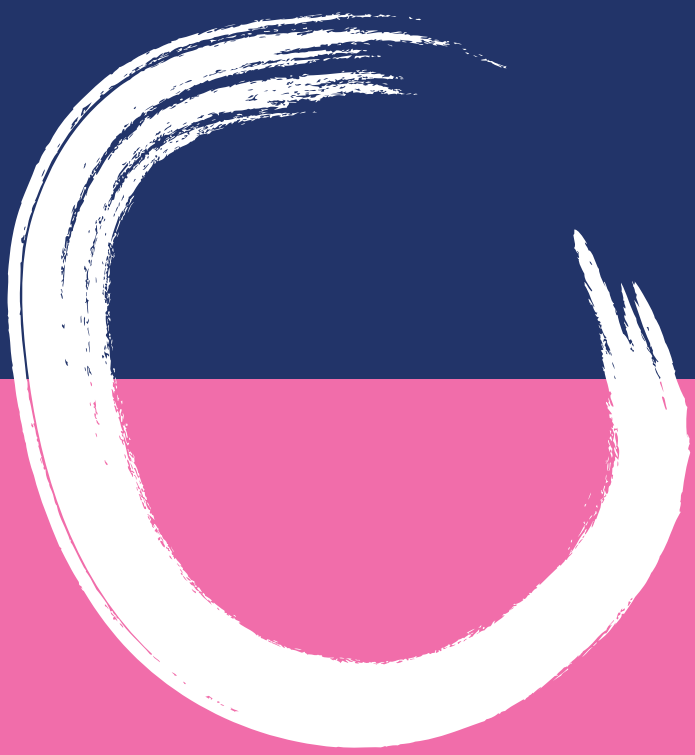
# MESENCHYMAL STROMAL CELL TREATMENT FOR COPD

*EXPERIMENTAL AND CLINICAL STUDIES*



WINIFRED BROEKMAN

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**WINIFRED BROEKMAN**

COLOPHON

**MESENCHYMAL STROMAL CELL TREATMENT FOR COPD**
*EXPERIMENTAL AND CLINICAL STUDIES*
Winifred Broekman
Thesis Leiden University Medical Center

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# MESENCHYMAL STROMAL CELL TREATMENT FOR COPD

*EXPERIMENTAL AND CLINICAL STUDIES*

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*"What if I've forgotten the most important thing?"*

HARUKI MURAKAMI – Norwegian Wood, 1987

Voor Els

**TABLE OF CONTENTS**

# GENERAL INTRODUCTION

*CHAPTER 1*

## DEFINITION AND CLINICAL PRESENTATION OF COPD

Chronic obstructive pulmonary disease (COPD) has been defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as "a common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases" [1]. Exposure to cigarette smoke is the major risk factor for development of COPD. COPD affects over 200 million people worldwide and it has remained listed the third major killer over the past decade, as reported by the WHO in 2012 [2]. Its prevalence and disease burden are expected to increase the coming decades due to continued exposure and aging of the world's population [1].

The major denominators of COPD are *i.* chronic bronchitis, i.e. the presence of a productive cough for most days over 3 months during 2 consecutive years; *ii.* remodelling and narrowing of small-airways; and *iii.* emphysema, i.e. enlarged airspaces distal to terminal bronchioles due to alveolar wall destruction. The presence of these features varies substantially among patients, which helps to explain the clinical heterogeneity of COPD. The diagnosis should be considered in patients presenting with dyspnea, chronic cough and/or sputum production who have a history of exposure to risk factors for COPD. To establish the diagnosis, a spirometric measurement showing a low ratio of forced expiratory volume in 1 second ($FEV_1$) to forced vital capacity (FVC) following inhalation of bronchodilating medication is required. A ratio below 0.70 confirms COPD, irrespective of patient age [1].

The primary goal of COPD treatment is to reduce symptoms and the risk of future events. Smoking cessation is the cornerstone of COPD treatment, in combination with pharmacologic therapy to reduce airway obstruction and exacerbations and regular physical activity or rehabilitation. Some patients with severe disease fulfil criteria for surgical interventions, such as lung volume reduction surgery or lung transplantation [1,3]. However, treatments that halt COPD progression or even cure the disease are not available. Moreover, it is unclear whether and how repair can be induced in order to restore lung architecture. As a potential repair treatment for COPD, cell therapy using mesenchymal stromal cells (MSCs) has generated interest based on promising results *in vivo* [4-6]. This thesis will focus on the potential of MSC-based cell therapy to induce repair in the lung, both *in vitro* and in human tissue, in the context of COPD. In this light, the introduction will address normal lung biology and physiological responses to airway epithelial damage, followed by the pathobiology of COPD and an overview on regenerative strategies.

## HUMAN LUNG DEVELOPMENT AND THE RESPIRATORY EPITHELIUM

Human lung development starts when the ventral foregut endoderm is formed from buds through a complex interplay between endoderm and mesoderm (embryonic stage, week 4). Subsequent branching and differentiation of epithelial tubes allows expansion of the respiratory tree (pseudoglandular stage, week 5-17), and angiogenesis and vascularization (canalicular stage, week 16-25). Once vascularization is established, terminal bronchioles are developed into respiratory bronchioles and alveolar ducts, and airway epithelial cells start to differentiate. This is followed by thinning of the interstitium, due to mesenchymal cell differentiation and apoptosis, and differentiation of alveolar epithelial cells (AECs) towards type I and type II AECs (terminal saccular stage, week 24-late fetal period). Meanwhile, capillary and lymphatic networks become well developed. Expansion of the surface area for gas exchange finalizes lung development (alveolar stage) and this process extends into childhood [7].

After birth, the lung is continuously challenged by air-borne insults such as particulates, pathogenic microorganisms and gaseous pollutants. Protection is provided by the epithelial lining that covers the airway and alveolar surface, constituting over 40 differentiated cell types. Within the airways, the predominating cell types are basal cells (BCs) that act as progenitor cells, ciliated cells, secretory cells (including goblet, serous and club cells), neuroendocrine cells and less well categorized intermediate cells. Their composition within the epithelial lining changes along the proximal to distal axis in order to meet local functional needs: in the proximal airways ciliated cells predominate, consisting mainly of pseudostratified ciliated columnar cells, mucus-secreting goblet cells and BCs. Towards the smaller airways these are gradually replaced by club cells, that contribute to airway clearance and reduction of surface tension, until a simple cuboidal epithelium largely lacking BCs remains in the respiratory bronchioles. At this level alveoli start to appear that compose the functional units of the respiratory system. Two types of AECs are discerned: type I AECs that facilitate gas exchange; and type II AECs that produce surfactant phospholipids and proteins to regulate alveolar surface tension [8,9].

## AIRWAY EPITHELIAL INJURY AND REPAIR

The airway epithelium protects the lung via barrier formation through cell-cell junctional complexes at the epithelial cells' apical side, mucus production and clearance, production of protective molecules (e.g. antioxidants, antiproteases, defensins, antimicrobial peptides and immune-regulating soluble factors) and plasticity of epithelial cells in response to changing environmental conditions [10]. In spite of these protective properties, epithelial barrier integrity can get disrupted. The degree of injury to the airway epithelium can be categorized into four

stages: reversible injury, in which healing occurs upon removal of the irritant and cell homeostasis is retained; exfoliation of individual cells without significant damage to non-ciliated and BCs; desquamation of a group of cells but with preservation of the basal cell layer; and finally desquamation of cells including loss of the basal cell layer [11].

The initial response to injury is grossly similar throughout the different regions of the lung and involves spreading and migration of neighbouring epithelial cells to cover the denuded area to re-establish an intact barrier. Mild injury is restored by epithelial hyperplasia and epidermoid metaplasia, followed by differentiation. In more severe injury, fibroblasts proliferate and differentiate into myofibroblasts to provide a temporary protective barrier and to support the expanding epithelial surface. Differentiation of progenitor cells subsequently restores epithelial function [12]. Although regional differences regarding cells responsible for maintenance and repair are not fully elucidated, it is a generally accepted view that the primary progenitor cell of the bronchial epithelium is the basal cell [13], whereas at the alveolar level type II AECs are considered progenitors for the recovery of both type I and type II AECs [14]. During resolution, hyperproliferation is reduced through apoptosis and the temporary supportive matrix is remodelled. It may take several more weeks for the establishment of ciliogenesis and complete regeneration of the pseudostratified mucociliary epithelium [12]. These dynamic changes of epithelial cell phenotypes during repair highlight the high plasticity of the airway epithelium, and show its ability to adapt to changing environmental conditions and to tolerate chronic stress.

## AIRWAY EPITHELIAL INJURY IN COPD

The dynamic process of wound repair may be affected by interruptions during the repair process, exaggerated responses to chronic stimuli or even aberrant responses to naive stimuli. This may lead to pathological remodelling of the airway epithelium and eventually to compromised pulmonary organ function [15]. Such aberrant repair responses are thought to underline the characteristic changes in the airway epithelium that are observed in patients with COPD and also frequently in smokers with a normal lung function, and include hyperplasia, particularly of basal and mucous cells, proliferation of mucous cells, metaplasia of squamous cells in small airways and submucosal gland hypertrophy. Besides, junctional barrier integrity is suppressed and differentiation of ciliated and nonmucous secretory cells is affected [16]. With progressive disease, fibrosis and hypertrophy of smooth muscle leads to narrowing of the small airways [17], which is implied to precede destruction of the alveolar structure [18]. At the alveolar unit itself, increased apoptosis, oxidative stress and excess proteases contribute to destruction of alveoli [19].

The most important risk factor for COPD development is exposure to cigarette smoke (CS). CS contains over 4700 chemical compounds, including high concentrations of free oxygen radicals [20]. Exposure to CS has direct negative effects on the integrity of the airway epithelium and its secretion of protective factors, besides evoking a physiological inflammatory response [21]. This response is usually self-limiting and does not appear to result in extensive airway epithelial injury in non-susceptible individuals. However, in individuals susceptible to COPD development, constituting about 1 in 5 smokers, the inflammatory response is enhanced and persists despite smoking cessation. Susceptibility factors for COPD are still being unravelled and likely include genetic and epigenetic factors, altered immune regulation, infections and abnormal repair mechanisms [19]. Apart from these, a more recent hypothesis on the pathogenesis of COPD focuses on the role of BCs. The theory that changes in BCs and their progeny contribute to airway epithelial remodelling in COPD is supported by observations that BCs contain the progenitor population that maintains a normally differentiated airway epithelium and that hyperplasia of the BC population and disordered BC differentiation are one of the earliest lesions observed in the airway epithelium in COPD (reviewed in [13,22]).

The enhanced inflammatory response following CS exposure encompasses increased secretion of cytokines, chemokines and other proinflammatory mediators by airway epithelial cells. This leads to recruitment of inflammatory cells, such as neutrophils and macrophages. The attraction and activation of dendritic cells to the site of inflammation links innate to adapted immune responses, predominantly increasing the numbers of $CD8^+$ cytotoxic T-cells in the airways and alveolar compartment, which secrete proteolytic enzymes that cause cell death of structural cells. Furthermore, the adaptive immune response includes Th1 $CD4^+$ T-cell responses that promote accumulation of inflammatory cells to the lung and contribute to $CD8^+$ T-cell activation; Th17 $CD4^+$ T-cell responses that regulate chronic tissue inflammation; and accumulation of T-regulatory cells and B-cells in the lung. In advanced stages of COPD, it has been suggested that autoimmune responses contribute to the perpetuation of inflammation via Th17 $CD4^+$ T-cell responses as well as formation of lymphoid follicles containing clusters of B-cells [21].

The inflammatory response is associated with an increase of proteolytic enzymes, an imbalance between oxidants and anti-oxidants and excess amounts of reactive oxygen species (ROS). Together, this further amplifies the inflammatory response, causes breakdown of the extracellular matrix, induction of mucus production and impairment of mucociliary clearance and leads to increased apoptosis of alveolar and endothelial cells. Insufficient phagocytosis of apoptotic cells results in secondary necrosis, which further increases the amounts of proinflammatory mediators that attract neutrophils and monocytes, thus augmenting inflammation. Moreover, inflammation is perpetuated by local tissue hypoxia and epigenetic changes caused by ROS, as well as by increased susceptibility and occurrence of respiratory tract infections due to impaired innate

immunity [19,21]. In addition, age-related changes in tissue maintenance and development of autoimmunity in advanced stages of the disease compromise tissue repair [23,24].

The sequel of events results in airway obstruction and tissue destruction, characteristic of the clinical phenotype of COPD. In order to restore tissue homeostasis, research has focused on manipulating endogenous repair and developing regenerative strategies, such as cell therapy or engineering of lung *ex vivo*.

## REGENERATIVE STRATEGIES FOR COPD

*Induction of endogenous repair*

Signaling pathways that drive airway epithelial development and maintenance as well as contribute to disease pathogenesis upon dysregulation are potential targets for therapies that manipulate endogenous epithelial repair. These include pathways involved in stem cell self-renewal and differentiation as well as in lung repair (e.g. Wnt, hedgehog, Notch, Retinoic Acid); molecules involved in epithelial and mesenchymal cell cross talk, particularly at repair niches (e.g. thrombospondin-1/bone morphogenetic protein); histone acetylation and deacetylation processes; and noncoding RNAs (reviewed in [25-27]). In addition to these targets, repair could be induced or enhanced following implantation of inductive extracellular matrix scaffolds at damaged areas, as these may attract local and circulating progenitor cells that have the potential to induce local repair or restore tissue structure [28]. Thus far, no new therapies or clinical trials have emerged from the identification of these elementary signaling pathways, apart from trials investigating the effect of retinoic acid on emphysematous lung, which did not appear to induce repair [29]. This lack of translation is related to observations that link altered signaling of these pathways to proliferative diseases, such as cancer and fibrosis [30].

