the remodelled lung all as a result of the inflammatory response. On the other hand, a good animal model for COPD should display a disruption of the lung epithelial barrier, production of pro-inflammatory factors by epithelial cells (consequently: inflammation) and small airway remodelling. Current research is devoted to optimizing these animal models, which shows progress, but the need for an optimized general animal model is definitely present.

Accurate animal models are needed in order to examine the influence of MSC-treatment on the body and to eventually be able to translate this to the human body. It is necessary to validate the working mechanism of MSCs in living organisms, since this could influence the outcomes. Also, toxicological research should be performed on animal models in order to test the safety before the treatment is performed in humans. Research regarding MSC-treatment and its underlying mechanisms and safety is rising. It is important to keep in mind that MSC isolation procedures and cell culture procedures differ per laboratory: these procedures have an influence on eventual results. Besides the difference in MSC-culture procedures and the need for adequate animal models in order to examine MSC-treatment comprehensively, the difficulty that remains is clinical translation from *in vivo* animal models to ARDS/COPD patients. It is never certain whether an animal model completely translates towards patient pathology, but the gap is made smaller with accurate animal models. Jointly, it can be stated that animal models are needed in research regarding ARDS and COPD and their possible MSC-treatment, but need to be optimized further.

Accurate animal models are needed, but in the meantime new *in vitro* models are identified that could help in validating and researching mechanisms related to ARDS or COPD. Current possibilities include using lung organoids or the lung-on-a-chip model in order to investigate ARDS and COPD and the possibilities of MSC-treatment in these diseases. Both *in vitro* techniques show the possibility to constitute a disease model for ARDS and COPD, but could also be used for drug validation and toxicological assessments. Specifically the lung organoid may be promising for examining MSCs niches and the working mechanism of MSCs.

However, these *in vitro* models are not yet where they should be in order to test MSC-treatment on diseased organoids or lungs-on-a-chip: also *in vitro* optimalization is needed. COPD and ARDS organoids might sound promising since they exhibit the 3D structure of the lung, but since organoids are often made from progenitor cells only, lung-organoids often lack the presence of immune cells and vasculature. These components are important in mimicking the pathological response of the body to inflammation and could therefore not be missed when examining ARDS or COPD and possible MSC-treatment. Next to this, lung-on-a-chip models sound promising because of the simulation of the human lung and the ability to perform multi-level tests, but in lung disease the most developed model so far concerns COPD: for ARDS there is no lung-on-a-chip model constituted yet. Jointly, it can be stated that lung organoids and lung-on-a-chip models are promising, but need to be optimized further in order to use them extensively in research related to lung disease and possible MSC-treatment.

This review aimed to identify whether the use of animal models as step in research towards stem cell therapy for ARDS and COPD is an utopia, a need, or a support in the quest to develop therapies. To conclude, there is an absolute need for improving and optimizing animal models and *in vitro* models for ARDS and COPD. Animal models for ARDS and COPD should be more general and most of all completely and adequately representing all the core problems of disease. *In vitro* models for ARDS and COPD should also be optimized for making a disease model comprising the core problems in disease, which could eventually be used to make progress in drug validation and toxicological assessments. Both *in vivo* and *in vitro* models should also be clinically translated in order to help ARDS/COPD patients, and this translation is thought to be difficult: research should also be devoted to optimizing this. All in all: there is a need for optimizing the *in vivo* and *in vitro* models for ARDS and COPD in order to examine the underlying mechanisms of MSC-treatment for these diseases. Thus, animal models (and *in vitro* models) are a support in the quest to develop therapies and the first step needed now is optimization of these models.

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