*Cell therapy*

Cell therapy is defined as the administration of a product intending to provide effector cells to treat a disease or in support of other therapy [31]. A well-known example is transplantation of hematopoietic stem cells in hematologic disorders, but the repertoire of cell therapy is expanding and includes administration of specific stem cell populations or effector cells, and induction and reprogramming of mature cells. The functionality of cell therapy is likely related to secretion of factors that impact the recipient's own (stem) cells, or may involve fusion of a donor cell with a recipient cell [32]. Besides, transdifferentiation and engraftment of exogenous cells has been observed [33,34]. Due to controversy and ethical issues surrounding the use of embryonic stem cells, most experience has been gained with adult cells, particularly those derived from the bone marrow. Apart from hematopoietic stem cells, the bone marrow contains a second

non-hematopoietic population of stromal cells, called mesenchymal stromal cells (MSCs), which have stem cell properties including the potential to self-renew and differentiate. MSCs exert regenerative as well as immunomodulatory properties and were shown to benefit tissue homeostasis [35]. A clinical trial performed in patients with moderate to severe COPD showed that MSC administration is safe but does not affect clinical outcome parameters [36]. It is however not yet investigated whether treatment with MSCs also has beneficial effects on human emphysematous lung tissue, as has been observed in several studies in rodents [4-6]. Given the topic of this thesis, an elaborate account on the therapeutic potential of MSC-based cell therapy in COPD is provided in chapter 6.

*Ex vivo bioengineering of the lung*

Bioengineering refers to the process of creating a functional, autologous organ that accurately mimics the properties of the native organ and is able to support the recipient's cells [28,37]. Creating a bioengineered lung is unarguably challenging, given the complex structure of the lung with its changing biomechanical properties and function, and its heterogeneous cell populations. Nevertheless, significant progress has been made in this field over the past years, resulting in the implantation of a 'breathing' engineered lung in rat. This was established by decellularization of a whole lung, followed by recellularization with lung and vasculature specific cells and by culturing in a bioreactor where chemical and mechanical properties mimicked the lung tissue environment. The engineered rat lung contributed to gas exchange when reimplanted in (syngeneic) rats, but its lifespan was short due to intravascular coagulation and defects in barrier function which resulted in lung failure [38,39]. Nevertheless, this result holds promise for the future of lung organ engineering. The realization of an engineered, functional human lung has the potency to replace its native counterpart, addressing the need for donor lungs for transplantation.

## OUTLINE OF THE THESIS

The topic of this thesis is airway epithelial injury and the potential of repair by mesenchymal stromal cells, in the context of the syndrome of COPD. The main objectives of this thesis are to investigate: 1) the effects of cigarette smoke and mesenchymal stromal cell-secreted factors on airway epithelial wound repair and 2) mesenchymal stromal cell characteristics and function in COPD patients, including clinical effects of MSCs in patients with severe emphysema.

This thesis starts with *in vitro* studies on airway epithelial wound repair. In **Chapter 2** we investigated airway epithelial wound closure and innate immunity in primary bronchial epithelial cell cultures and its modulation by exposure to whole cigarette smoke. In **Chapter 3** the ability of MSC-secreted factors to induce airway epithelial wound closure was investigated.

The next chapters focus on the clinical application of MSCs from COPD patients. **Chapter 4** addresses the question whether MSCs from COPD patients are phenotypically and functionally comparable to MSCs from healthy controls. **Chapter 5** describes the results from a clinical trial in which we administered MSCs to patients with severe emphysema, which includes data obtained from lung tissue analysis following MSC administration. Finally, **Chapter 6** provides an overview of the (pre)clinical studies that investigated the efficacy of MSCs as a new treatment for COPD.

The results from the *in vitro* studies and clinical trial are discussed and put into a broader perspective in **Chapter 7**. We focus on models to investigate airway epithelial injury, optimization of MSC-based treatments and alternative strategies to target tissue repair in the lung. A general conclusion and suggestions for future studies on novel COPD therapies is provided.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AEC | alveolar epithelial cell |
| BC | basal cell |
| CD | cluster of differentiation |
| COPD | chronic obstructive pulmonary disease |
| CS | cigarette smoke |
| $FEV_1$ | forced expiratory volume in 1 second |
| FVC | forced vital capacity |
| GOLD | global initiative for chronic obstructive lung disease |
| MSC | mesenchymal stromal cell |
| ROS | reactive oxygen species |

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# CIGARETTE SMOKE MODULATES REPAIR AND INNATE IMMUNITY FOLLOWING INJURY TO AIRWAY EPITHELIAL CELLS

*CHAPTER 2*

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[1] both authors contributed equally to this work

**ABSTRACT**

Cigarette smoking is the main risk factor associated with chronic obstructive pulmonary disease (COPD), and contributes to COPD development and progression by causing epithelial injury and inflammation. Whereas it is known that cigarette smoke (CS) may affect the innate immune function of airway epithelial cells and epithelial repair, this has so far not been explored in an integrated design using mucociliary differentiated airway epithelial cells.

In this study, we examined the effect of whole CS exposure on wound repair and the innate immune activity of mucociliary differentiated primary bronchial epithelial cells, upon injury induced by disruption of epithelial barrier integrity or by mechanical wounding.

Upon mechanical injury, CS caused a delayed recovery in the epithelial barrier integrity and wound closure. Furthermore, CS enhanced innate immune responses, as demonstrated by increased expression of the antimicrobial protein RNase 7. These differential effects on epithelial repair and innate immunity were both mediated by CS-induced oxidative stress.

Overall, our findings demonstrate modulation of wound repair and innate immune responses of injured airway epithelial cells that may contribute to COPD development and progression.

## INTRODUCTION



Smoking has been shown to increase epithelial inflammation and injury, and has been suggested to disrupt the host defense function of the airway epithelium [1,2]. These effects may be highly relevant for our understanding of the development of smoking-induced lung diseases [3], including chronic obstructive pulmonary disease (COPD), an inflammatory lung disorder that is characterized by a progressive and irreversible obstruction of airflow [4]. Changes in the airway epithelium resulting from exposure to smoke are early and key events in the development and progression of COPD [5,6]. Airway epithelial cells, which line the surface of the respiratory tract, normally function as the first host defense barrier against respiratory pathogens [2]. However, extensive epithelial injury, for instance caused by cigarette smoking, respiratory pathogens and inflammation, may lead to disruption of the epithelial barrier integrity and cell death [7-9]. Upon injury, a rapid wound repair process is initiated during which airway epithelial cells produce innate immune mediators to enhance host defenses at the wounded area [10]. These repair responses are essential for restoration of the barrier function of the epithelium, and subsequent regeneration of a pseudostratified layer of epithelial cells. However, the repair process might be altered directly by CS exposure or indirectly by CS-induced inflammation, and this modulation of repair might contribute to COPD development and progression by promoting epithelial remodeling and persistent airway inflammation.

The direct effects of CS on wound repair of airway epithelial cells have been primarily studied by applying an aqueous extract of CS on undifferentiated submerged cultures of airway or alveolar epithelial cell lines or primary airway epithelial cells [9,11,12]. However, to gain more insight in the effect of smoking on airway epithelial repair, further research is required using conditions that better reflect the local conditions in lungs of smokers. Air-liquid interface cultures of mucociliary differentiated primary bronchial epithelial (ALI-PBEC) represent a widely accepted model to investigate airway epithelial cell functioning in lung diseases [2,5]. These cultures are highly similar to the airway epithelium of the small and large conducting airways, and display a pseudostratified morphology including ciliated, secretory, intermediate columnar and basal cells (BCs) [13,14]. We have used exposure of ALI-PBEC cultures to whole CS to better mimic smoke exposure *in vivo* [7,15]. Using this model, we have previously shown epithelial injury and transient disruption of the epithelial barrier integrity upon acute exposure to whole CS [7]. This response was accompanied by epidermal growth factor receptor (EGFR)-mediated expression of innate immune mediators, including expression of the neutrophil chemoattractant C-X-C Ligand 8 (CXCL8, or IL-8) and selective expression of the antimicrobial protein Ribonuclease 7 (RNase 7) by BCs present in ALI-PBEC cultures. These findings provided important evidence for a dual function of airway BCs in epithelial repair and innate immunity that requires further investigation [16]. Especially, the influence of CS on the dual function of airway epithelial BCs in mediating

wound repair and innate immunity is of interest in view of the development of smoking related diseases such as COPD.

In the present study we examined the effect of epithelial exposure to whole CS on repair and induction of innate immune responses by wounded ALI-PBEC cultures. Epithelial injury was induced by disrupting the epithelial barrier integrity, via disruption of cell junctions or via mechanical wounding of epithelial layers. EGFR-induced innate immune responses were examined by determining the expression of the BC-specific mediator RNase 7 and the luminal airway epithelial cell- and BC-expressed chemokine IL-8. In addition, the role of BCs in wound repair after mechanical injury was determined by assessment of the number of BCs at the wound edge. Moreover, we studied the contribution of oxidative stress and EGFR signal transduction to wound repair and innate immune responses.

## METHODS

*ALI-PBEC and whole cigarette smoke exposure model*

Primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal lung tissue obtained from patients undergoing resection surgery for lung cancer at the Leiden University Medical Center. Use of such lung tissue that became available for research within the framework of patient care was in line with the "Human Tissue and Medical Research: Code of conduct for responsible use" (2011) (www.federa.org), that describes the no-objection system for coded anonymous further use of such tissue. PBEC were cultured and differentiated at the air-liquid interface as previously described [7,17]. In short, cells at passage 2 were seeded at a density of 40.000 cells/0.9 $cm^2$ on 0.4 µm pore sized semi-permeable transwell membranes (Corning Costar, Cambridge, MA, USA) that were coated with a mixture of 30 µg/ml PureCol (Advanced BioMatrix, San Diego, CA, USA), 10 µg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/ml fibronectin (isolated from human plasma) in PBS. Cells were cultured in bronchial epithelial growth medium (BEGM) (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Bleiswijk, The Netherlands) (1:1 mixture) containing 1 mM Hepes (Lonza) and supplemented with SingleQuot supplements and growth factors according to the manufacturer's instructions (bovine pituitary extract [BPE], hydrocortisone, human epidermal growth factor [hEGF], epinephrine, transferrin, insulin, T3 and retinoic acid; all from Lonza), and additional 15 ng/ml retinoic acid (Sigma-Aldrich), 1 mg/ml BSA (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/ml streptomycin (Lonza). Cells were first cultured in submerged conditions until confluence, followed by air-exposed culturing during 2-3 weeks to allow mucociliary differentiation. Exposure of ALI-PBEC to whole cigarette smoke (CS) was done according to a previously described model [7]. In this model, ALI-PBEC were placed in exposure chambers and

exposed to air (control) or whole cigarette smoke (CS) derived from one cigarette (3R4F reference cigarettes, University of Kentucky, Lexington, KY, USA) during a period of 15 minutes. Following exposure, the culture medium was refreshed.



*Calcium switch assay*

The effect of disruption of the epithelial barrier integrity was examined using the calcium switch assay [18]. In brief, ALI-PBEC were incubated for 15-30 minutes with 700 µL of calcium-free minimum essential medium (Gibco) that was added at the apical side and 1000 µL in the basolateral compartment to deprive cells of calcium to disrupt cell-cell contacts. Epithelial barrier disruption was determined by measuring the transepithelial electrical resistance (TEER) using MilliCell-ERS (Millipore, Bedford, MA, USA). After a complete loss of the barrier integrity, the apical medium was removed and cells were exposed to air or CS. Following exposure, the basal medium was replaced with calcium-containing culture medium with growth factors to allow reformation of junctions.

*Wound healing assay*

Wound healing assays were performed according to a previously described protocol [9], adapted for use in ALI-PBEC. In brief, the apical side of ALI-PBEC cultures was washed with PBS, and cells were starved for growth factors overnight in starvation medium (supplemented BEGM:DMEM without BPE and hEGF). 500 µL PBS was added to the apical surface of ALI-PBEC to facilitate mechanical injury, which was induced by scraping the cell layer with a sterile Pasteur pipette with a soft tip, creating a wound with a diameter of 3 mm. After wounding, the apical surface of the cultures was washed with 200 µL PBS to remove cellular debris.

In designated experiments, 10 mM of N-acetylcysteine (NAC) (Sigma Aldrich) was used to determine the role of oxidative stress. To investigate EGFR and ERK signaling, cells were incubated with AG1478 (EGFR tyrosine kinase inhibitor) or U0126 (MEK1/2 inhibitor) (both Calbiochem, Darmstadt, Germany). After wounding, ALI-PBEC cultures were exposed to whole CS, and culture medium was replaced by fresh starvation medium, including additional inhibitors as indicated. For live imaging experiments, images of wounded ALI-PBEC were acquired using a Leica DM16000 phase-contrast light microscope (Leica Microsystems, Wetzlar, Germany), collecting digital images of the wound every 15 minutes up to 48 hours. During this period, cells were placed inside a micro cell incubator at 37°C in a 7.5% $CO_2$ humidified atmosphere. The acquired images were used to create a time-lapse movie. For other wound healing experiments, digital images were collected on a digital camera connected to an inverted phase-contrast light microscope using Cell Sense Entry imaging software (both Olympus, Tokyo, Japan), at time 0, 6, 24 and 48 h after wounding. The surface of the wound area was measured using Photoshop CS6 (Adobe, San Jose, California, USA) in order to assess remaining wound size and wound closure rates.

*Immunofluorescence confocal imaging*

Immunofluorescence staining of wounded ALI-PBEC was conducted as previously described [7]. Cells were stained with a monoclonal anti-rabbit p63 antibody (ab124762, Abcam, Cambridge, UK) (1:100) to detect BCs, and DAPI to stain all nuclei. Z-stack images of the wound edge were made using a Leica TCS SP5 confocal inverted microscope (Leica Microsystems, Wetzlar, Germany) and processed using the Leica Application Suite Advanced Fluorescence software (LAS AF; Leica Microsystems). Five random images of air and CS-exposed wounded ALI-PBEC were used from independent donors to determine the number of $p63^+$ cells. The percentage of $p63^+$ nuclei was determined at the leading wound edge and at a randomly selected unwounded area. Moreover, the average number of $p63^+$ cells at the wound edge was calculated per 400 µm, and the internuclear distance between a $p63^+$ cell at the leading wound edge and its first adjacent $p63^+$ cell was quantified in approximately 20-30 nuclei per image. Further explanation of this method is provided in Supplementary Figure S1.

*Quantitative real-time PCR*

RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR) was conducted as described previously [7]. mRNA expression was examined for the genes described in Table S1. Relative gene expression compared to reference genes *ATP5B* and *RPL13A* was calculated according to the standard curve method. Reference genes were selected using the "Genorm" software [19].

*Western blot*

Western blot analysis of EGFR and ERK1/2 phosphorylation was done as previously described [7]. The following primary antibodies were used: rabbit monoclonal Ab EGFR #D38B1 (1:1000), rabbit polyclonal phospho-EGFR #2234, rabbit polyclonal ERK1/2 #9102 (1:1000), and rabbit polyclonal phospho-ERK1/2 #9101 (all Cell Signalling, Leiden, The Netherlands). Protein bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

*ELISA*

Secretion of IL-8 (R&D, Minneapolis, MN, USA) was determined according to the manufacturer's protocol.

*Statistics*

Data were analyzed using GraphPad Prism 6.0 (GraphPad Inc., La Jolla, CA, USA). Statistical tests used for data analysis were 2-way ANOVA, with post-hoc Bonferroni correction for multiple analyses. Differences with a p-value $< 0.05$ were considered as statistically significant.

## RESULTS

*Cigarette smoke delays barrier recovery and enhances innate immune responses*

We first used a calcium switch assay to determine the effect of CS on recovery of the airway epithelial barrier, and to explore the importance of the loss of barrier integrity for the induction of RNase 7 and IL-8. In this assay, calcium-depleted culture medium was applied at the apical surface and basal compartment of ALI-PBEC, resulting in a complete impairment of the airway epithelial barrier integrity as determined by measuring the transepithelial electrical resistance (TEER). Subsequently, cells were exposed to CS or air as negative control. The effect of CS on barrier recovery was determined at different time points, and induction of innate immune responses was assessed at 24 h. Both air- and CS-exposed ALI-PBEC displayed complete recovery of the airway epithelial barrier integrity 24 h after barrier disruption (Figure 1A). However, CS exposure caused a delay in this recovery at 6 h after exposure, which was significantly different compared to air-exposed cells. Assessment of RNase 7 mRNA expression demonstrated a significantly higher expression in CS-exposed ALI-PBEC incubated with calcium-depleted medium (Figure 1B). In control cultures, we did not detect CS-induced expression of RNase 7 at 24 h; in a previous study we also no longer detected CS-induced expression of RNase 7 at this time point [7]. Similar to RNase 7, we observed enhanced secretion of IL-8 in the basal medium upon barrier disruption and CS exposure (Figure 1C). Overall, these findings suggest that CS delays restoration of epithelial barrier function following calcium deprivation, while further increasing innate immune responses.

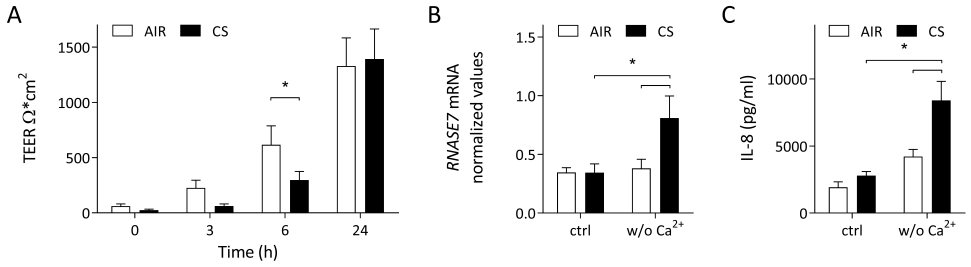


**Figure 1. Effects of CS on airway epithelial barrier recovery and innate immunity.** Barrier integrity in ALI-PBEC was disrupted using calcium depletion, and cells were subsequently exposed to air or CS. (A) The transepithelial electrical resistance (TEER) was subsequently measured at 0, 3, 6, and 24 h after exposure to assess loss and recovery of barrier integrity in air- and CS-exposed cultures. TEER values in ohm (Ω). n = 7 independent donors. (B) At 24 h, mRNA expression of *RNASE7* was assessed in ALI-PBEC that were incubated with calcium-depleted medium (w/o $Ca^{2+}$) versus control medium (ctrl), and subsequently exposed to either air or CS and further incubated in calcium containing medium. Normalized mRNA expression compared to *RPL13A* and *ATP5B* is depicted in the graph. n = 4 independent donors. (C) Secretion of IL-8 in the basal culture medium was assessed by ELISA. n = 5 independent donors. Data are shown as mean; error bars represent SEM; experiments were conducted using duplicate exposures in all donors, * $p < 0.05$.

*Cigarette smoke delays repair and further increases RNase 7 expression*

To further examine epithelial repair and induction of innate immune responses in wounded airway epithelial cells, we used a wound healing model in which ALI-PBEC cultures were mechanically injured by applying circular wounds. In this model, ALI-PBEC displayed intrinsic wound healing, and full wound closure was observed within approximately 48 h, as measured by live imaging (Figure S2). Whole CS exposure directly following epithelial injury impaired wound healing of ALI-PBEC during the first 24 h after exposure (Figure 2A and B), with significantly decreased wound closure rates during the first 6 h after CS exposure, but not at later time intervals (Figure 2C). Live imaging experiments further demonstrated impaired wound repair at early time points, with recovery of wound closure rates approximately 6 h after CS exposure (Figure 2D).

Next, we determined the effect of CS exposure following mechanical injury on mRNA expression of RNase 7 and protein secretion of IL-8. Comparison between intact and wounded cultures demonstrated significantly higher CS-induced mRNA expression of RNase 7 in wounded ALI-PBEC at 6 h after exposure (Figure 2E). In contrast, we did not observe such an effect on IL-8 protein secretion (Figure 2F). Taken together, these findings further demonstrate impairment of epithelial repair upon CS exposure, which was accompanied by induction of RNase 7, but not of IL-8.

*$P63^+$ cells at the wound edge are increased in CS-exposed ALI-PBEC*

Previously, we reported cell-type specific expression of RNase 7 by BCs in response to CS, whereas expression of IL-8 was observed in both luminal cells and BCs [7]. The selective increase in RNase 7 expression in CS-exposed wounded cells suggests that CS in particular affects the activity of BCs in wounded ALI-PBEC. Therefore, we next examined the contribution of BCs to wound repair of ALI-PBEC. This was determined by assessing the number of cells at the wound edge that stained positive for the nuclear BC-marker p63 (Figure 3A and Figure S1). The majority of cells (approximately 80%) directly located at the wound edge stained $p63^+$, which was a significantly higher proportion compared to intact areas of the same culture (appr. 35%) (Figure 3B). In CS-exposed cultures, $p63^+$ cells appeared to accumulate in higher numbers at the wound edge (Figure 3C). Moreover, we observed in CS-exposed cells significantly smaller internuclear distances between $p63^+$ cells located at the leading wound edge and located directly adjacent to the wound edge (Figure 3D and Figure S1 for explanation of the method). These observations suggest that CS impairs spreading and migration of BCs, a process that is important especially in the initial phase of wound repair. Collectively, these findings suggest that CS not only affects the innate immune function of BCs during repair, as shown by increased expression of RNase 7, but also affects the wound repair activity.

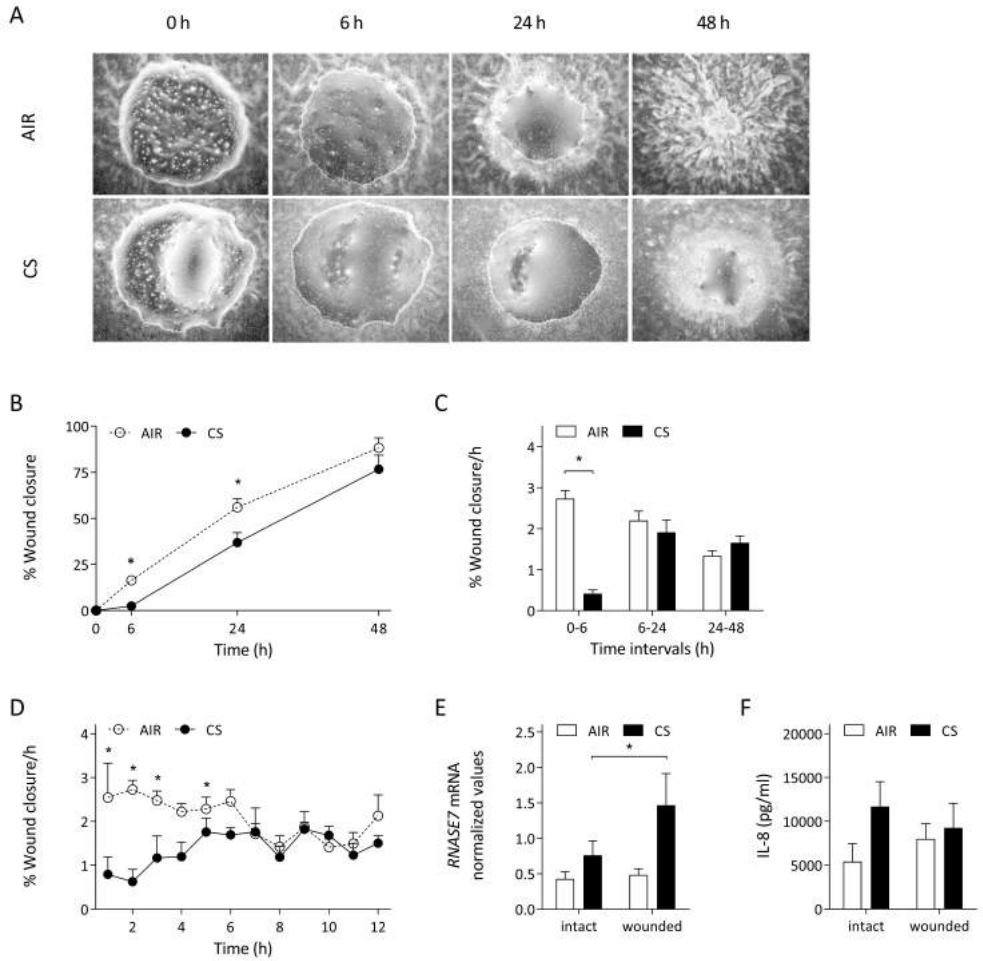


**Figure 2. Effect of CS on airway epithelial wound healing and innate immunity.** ALI-PBEC were mechanically injured and subsequently exposed to air (control) or whole cigarette smoke (CS). (A) Phase-contrast light microscopy images were made of air- and CS-exposed ALI-PBEC at 0, 6, 24 and 48 h after exposure. (B) Wound closure is shown in percentage in air- versus CS-exposed cells and (C) wound closure rate in percentage per hour at different time intervals was calculated. n = 8 independent donors. (D) Wound closure rates per hour in air- and CS-exposed ALI-PBEC up to 12 h after exposure were determined using live imaging. n = 7 independent donors. (E) *RNASE7* mRNA expression was determined in intact or wounded ALI-PBEC exposed to air or CS, at 6 h after exposure. Values shown represent normalized mRNA expression compared to *RPL13A* and *ATP5B*. n = 7 independent donors. (F) IL-8 secretion was determined in the basal culture medium. n = 9 independent donors. Data are shown as mean; error bars represent SEM; experiments were conducted in duplicate, * $p < 0.05$.

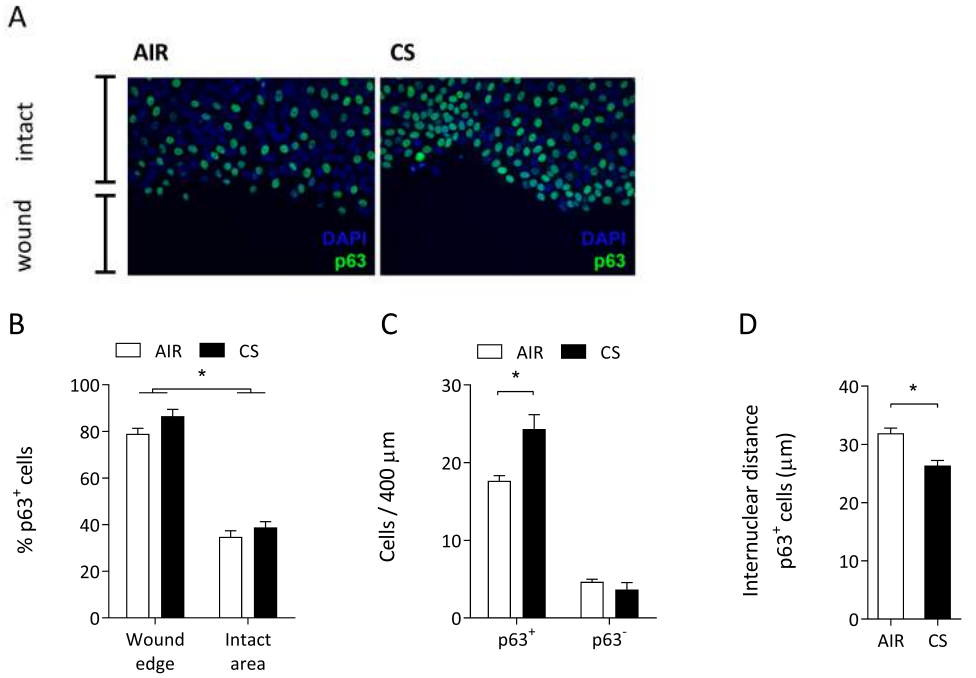


**Figure 3. p63⁺ cells at the wound edge of ALI-PBEC.** (A) Immunofluorescence staining for p63 (green) and nuclei (blue (DAPI)) of mechanically injured ALI-PBEC. (B) Percentage of p63⁺ cells at the first line of cells directly at the wound edge or in intact areas, in air- versus CS-exposed cells. (C) Number of p63⁺ cells and p63⁻ cells at the wound edge per 400 µm length of wound edge, in air- versus CS-exposed cells. (D) Internuclear distance in µm between p63⁺ cells located directly at the leading wound edge and the first adjacent p63⁺ cell. All graphs: n = 3 independent donors. Data are shown as mean; error bars represent SEM, experiments were conducted in duplicate, * $p < 0.05$.

*CS affects wound repair and innate immune responses through oxidative stress*

To understand the mechanism of CS-mediated modulation of wound repair and innate immune responses we next examined the role of oxidative stress. The presence of oxidative stress upon CS exposure was demonstrated indirectly by showing CS-induced mRNA expression in wounded ALI-PBEC of heme oxygenase (decycling) 1 (*HMOX1*) and smoke and cancer-associated lncRNA-1 (*SCAL1*), both target genes of the oxidative stress-dependent Nrf2 pathway [20,21]. This induction by CS was blunted by treatment with the antioxidant N-acetylcysteine (NAC), suggesting involvement of oxidative stress (Figure 4A and B). Treatment with NAC in CS-exposed cultures partially restored wound repair (Figure 4C). In contrast, CS-induction of RNase 7 in wounded ALI-PBEC was completely inhibited by NAC (Figure 4D), and also CS-induced IL-8 mRNA expression was significantly inhibited (Figure 4E). These findings demonstrate a differential effect of CS-induced oxidative stress on wound repair and innate immune responses, which are suppressed and enhanced respectively.

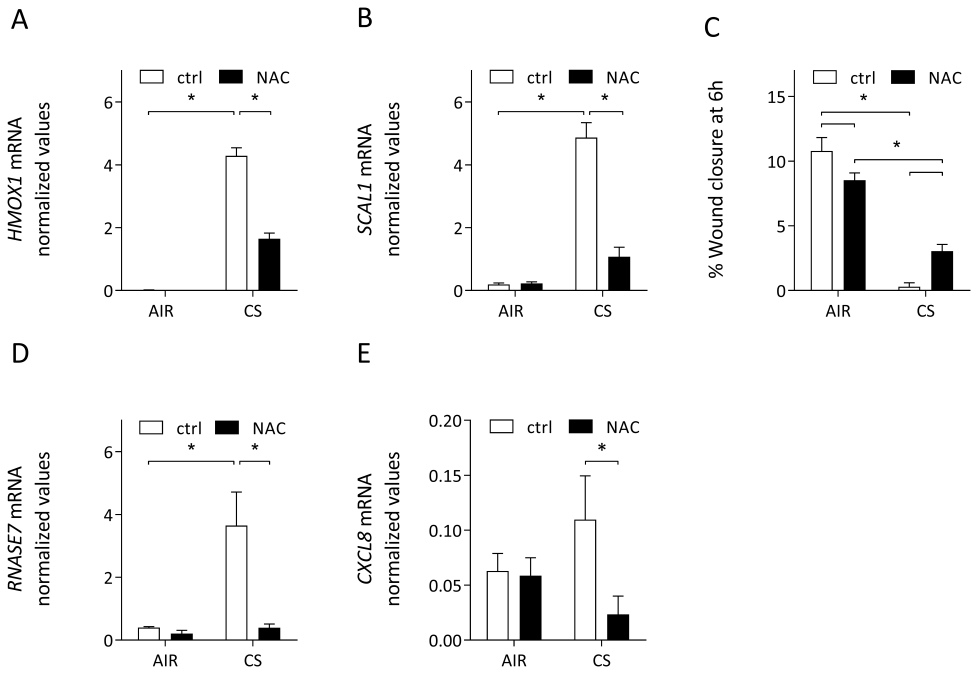


**Figure 4. Role of CS-induced oxidative stress in modulating airway epithelial repair and innate immunity.** Wounded ALI-PBEC were pre-incubated with NAC (10 mM) and subsequently exposed to air or CS. mRNA expression of the oxidative stress-induced genes (A) *HMOX1* and (B) *SCAL1* was determined 6 h after exposure. Values shown represent normalized mRNA expression compared to *RPL13A* and *ATP5B*. n = 3 independent donors, * $p < 0.05$. (C) Wound closure in presence or absence of NAC (10 mM) 6 h after wounding, in air- versus CS-exposed cells. Data are shown as percentage wound closure compared to t = 0 h. n = 7 independent donors. (D) mRNA expression of *RNASE7* and (E) *CXCL8* was assessed in wounded ALI-PBEC incubated with NAC (10 mM), at 6 h after exposure to air or CS. Data are shown as normalized mRNA expression compared to *RPL13A* and *ATP5B*. n = 3 independent donors. In all graphs data are shown as mean; error bars represent SEM; experiments were conducted in duplicate; * $p < 0.05$.

*EGFR & ERK1/2-signaling are required for wound repair and innate immune responses*

The epidermal growth factor receptor (EGFR) and downstream MAP-kinase/extracellular signal-regulated kinase (ERK)1/2 signaling pathway are important in both wound repair and induction of innate immune responses [10]. Therefore, we examined the role of EGFR and ERK1/2 in the repair and induction of innate immune responses in wounded ALI-PBEC. First, the contribution to the intrinsic wound repair of ALI-PBEC was demonstrated by inhibitor experiments, showing impaired wound healing in the presence of AG1478 (EGFR tyrosine kinase inhibitor) and U0126 (Mitogen activated protein kinase/ERK kinase (MEK)1/2 inhibitor) (Figure 5A). As CS completely impaired wound healing at 6 h, we did not observe an additional effect of EGFR or MEK1/2 inhibition. In agreement with our previous study [7], CS-induced mRNA expression of RNase 7 in wounded ALI-PBEC cultures was significantly inhibited upon EGFR and ERK1/2 inhibition (Figure 5B).

We subsequently determined EGFR and ERK1/2 phosphorylation in wounded ALI-PBEC. Phosphorylation of both proteins was observed in CS-induced wounded ALI-PBEC (Figure 5C-E). EGFR inhibition completely suppressed ERK1/2 phosphorylation in air-exposed cells, whereas in CS-exposed cells only a partial inhibition was observed. In contrast, inhibition of ERK1/2 phosphorylation did not affect EGFR phosphorylation. The contribution of CS-induced oxidative stress to EGFR and ERK1/2 phosphorylation was determined by examining the effect of antioxidant treatment. CS-induced EGFR phosphorylation in wounded ALI-PBEC was not altered by NAC (Figure 5C-E), whereas NAC did decrease CS-induced ERK1/2 phosphorylation. This suggests an EGFR-independent activation of ERK1/2 mediated by oxidative stress. In summary, these findings indicate involvement of EGFR- and ERK1/2 signaling in intrinsic wound healing, and in CS-induced innate immune responses during repair. Moreover, CS increases ERK1/2 phosphorylation in both an EGFR-dependent and -independent pathway mediated in part by oxidative stress (Figure 6).

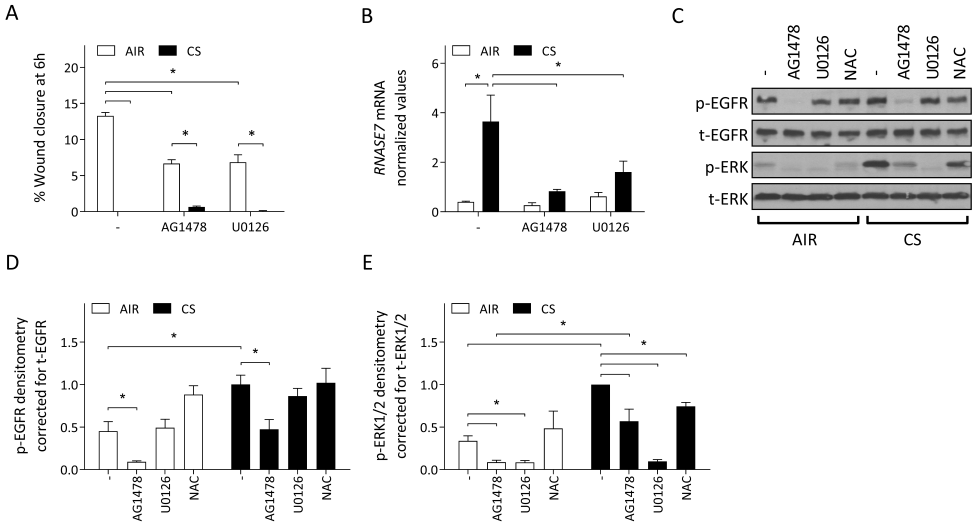


**Figure 5. EGFR and ERK1/2 signaling in wounded ALI-PBEC.** (A) Intrinsic wound healing of ALI-PBEC was determined in the presence of the EGFR tyrosine kinase inhibitor AG1478 (1 μM) or the MEK1/2 inhibitor U0126 (25 μM) at 6 h after exposure with either air or CS. Data are shown as percentage wound closure compared to t = 0 h. (B) mRNA expression of *RNASE7* was determined by qPCR. Data are shown as normalized mRNA expression compared to *RPL13A* and *ATP5B*. (C) Western blot analysis of EGFR and ERK1/2 phosphorylation of wounded ALI-PBEC exposed to air or CS in the presence of AG1478, U0126, and NAC, at 6 h after exposure. Bands were quantified by densitometry for analysis of (D) EGFR and (E) ERK1/2 phosphorylation and corrected for total-EGFR and total-ERK1/2, respectively. For all graphs data are shown as mean; error bars represent SEM; experiments were conducted in duplicate; n = 3 independent donors; * $p < 0.05$.

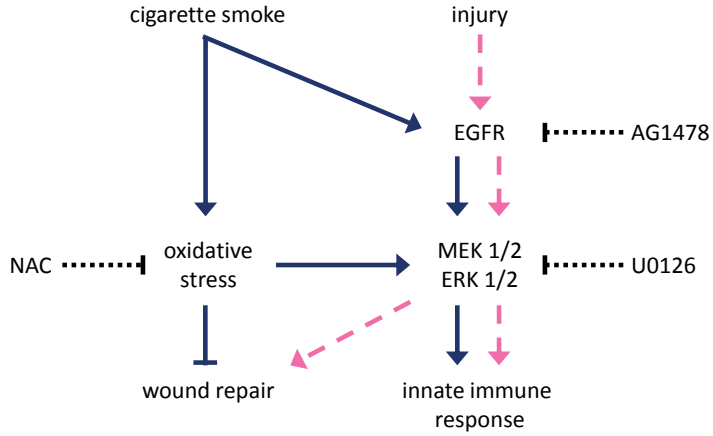


**Figure 6. Proposed model.** EGFR signaling is activated by CS and injury, and this leads to MEK1/2-mediated phosphorylation of downstream ERK1/2. CS furthermore directly causes phosphorylation and activation of ERK1/2 via oxidative stress, which is independent of EGFR signaling. EGFR/ERK1/2-mediated wound repair is suppressed by CS via oxidative stress. In contrast, activation of ERK1/2 due to a combined effect of CS-induced oxidative stress and injury, results in an enhanced innate immune response. Solid blue lines represent the effect of CS, dashed pink lines the effect of injury. NAC, AG1478 and U0126 were used to inhibit oxidative stress, EGFR phosphorylation, and ERK/12 phosphorylation respectively.



## DISCUSSION

In this study we examined the effect of whole CS exposure on wound repair and innate immune responses of injured ALI-PBEC cultures. We observed a detrimental effect of acute CS exposure on the restoration of the epithelial barrier integrity and wound closure after mechanical wounding. In contrast, induction of innate immune responses, in particular expression of RNase 7, was further enhanced in CS-exposed injured ALI-PBEC. The impairment of epithelial repair in the mechanical injury model was accompanied by an accumulation of BCs at the wound edge. Moreover, oxidative stress contributed to the CS-induced attenuation of wound repair and the induction of innate immune responses. Both intrinsic wound repair and CS-induced innate immune responses required EGFR- and ERK1/2 mediated signal transduction.

Whole CS exposure of ALI-PBEC attenuated airway epithelial repair, in particular during the first 6 h following CS exposure. Such a transient effect of CS is in line with our previous study, in which CS was shown to transiently affect the epithelial barrier integrity followed by restoration within 24 h [7]. Oxidative stress contributed to the observed effects of whole CS exposure on epithelial wound repair, as NAC partly reversed the observed induction of an anti-oxidant response and impairment of epithelial wound repair. This is in line with our previous findings in undifferentiated (submerged cultured) PBEC, using an aqueous extract of cigarette smoke [9], and suggest that findings made in these less physiological conditions remain relevant.

In contrast to the suppressive effect of CS on epithelial repair, CS-exposure resulted in increased innate immune responses in injured airway epithelial cell layers. Following CS exposure, increased IL-8 secretion was observed in the calcium switch model, whereas increased expression of RNase 7 was detected in the calcium switch as well as the mechanical wound model. Previously, we reported cell type-specific expression of RNase 7 by BCs present in ALI-PBEC cultures [7]. The current observation further suggests that BCs are particularly affected upon injury of ALI-PBEC. Indeed, BCs are regarded as a heterogenic population including epithelial progenitor cells of the pseudostratified airway epithelium [22,23], and it is assumed that BCs repopulate denuded wound areas through cell spreading and migration after injury [24]. In agreement with this, the majority of cells at the leading wound edge of injured ALI-PBEC stained positive for the BC marker p63. In line with earlier reports [9,25], this could not be explained by increased proliferation, as only limited numbers of proliferating cells were observed at the wound edge as assessed by BrdU incorporation (*data not shown*). However, $p63^+$ cells displayed smaller internuclear distances upon CS exposure, suggesting that spreading and migration of BCs is impaired. Further studies are needed to determine whether cigarette smoke specifically targets subpopulations of BCs.

We speculate that the transient effect of CS in our model reflects the acute effects of smoking on the airway epithelium that is in a process of repair. Normally, BCs of the airway epithelium will close denuded wound areas through cell spreading before starting cell proliferation [26]. However, primarily under the influence of oxidative stress caused by smoke exposure, the cells shift towards a different function, displaying reduced repair-promoting migratory activity but increased innate immune and cytoprotective anti-oxidant responses. The transient effect of CS on wound repair suggests recovery of airway epithelial cells from mild damage that does not cause extensive cell injury and promote cell death. Indeed, CS exposure induced the expression of genes involved in the Nrf2-mediated antioxidant and survival response, which suggests that this response is involved in the restoration of wound repair following CS exposure. This mechanism may be impaired in COPD, since previous studies have reported attenuated Nrf2-dependent antioxidant responses in the bronchial tissues from COPD patients compared to non-COPD smokers [27,28]. Moreover, it has been shown that COPD airway epithelial cells display reduced wound repair and epithelial barrier properties [29,30]. Therefore, it can be speculated that an impaired oxidative stress response is related to epithelial dysfunction during COPD disease progression. It needs to be noted that our experimental design was adapted to mimic the effects of acute cigarette smoke exposure, and not that of the repeated exposures that are typical from smoker's lungs. Further studies are needed to explore such effects during repeated CS exposure. Although the airway epithelial cells start to recover from the effects of CS in both the calcium switch and wound repair model at 6 h after exposure, induction of RNase 7 in BCs persisted at later time points. In particular, in the calcium switch assay enhanced expression of RNase 7 was observed at 24 h after smoke exposure, when the epithelial barrier integrity had recovered. This suggests that BCs display innate immune responses after the epithelium has recovered from injury. Epithelial injury

results in activation of the EGFR signalling pathways in BCs, which is important for both wound closure and RNase 7 expression.

We propose that the reduced wound repair activity of the airway epithelium upon CS exposure increases the susceptibility of the epithelium and underlying tissues to microbial colonization and infections. The increased expression of the antimicrobial RNase 7 by BCs that occurs in parallel with impaired wound repair might be a compensatory mechanism to provide a last-resort antibacterial defense against invading microbes. We did not study the host defence activity of CS-exposed and injured airway epithelial cells using functional assays. Therefore, further research is required to determine the additional effects of adding live microbes in our wound healing model, and the putative modulating effect of increased RNase 7 expression. Antimicrobial proteins and peptides such as RNase 7 display immunomodulatory and wound repair enhancing properties [31,32]. These responses might contribute to the wound repair process but might also contribute to cell injury when these mediators are produced in high amounts and/or during prolonged periods. There is however currently no evidence for other activities of RNase 7, and therefore further research is required to demonstrate this. We used mechanical wounding of the epithelial layer by scraping, which is widely used in studies on repair but is obviously a less physiologically relevant model of injury than e.g. bacterial or viral infection or repeated smoke exposure. An important advantage of the model is, however, that it allows creation of a defined wound and quantification of its repair. Another advantage is that it allows an analysis of the interaction between microbial infection or smoke exposure and wound repair.

Previously, we reported the importance of EGFR signaling in induction of innate immune responses by CS, which was mediated by downstream ERK1/2 activation [7]. Antioxidant treatment did not reduce EGFR signaling, but did decrease ERK1/2 phosphorylation, suggesting an EGFR-independent activation of ERK1/2 by oxidative stress (Figure 6). Thus, although EGFR-signal transduction is critical in airway epithelial wound repair and innate immunity [10], these findings demonstrate that other signaling transduction pathways contribute to repair and might be affected by CS exposure. Further research on CS effects on other repair pathways is required, and might also elucidate the differential regulation of epithelial wound repair and RNase 7 expression in mechanically wounded ALI-PBEC.

In summary, our findings demonstrate disturbances in the repair of injured airway epithelium and epithelial innate immunity upon cigarette smoke exposure. Oxidative stress caused by smoking is a key mechanism in modulating these responses, and in particular affects the activity of basal cells. These findings contribute to our understanding of how the repair and innate immune activity of wounded airway epithelial cells can be affected by cigarette smoking and might contribute to the development and progression of COPD.

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A supplementary video of airway epithelial wound repair is available online via PlosOne at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0166255#sec018

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ALI | air-liquid interface cultured |
| BCs | basal cells |
| BEGM | bronchial epithelial growth medium |
| BPE | bovine pituitary extract |
| BSA | bovine serum albumine |
| COPD | chronic obstructive pulmonary disease |
| CS | cigarette smoke |
| CXCL8 | chemoattractant C-X-C Ligand 8 |
| DMEM | Dulbecco's modified Eagle's medium |
| EGFR | epidermal growth factor receptor |
| ERK1/2 | extracellular signal-regulated kinase 1/2 |
| hEGF | human epidermal growth factor |
| HMOX1 | heme oxygenase (decycling) 1 |
| IL | interleukin |
| MAP | mitogen-activated protein kinase |
| MEK1/2 | MAPK/ERK kinase 1/2 |
| NAC | N-acetylcysteine |
| Nrf2 | nuclear factor (erythroid-derived 2)-like 2 |
| P63 | tumor protein 63 |
| PBEC | primary bronchial epithelial cells |
| RNase7 | ribonuclease 7 |
| SCAL1 | smoke and cancer-associated lncRNA-1 |
| SEM | standard error of the mean |
| TEER | transepithelial electrical resistance |
| w/o $Ca^{2+}$ | calcium-depleted culture medium |

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## SUPPLEMENTARY FIGURES

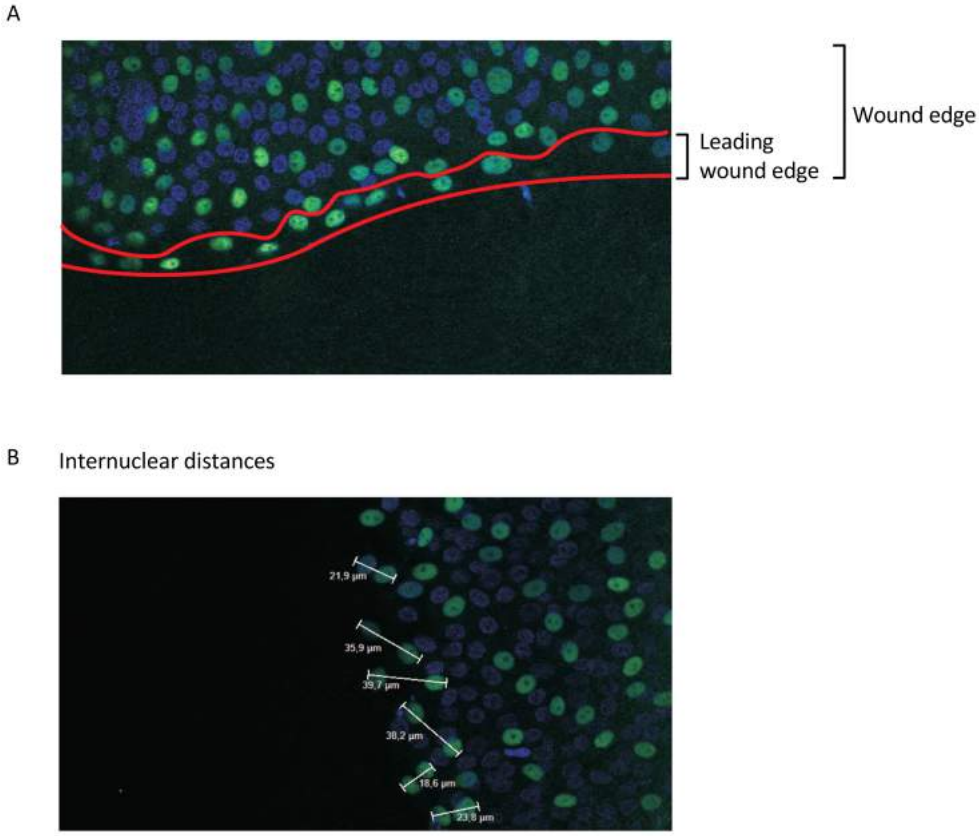


**Figure S1. Quantification methods of p63⁺ cells in wounded ALI-PBEC**. (A) Analysis of p63⁺ and p63⁻ DAPI-stained nuclei at the leading wound edge. (B) Graphic example of how the internuclear distances were determined between p63⁺ cells located at the leading wound edge and p63⁺ cells that were perpendicular to the wound. p63⁺ cells at the leading wound edge were defined by the absence of other p63⁺ cells in the 45°-135° angle in its front perpendicularly to the wound edge. The most proximate p63⁺ cell that did not fulfill this definition was considered the reference cell to be selected for the measurement of the internuclear distance between adjacent p63⁺ cells. The distance between the outside edges of these two cells was regarded the internuclear distance. To prevent underestimation of distances in p63⁺ denser areas, each non-wound edge cell could be used only once for internuclear distance assessment, targeting overall at the lowest mean distance. The measurements were done in 5 randomly taken images of air- and CS-exposed ALI-PBEC. This analysis was performed in cultures derived from 3 independent donors.

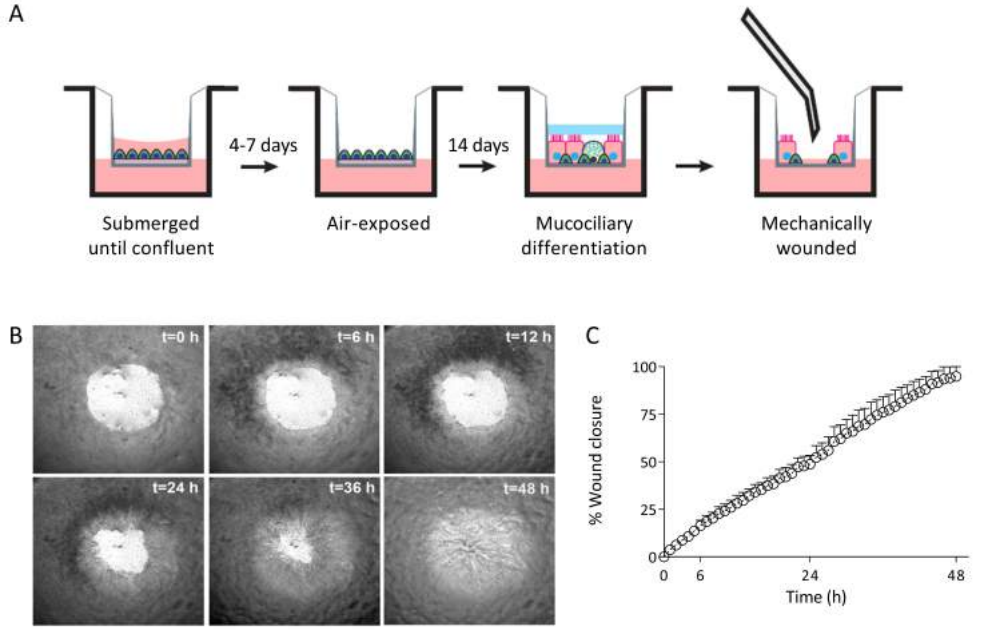


**Figure S2. Live imaging of intrinsic airway epithelial wound repair.** (A) Primary bronchial epithelial cells (PBEC) were cultured and differentiated in an air-liquid interface (ALI) model, and subsequently mechanically wounded to assess wound repair. (B) The wound closure of ALI-PBEC was followed by live imaging at 0, 6, 12, 24, 36 and 48 h after wounding. (C) Wound closure was determined each hour, up to 48 h, by live imaging. Data are shown as the percentage wound closure compared to t = 0. Data are shown as mean; error bars represent SEM; experiments were conducted in duplicate. N = 3 independent donors.

**SUPPLEMENTARY TABLE S1. qPCR primer sequences**

| Gene | Primer sequence forward | Primer sequence reverse |
|---|---|---|
| *HMOX1* | 5′-AACCCTGAACAACGTAGTCTGCGA-3′ | 5′-ATGGTCAACAGCGTGGACACAAA-3′ |
| *SCAL1* | 5′-GGCATTTACCAGCTGAGGGA-3′ | 5′-TACCCCTACCTAGCACAGCA-3′ |
| *RNASE7* | 5′-CCAAGGGCATGACCTCATCAC-3′ | 5′-ACCGTTTTGTGTGCTTGTTAATG-3′ |
| *IL8* | 5′-CAGCCTTCCTGATTTCTG-3′ | 5′-CACTTCTCCACAACCCTCTGC-3′ |
| *RPL13A* | 5′-AAGGTGGTGGTCGTACGCTGTG-3′ | 5′-CGGGAAGGGTTGGTGTTCATCC-3′ |
| *ATP5B* | 5′-TCACCCAGGCTGGTTCAGA-3′ | 5′-AGTGGCCAGGGTAGGCTGAT-3′ |

# TNF-α AND IL-1β-ACTIVATED MESENCHYMAL STROMAL CELLS INCREASE AIRWAY EPITHELIAL WOUND HEALING *IN VITRO* VIA ACTIVATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR

*CHAPTER 3*

**RESPIRATORY RESEARCH, 2016 Jan 11; 17:3**

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**ABSTRACT**

**Background:** Mesenchymal stromal cells (MSCs) are investigated for their potential to reduce inflammation and to repair damaged tissue. Inflammation and tissue damage are hallmarks of chronic obstructive pulmonary disease (COPD) and MSC infusion is a promising new treatment for COPD. Inflammatory mediators attract MSCs to sites of inflammation and affect their immune-modulatory properties, but little is known about their effect on regenerative properties of MSCs. This study investigates the effect of the pro-inflammatory cytokines TNF-$\alpha$ and IL-1$\beta$ on the regenerative potential of MSCs, using an *in vitro* wound healing model of airway epithelial cells.

**Methods:** Standardized circular wounds were created by scraping cultures of the airway epithelial cell line NCI-H292 and primary bronchial epithelial cells cultured at the air-liquid interface (ALI-PBEC), and subsequently incubated with MSC conditioned medium (MSC-CM) that was generated in presence or absence of TNF-$\alpha$/IL-1$\beta$. Remaining wound size was measured up to 72 hours. Phosphorylation of ERK1/2 by MSC-CM was assessed using Western blot. Inhibitors for EGFR and c-Met signaling were used to investigate the contribution of these receptors to wound closure and to ERK1/2 phosphorylation. Transactivation of EGFR by MSC-CM was investigated using a TACE inhibitor, and RT-PCR was used to quantify mRNA expression of several growth factors in MSCs and NCI-H292.

**Results:** Stimulation of MSCs with the pro-inflammatory cytokines TNF-$\alpha$ and IL-1$\beta$ increased the mRNA expression of various growth factors by MSCs and enhanced the regenerative potential of MSCs in an *in vitro* model of airway epithelial injury using NCI-H292 airway epithelial cells. Conditioned medium from cytokine stimulated MSCs induced ERK1/2 phosphorylation in NCI-H292, predominantly via EGFR; it induced ADAM-mediated transactivation of EGFR, and it induced airway epithelial expression of several EGFR ligands. The contribution of activation of c-Met via HGF to increased repair could not be confirmed by inhibitor experiments.

**Conclusion:** Our data imply that at sites of tissue damage, when inflammatory mediators are present, for example in lungs of COPD patients, MSCs become more potent inducers of repair, in addition to their well-known immune-modulatory properties.

## INTRODUCTION

In chronic obstructive pulmonary disease (COPD), the release of proteases and other mediators by a variety of inflammatory and resident cells is thought to cause tissue damage within the lung [1-3]. The endogenous regenerative capacity of the lung to restore damaged structures is limited, and the resulting imbalance between insufficient repair mechanisms and excess tissue damage will lead to irreversible tissue damage [4], ultimately causing organ failure.

Current COPD treatment targets symptoms, and there is a lack of treatments that halt disease progression and/or restore lung structure. The only current option for patients with chronic respiratory failure due to severe emphysema is lung transplantation, but the availability of donor lungs is limited and the success of lung transplantation varies. Therefore, new approaches to restore damaged lung tissue in COPD are needed.

A promising therapeutic approach that targets restoration of destructed lung tissue as well as reduction of inflammation is the administration of mesenchymal stromal cells (MSCs). MSCs are multipotent progenitor cells of non-hematopoietic origin defined by their capacity to differentiate into multiple lineages of the mesenchyme [5]. Besides their differentiation capacity, MSCs can favour repair of wounded tissue by modulating cellular responses in structural and immune cells, creating a regenerative and anti-inflammatory environment (reviewed in [6,7]). The main mechanisms by which MSCs exert these effects are via cell-cell interactions and secretion of soluble factors.

Indeed, MSCs can reduce inflammation and repair alveolar structures as has been demonstrated in *in vivo* rodent models of cigarette smoke or elastase-induced emphysema [8-10]. It has been suggested that MSCs mediate this effect in part via the release of soluble factors, including hepatocyte growth factor (HGF) and epidermal growth factor receptor (EGFR) ligands, which can both increase proliferation of epithelial cells [11-15]. The receptors for these growth factors, c-Met and EGFR respectively, can activate extracellular signal-regulated kinase 1/2 (ERK1/2), one of the mitogen activated protein kinases (MAPK). Activation of this signaling pathway results in proliferation, differentiation and migration, processes that are fundamental for wound repair [16]. Whereas conditioned medium from MSCs has been shown to enhance airway epithelial wound healing *in vitro* [17], the contribution of HGF or EGFR ligands and underlying ERK1/2 signaling has not yet been investigated.

Another interesting and yet unanswered issue is whether pro-inflammatory cytokines can affect the potential of bone-marrow derived MSCs to repair damaged pulmonary epithelium at sites of inflammation. It is known that inflammatory mediators can attract MSCs (reviewed in [18,19]) and alter their secretome [20-24], which is beneficial for the immune response [22] and for skin wound healing [25]. However, whether inflammatory mediators also increase the potential of bone marrow-derived MSCs to repair damaged pulmonary epithelium remains to be elucidated. Moreover, the cellular and molecular mechanisms that underlie such a repair potentiating effect within the airway epithelium are largely unknown.

Therefore, in the present study we investigated the effect of pro-inflammatory cytokines involved in the pathogenesis of COPD (i.e. Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β)) [26-29] on the expression of growth factors by MSCs. We explored the effect of the conditioned medium from these stimulated MSCs on airway epithelial wound repair *in vitro*, and the contribution of the ERK1/2 signaling pathway, c-Met and EGFR to this effect. Our results show that stimulation of MSCs with TNF-α and IL-1β increases their regenerative potential as assessed in an *in vitro* model of airway epithelial repair. Furthermore, we demonstrate the crucial involvement of EGFR-activation in this process.

## MATERIALS AND METHODS

*Cell culture*

Cells from the NCI-H292 human lung mucoepidermoid carcinoma epithelial cell line (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA), supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (all from Bio Whittaker, Walkersville, MD, USA) and 10% [v/v] heat inactivated fetal calf serum (FCS) (Bodinco, Alkmaar, The Netherlands). Human primary bronchial epithelial cells (PBEC) isolated from tumor-free bronchial tissue [30] were cultured on semi-permeable transwell membranes with a 0.4 µm pore size (Corning Costar, Cambridge, MA, USA). Transwells were coated with 30 µg/ml PureCol (Advanced BioMatrix, San Diego, CA, USA), 10 µg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/ml fibronectin diluted in PBS. Upon establishment of a confluent cell layer, PBEC were cultured at the air-liquid interface (ALI) during 2 weeks for differentiation. Culture medium consisted of a 1:1 mixture of bronchial epithelial growth medium (BEGM) (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 0.4% (w/v) bovine pituitary extract (BPE), 1 µM hydrocortisone (HC), 0.5 ng/ml human epidermal growth factor (hEGF), 0.5 µg/ml epinephrine, 10 µ/ml transferrin, 5 µg/ml insulin, T3, 0.1 ng/ml retinoic acid (RA), 1 mM Hepes (all Lonza), 1 mg/ml BSA (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza), and additional supplementation of 15 ng/ml RA (Sigma-Aldrich) for mucociliary differentiation.

Mesenchymal stromal cells (MSCs) were isolated from bone marrow from healthy donors and expanded in culture following a previously described protocol of the department of Immunohematology and Blood Transfusion at Leiden University Medical Center [31]. MSC characterization was based on morphology and immunophenotyping using flow cytometry for the following markers: HLA-DR, CD73, CD90, CD31, CD34, CD45, CD80 (Becton Dickinson (BD) Bioscience, Franklin Lakes, NJ, USA) and CD105 (Ancell, Bayport, MN, USA), using FACSCalibur and CellQuest Pro Software (BD Bioscience). MSCs were cultured in DMEM GlutaMAX™ (Gibco), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, and 10% [v/v] heat-

inactivated FCS (Thermo Fisher Scientific, UT, USA). All cells were cultured at 37°C in a 5% $CO_2$ humidified incubator. Before experiments, NCI-H292 and MSCs were starved for growth factors overnight using serum-free (SF) culture medium; ALI-PBEC were starved for growth factors in B/D medium lacking BPE, HC, hEGF, RA and BSA. Prior to experiments, the apical side of ALI-PBEC cultures was washed with 100 µL of PBS to remove excess mucus.

*Preparation of conditioned medium*

MSCs were grown until 80-90% confluence and starved overnight in serum-free medium after washing with PBS. To generate MSC conditioned medium (MSC-CM), cells were cultured for 24 hours in either serum-free medium (LG-DMEM) containing TNF-α and IL-1β (both 20 ng/ml; Peprotech, Rocky Hill, NJ, USA) (to generate MSC-CM$^{STIM}$) or serum-free medium alone (to generate MSC-CM$^{CTRL}$). MSC-CM from different donors and generated at different passages was pooled before experiments to reduce effects caused by donor and/or passage variation. All wound healing experiments were performed with MSC-CM at passage 4-6. A part of the western blot experiments were performed with MSC-CM at passage 2-4. Control DMEM medium with TNF-α and IL-1β (DMEM$^{STIM}$) or without (DMEM$^{CTRL}$) was obtained by incubating medium in culture flasks not containing cells in the same incubator for 24 hours. MSC-CM and DMEM control medium were harvested and centrifuged for 7 minutes at 230xg to remove debris, and stored in 2 ml aliquots at -80°C until further use.

*Wound repair model*

A confluent monolayer of NCI-H292 cells or differentiated ALI-PBEC was mechanically injured by scraping the cell layer with a sterile Pasteur pipette with a soft tip (essentially as described in [32]). Two wounds with a diameter of 3 mm each were made in each well in a 12 wells plate for NCI-H292, or one wound per Transwelll insert for ALI-PBEC. Each experiment was performed in duplicate. After wounding, medium was replaced by the following stimuli: serum free standard culture medium (negative control); 20 ng/ml Transforming Growth Factor-α (TGF-α) (Sigma-Aldrich, St. Louis, MO, USA) in serum free standard culture medium (positive control [32]); or MSC-CM and the corresponding DMEM controls, which were all diluted 1:2 in serum free standard culture medium (based on dose response experiments), unless otherwise specified. For inhibitor experiments exploring the role of c-Met and the Epidermal Growth Factor receptor (EGFR) in wound healing, 0.05 µM PF04217903 (c-Met inhibitor; Sigma-Aldrich) and/or 0.2 µM AG1478 (EGFR tyrosine kinase inhibitor; Calbiochem, Darmstadt, Germany) were added to relevant stimuli for the full culturing period. Hepatocyte Growth Factor (HGF) (Peprotech) and TGF-α (both at 20 ng/ml in LG-DMEM) served as positive controls, whereas LG-DMEM was used as negative control (DMEM$^{CTRL}$).

Digital images of the wounds were collected every 24 hours up to 72 hours maximum (NCI-H292) or at 6, 24 and 48 hours (ALI-PBEC), on an inverted phase-contrast light microscope using Cell

Sense Entry imaging software (both from Olympus, Tokyo, Japan). The surface of the wound area was measured using Image J software (National Institutes of Health, USA), and residual wound area in percentage was assessed by comparing the remaining wound size at different time points with the wound size at the start of the experiment ((1-wound size t=x/wound size t=0)*100).

*Phosphorylation of ERK 1/2*

NCI-H292 cells used for assessment of ERK1/2 phosphorylation were cultured to 70% confluence in a 12 wells plate. All stimuli were diluted 1:2 in RPMI. Cells were stimulated with either MSC-$CM^{STIM}$ or $DMEM^{STIM}$. HGF or TGF-$\alpha$ in a final concentration of 20 ng/ml diluted in LG-DMEM and $DMEM^{CTRL}$ were used as positive resp. negative controls.

The role of c-Met and EGFR activation was investigated by pre-incubation of cells during 1 hour with either 0.05 µM PF04217903, or 10 µM AG1478, or 2 µg/ml neutralizing antibodies against EGFR (Calbiochem) dissolved in RPMI in assigned wells before addition of stimuli. The role of transactivation was evaluated using the TNF-$\alpha$ converting enzyme (TACE)/ADAM17 inhibitor TAPI-1 (10 µM) (Santa Cruz Biotechnology, Dallas, TX, USA). Controls were treated with an equal volume of RPMI for 1 hour.

Cells were incubated with stimuli for 15 minutes or various time periods for time-series experiments, and immediately cooled down on ice and washed with cold ERK washing buffer (5 mM Tris pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Cell lysates were generated by incubation with lysis buffer (0.5% [v/v] Triton X-100, 1mM $Na_3VO_4$ and Mini complete protease cocktail (Roche, Basel, Switzerland) in ERK washing buffer) for 15 minutes. Samples from duplicate wells were pooled. After dilution in 2 times concentrated reducing sample buffer (4% [v/v] SDS, 10% [v/v] beta-Mercaptoethanol, 20% [v/v] glycerol, 0.5 M Tris pH 6.8 and 0.003% [w/v] Bromphenol blue) samples were boiled for 5 minutes and spun down at 20780xG for 5 minutes, before loading and running on a 10% SDS-PAGE gel. Proteins were transferred onto a polyvinylidene difluoride membrane using the Mini-transblot system (both from Bio-Rad, Hercules, CA, USA).

Non-specific binding sites on the blots were blocked with PBS/0.05% [v/v] Tween 20/0.5% [w/v] casein (Sigma-Aldrich) for at least one hour followed by overnight incubation at 4°C with antibodies directed against total and phospho-ERK1/2 (Cell Signaling, Beverly, MA, USA) in PBS/0.05% Tween 20. After washing of the blot with PBS/0.05% Tween 20, secondary HRP labeled goat antibodies (BD Bioscience) were added for 1 hour at room temperature, followed by extensive washing. The membranes were developed on film (FujiFilm Corporation, Tokyo, Japan) using enhanced chemiluminescent (ECL) detection system (ThermoScientific, Rockford, IL, USA).

*mRNA expression*

NCI-H292 cells were grown to near confluence and exposed to MSC-$CM^{STIM}$ or $DMEM^{STIM}$ or $DMEM^{CTRL}$ (neg ctrl) 1:2 in RPMI. MSCs were grown to 80-90% confluence and stimulated with TNF-$\alpha$ and IL-1$\beta$ (20 ng/ml each) or serum free medium. Incubation times were based on initial

experiments on limited samples revealing the largest increase of mRNA expression for several genes after 9 hours (NCI-H292) and 6 hours (MSCs) of stimulation. Sample triplicates from a 24 wells plate were pooled and RNA was extracted using Maxwell® 16 RNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturers protocol, and quantified using the Nanodrop ND-1000 UV-visible spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Complementary DNA was generated by adding Oligo(dT) primers (Qiagen, Düsseldorf, Germany) and 10 nM dNTP mix (Promega) to the RNA sample, and heating this to 65°C for 5 minutes. Subsequently, 5x 1st strand RNA buffer, RNasin and M-MLV (all from Promega) were added, and the samples were incubated at 37°C during 50 minutes followed by heat inactivation of M-MLV at 70°C during 15 minutes. Primers were designed using PubMed Gene Database and Primerbank (Table 1) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) (http://pga.mgh.harvard.edu/primerbank). Quantitative real-time PCR was performed in triplicate in a 384 wells plate (Bio-Rad CFX384™), with samples mixed with respective primers and SYBR Green supermix (Bio-Rad) in a final volume of 8 µl. Results were checked for outliers: outliers were removed if the variance within triplicates was above 10%.

Expression of *ACTB* and *GAPDH* was used to normalize mRNA expression in MSCs, whereas *RPL13A* and *ATP5B* were used for NCI-H292 (Table 1).



**TABLE 1. qPCR primer sequences**

| Gene | Primer sequence forward | Primer sequence reverse |
|---|---|---|
| *ACTB* | 5'-TTCCAGGAGCGAGATCCCT-3' | 5'-CACCCATGACGAACATGGG-3' |
| *ATP5B* | 5'-TCACCCAGGCTGGTTCAGA-3' | 5'-AGTGGCCAGGGTAGGCTGAT-3' |
| *GAPDH* | 5'-TTCCAGGAGCGAGATCCCT-3' | 5'-CACCCATGACGAACATGGG-3' |
| *RPL13A* | 5'-AAGGTGGTGGTCGTACGCTGTG-3' | 5'-CGGGAAGGGTTGGTGTTCATCC-3' |
| *AREG* | 5'-GGTGGTGCTGTCGCTCTTG-3' | 5'-AGGTGTCATTGAGGTCCAATCC-3' |
| *CCDN1* | 5'-CAATGACCCCGCACGATTTC-3' | 5'-CATGGAGGGCGGATTGGAA-3' |
| *EGF* | 5'-TGCAGAGGGATACGCCCTAA-3' | 5'-CAAGAGTACAGCCATGATTCCAAA-3' |
| *FGF2* | 5'-TGGCTATGAAGGAAGATGGAAGA-3' | 5'-TCCAATCGTTCAAAAAAGAAACAC-3' |
| *HB-EGF* | 5'-TGGACCTTTTGAGAGTCACTTTATCC-3' | 5'-CGTGCTCCTCCTTGTTTGGT-3' |
| *HGF* | 5'-TCCAGAGGTACGCTACGAAGTCT-3' | 5'-CCCATTGCAGGTCATGCAT-3' |
| *IL6* | 5'-CAGAGCTGTGCAGATGAGTACA-3' | 5'-GATGAGTTGTCATGTCCTGCAG-3' |
| *PDGFA* | 5'-CACCACCGCAGCGTCAA-3' | 5'-CCTCACCTGGACTTCTTTTAATTTTG-3' |
| *TGFA* | 5'-AGGTCCGAAAACACTGTGAGT-3' | 5'-AGCAAGCGGTTCTTCCCTTC-3' |
| *VEGF* | 5'-CGAGGGCCTGGAGTGTGT-3' | 5'-TGGTGAGGTTTGATCCGCATA-3' |

*Statistics*

The data are expressed as mean ± standard error of the mean unless depicted otherwise. GraphPad Prism 6.0 (GraphPad Inc., La Jolla, CA, USA) was used for statistical analysis. For comparison between groups, the Mann-Whitney test was used, for comparison of three or more groups the Kruskall Wallis test. Differences were considered significant when $p < 0.05$.

## RESULTS

*Stimulation of MSCs with pro-inflammatory cytokines induces mRNA expression of several growth factors and leads to increased protein levels of HGF*

To investigate the effect of pro-inflammatory cytokines on growth factor expression in MSCs, the mRNA expression of a variety of growth factors was analyzed in MSCs stimulated with TNF-α and IL-1β (20 ng/ml each).

The mRNA expression of the growth factors Fibroblast Growth Factor 2 (*FGF2*), Hepatocyte Growth Factor (*HGF*), Heparin-binding EGF-like growth factor (*HBEGF*) and Interleukin-6 (*IL6*) was significantly increased ($p<0.05$), and a non-significant increase was observed in Amphiregulin (*AREG*) ($p=0.06$). mRNA expression of the other growth factors evaluated did not change significantly (Figure 1).

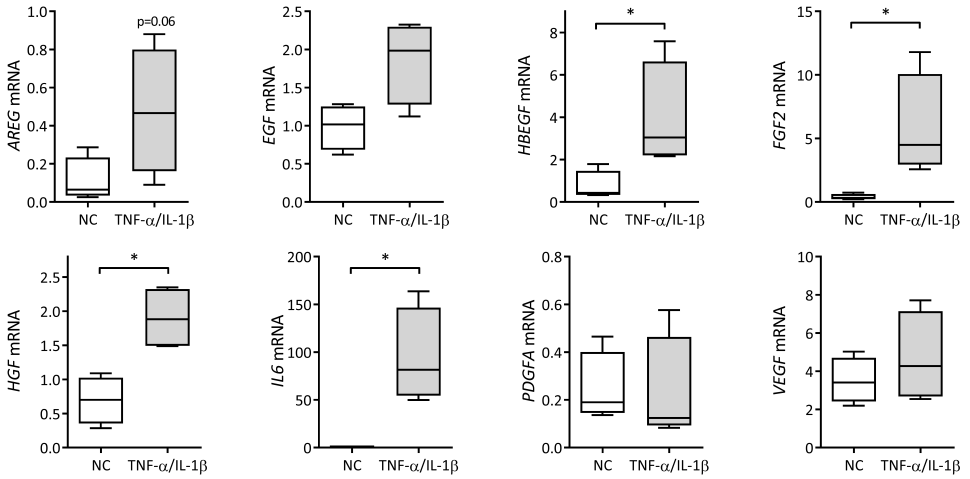


**Figure 1. Stimulation of MSCs with TNF-α and IL-1β increases the expression of several growth factors.** MSCs were stimulated with TNF-α and IL-1β 20 ng/ml each and harvested for RNA extraction after 6 hours. mRNA expression of various growth factors (*AREG*, *EGF*, *HBEGF* (all EGFR ligands), *FGF2, HGF, IL6, PDGFA*, and *VEGF*) was determined by qPCR, which showed a significant increase of FGF2, HBEGF, HGF and of IL6, and an increase of AREG and EGF. Values were normalized to *ACTB* and *GAPDH* reference genes. Box and whiskers represent median, interquartile range and minimum and maximum for n=4 obtained from three different donors; (*) $p<0.05$.

*MSC-conditioned medium increases wound closure in NCI-H292 airway epithelial cells*

Exposure of MSCs to the pro-inflammatory cytokines TNF-α and IL-1β resulted in increased mRNA expression of several growth factors. To assess whether this observation has functional relevance, a wound closure model was used to investigate the effect of MSC-CM$^{STIM}$ on wound closure in NCI-H292 airway epithelial cell monolayers. No significant differences were observed when

wounded NCI-H292 cells were incubated with conditioned medium from unstimulated MSCs (MSC-CM$^{CTRL}$), compared to the control medium (DMEM$^{CTRL}$) (Figure 2A). In contrast, MSC-CM from TNF-α and IL-1β stimulated MSCs (MSC-CM$^{STIM}$) significantly enhanced wound closure compared to DMEM$^{STIM}$ (also containing TNF-α and IL-1β) after 24 and 48 hours and was even more effective than the positive control TGF-α (Figure 2B and C) (p=0.002 at 48 hours). The effect of MSC-CM$^{STIM}$ was dose-dependent and still detectable at a 1:10 dilution (Figure 2D). Next the effect of MSC-CM on wound closure in well-differentiated cultures of ALI-PBEC was investigated. Wound closure in ALI-PBEC cultures was faster than in NCI-H292, and full wound closure was observed within 48 h. In ALI-PBEC, MSC-CM$^{STIM}$ also significantly increased epithelial wound closure, however to a similar extent as its control (DMEM$^{STIM}$) (Figure 2E and F).



*MSC-CM$^{STIM}$ activates ERK1/2 signaling in NCI-H292 via (trans)activation of EGFR*

The observation that MSC-CM$^{STIM}$ enhanced wound closure in NCI-H292 monolayers prompted further investigation into the underlying cellular response accountable for this effect. Epithelial wound healing is regulated by activation of mitogen activated protein kinases (MAPKs), and in particular via activation of the MAPK Extracellular signal-Regulated Kinase (ERK)1/2, which is known to be involved in cell proliferation, differentiation and migration [16,33]. Therefore we further examined the role of this pathway in MSC-CM induced wound healing. In NCI-H292 monolayers, MSC-CM$^{STIM}$ increased ERK1/2 phosphorylation, when compared to DMEM$^{STIM}$ (Figure 3A and B). This effect was more pronounced at early time points (15-30 min), but could be observed up to 6 hours of stimulation (Figure 3C and D). Next, the involvement of two upstream receptors, c-Met (HGF-receptor) and Epidermal Growth Factor Receptor (EGFR), in ERK1/2 phosphorylation by MSC-CM$^{STIM}$ was explored using a tyrosine kinase inhibitor to block c-Met and EGFR, or neutralizing anti-EGFR antibodies. Control experiments showed that ligands for both c-Met (HGF) and EGFR (TGF-α) are potent activators of ERK1/2, and that the respective inhibitors block this activation effectively and specifically (data not shown). At 15 minutes, phosphorylation of ERK1/2 by MSC-CM$^{STIM}$ was inhibited by the EGFR inhibitor (AG) and EGFR neutralizing antibodies (αEGFR) (Figure 4A-D), but was not affected by the c-Met inhibitor (PF) (Figure 4A and B). This shows that although both EGFR and c-Met are potent activators of ERK1/2, the increase in ERK1/2 phosphorylation as observed upon stimulation with MSC-CM$^{STIM}$ is mainly mediated via the EGFR pathway.

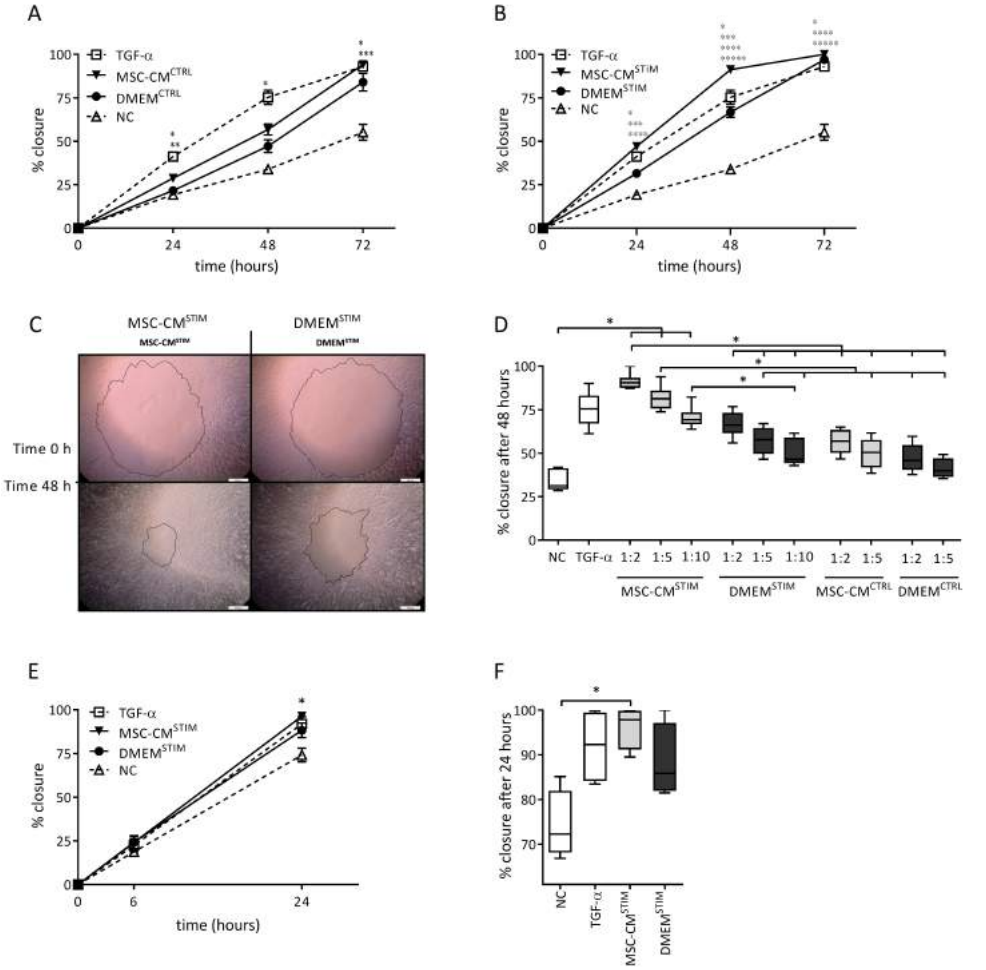


**Figure 2. MSC-CM increases wound closure.** (A, B) NCI-H292 cells were injured by making a circular wound with a diameter of 3 mm, and subsequently incubated with MSC-CM$^{CTRL}$, MSC-CM$^{STIM}$, DMEM$^{CTRL}$ and DMEM$^{STIM}$, and a negative control (NC, RPMI only) and positive control (RPMI supplemented with TGF-α 20 ng/ml). The wound size was measured at 0, 24, 48 and 72 hours after wounding. MSC-CM$^{STIM}$ significantly increased wound closure. The effect of MSC-CM$^{STIM}$ was dose-dependent. Error bars represent standard error of the mean (SEM). n=4-6; (*) $p<0.05$ TGF-α compared to NC and (**) compared to DMEM; (***) $p<0.05$ MSC-CM compared to NC and (****) compared to DMEM; (*****) $p<0.05$ DMEM$^{STIM}$ compared to NC. (C) Morphology of the closure of the wound: photos in the upper panel are taken at t=0 hours, the lower panel shows the same wounds photographed 48 hours later. The two photos on the left side are obtained from MSC-CM$^{STIM}$ stimulated cells, whereas photos at the right represent its control, DMEM$^{STIM}$. (D) Dose response. Box and whiskers represent median, interquartile range and minimum and maximum. n=4-6; (*) $p<0.05$. (E) Wound closure in ALI-PBEC measured at 6 and 24 (at 48 hours all wounds were closed). At 24 hours, MSC-CM$^{STIM}$ significantly enhanced wound healing compared to the NC, but no significant differences were observed compared to its control, DMEM$^{STIM}$. Error bars represent SEM, n=4; (*) $p<0.05$ for MSC-CM$^{STIM}$ compared to NC. (F) Wound closure in ALI-PBEC at 24 hours. Box and whiskers represent median, interquartile range and minimum and maximum, for n=4 as in fig. 2E; (*) $p<0.05$.

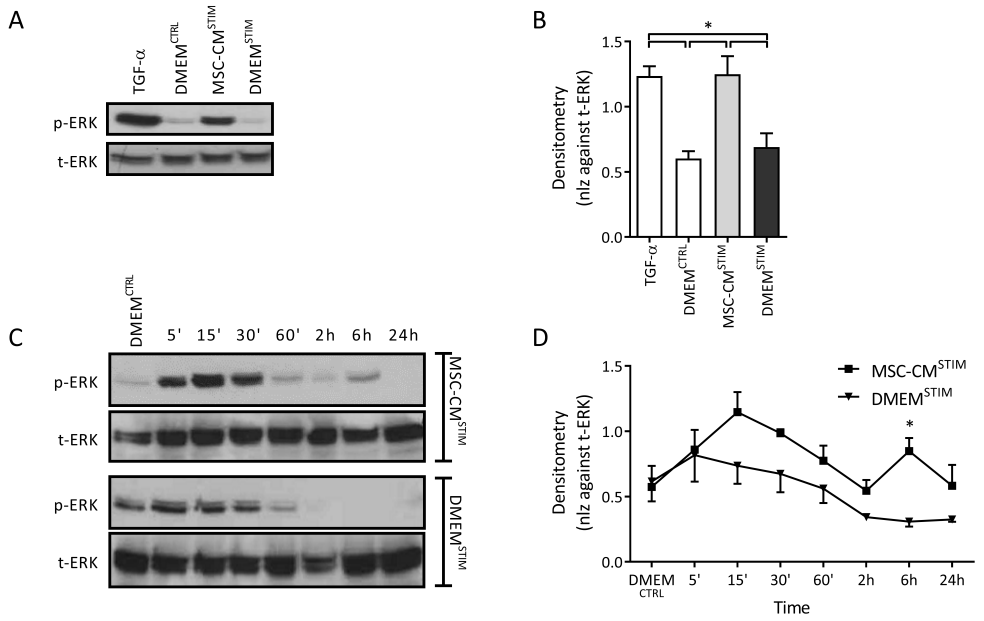


**Figure 3. MSC-CM$^{STIM}$ increases ERK1/2 phosphorylation.** NCI-H292 cells were incubated with MSC-CM$^{STIM}$, DMEM$^{STIM}$, DMEM$^{CTRL}$ or TGF-α 20 ng/ml (pos ctrl). ERK1/2 phosphorylation and total ERK was determined in cell lysates using Western blot. (A) After 15 minutes incubation time MSC-CM$^{STIM}$ increased ERK1/2 phosphorylation compared to its control. (B) Densitometry for figure 3A. Error bars represent SEM, n=6; (*) p<0.05. (D) Time course experiment, demonstrating that the effect of MSC-CM$^{STIM}$ was most prominent up to 30 minutes, but could still be observed up to 6 hours. DMEM$^{CTRL}$ was obtained at 15 minutes. (D) Densitometry for figure 3C. Error bars represent SEM, n=4-5; (*) p<0.05.

Besides direct EGFR-dependent activation of ERK1/2 by constituents of the MSC-CM, it is also known that airway epithelial cells promote wound healing in an autocrine manner, via transactivation of EGFR. In this process, matrix metalloproteinases, predominantly TACE/ADAM17, mediate the shedding of cell surface-bound EGFR ligands [34]. These ligands in turn activate ERK1/2 via EGFR. Addition of a TACE/ADAM17 inhibitor (TAPI-1) decreased ERK1/2 phosphorylation induced by MSC-CM$^{STIM}$ (Figure 4C and D). This suggests that EGFR transactivation through TACE/ADAM17 contributes to ERK1/2 phosphorylation in NCI-H292 monolayers, in addition to direct effects of MSC-CM$^{STIM}$. This raised the question whether MSC-CM$^{STIM}$ could induce EGFR ligand expression in airway epithelial cells. To investigate this, NCI-H292 were incubated with MSC-CM$^{STIM}$ and gene expression of several EGFR ligands was assessed. A significant increase of mRNA expression of both *AREG* and *HBEGF* was observed upon stimulation with MSC-CM$^{STIM}$ compared to its control (Figure 4E). Enhanced ERK1/2 activation promotes in part cell proliferation by increasing expression of Cyclin D1 (*CCDN1*) [35], a cell cycle regulator. In line with MSC-CM$^{STIM}$ induced ERK1/2 phosphorylation, the mRNA expression of *CCDN1* was significantly higher compared to its control, suggesting an increase in cell proliferation (Figure 4F).

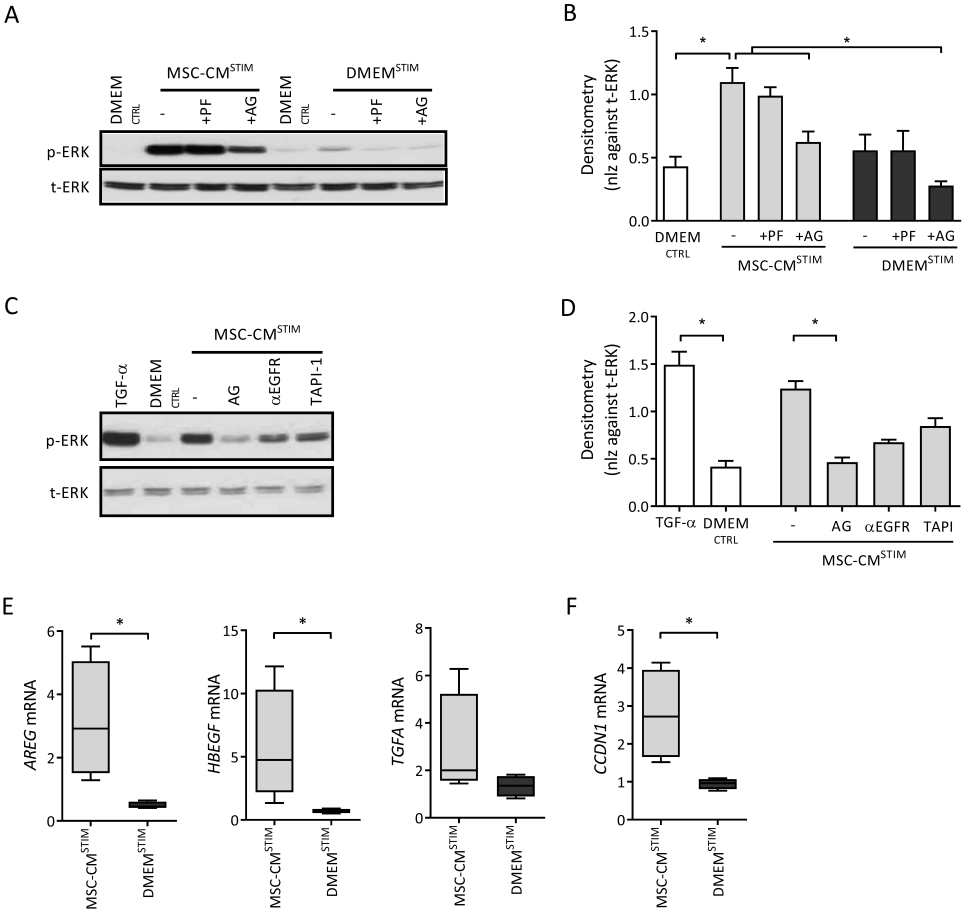


**Figure 4. MSC-CM$^{STIM}$ induced ERK1/2 phosphorylation is mediated via (trans)activation of EGFR.** (A) 1 hour pre-incubation of NCI-H292 with 0.05 μM PF04217903 and/or 10 μM AG1478, followed by 15 minutes stimulation with MSC-CM$^{STIM}$ or DMEM$^{STIM}$, or DMEM$^{CTRL}$ showed that inhibition of EGFR decreased ERK1/2 phosphorylation as determined using Western blot. (B) Densitometry for figure 4A. Error bars represent SEM, n=5; (*) p<0.05. (C) 1 hour pre-incubation with 2 μg/ml neutralizing anti-EGFR antibodies and 10 μM TAPI-1 followed by incubation with MSC-CM$^{STIM}$ during 15 minutes showed that these agents decreased ERK1/2 phosphorylation. (D) Densitometry for figure 4C. Error bars represent SEM, n=3; (*) p<0.05. (E) NCI-H292 cells were incubated with MSC-CM$^{STIM}$ or DMEM$^{STIM}$ and harvested after 9 hours for mRNA analysis. mRNA expression of the EGFR ligands *AREG*, *HBEGF* and *TGFA* was determined by qPCR. MSC-CM$^{STIM}$ significantly increased the expression of *AREG* and *HBEGF*. Expression of *TGFA* increased but this was not significant. (F) mRNA expression of the proliferation marker *CCDN1* was significantly increased. Values were normalized against *RPL13A* and *ATP5B* reference genes. Box and whiskers represent median, interquartile range and minimum and maximum. n=4; (*) p<0.05.

*Blocking of EGFR reduces the stimulatory effect of MSC-CM$^{STIM}$ on wound healing*

In NCI-H292 monolayers, stimulation with MSC-CM$^{STIM}$ resulted in increased ERK1/2 phosphorylation, and this process appeared to be predominantly regulated via EGFR signaling. To assess if this observation could be translated into a functional effect, wound healing experiments were repeated using the before mentioned tyrosine kinase inhibitors. To limit toxic side effects after prolonged exposure to high doses of AG, we adjusted the concentration of AG to 0.2 μM based on dose-response experiments; for the c-Met inhibitor PF a concentration of 0.05 μM sufficed (data not shown). The EGFR ligand TGF-α and the c-Met ligand HGF both significantly enhanced wound healing in NCI-H292 monolayers, indicating that both their corresponding receptors could be involved in MSC-mediated wound repair. Inhibition of EGFR as well as of c-Met blocked the effect of TGF-α and HGF, respectively (Figure 5A). In the presence of the EGFR inhibitor the effect of MSC-CM$^{STIM}$ on wound closure was significantly reduced to a level similar to that observed in the negative control. Blocking of the HGF receptor c-Met alone had no effect on wound closure induced by MSC-CM$^{STIM}$ (Figure 5B). These data indicate that signaling through EGFR is the predominant pathway by which MSC-CM$^{STIM}$ increased wound healing in NCI-H292 epithelial cells.

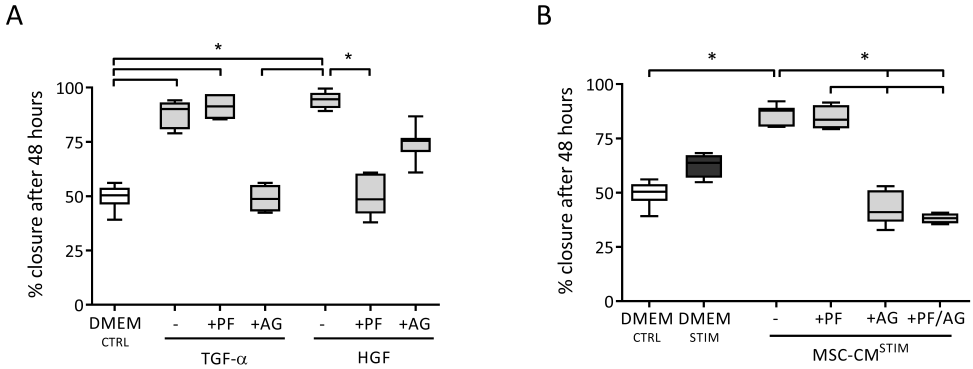


**Figure 5. Blocking of EGFR reduces the stimulatory effect of MSC-CM$^{STIM}$ on wound closure.** (A) NCI-H292 wounded cell layers were stimulated with MSC-CM$^{STIM}$ or DMEM$^{STIM}$, DMEM$^{CTRL}$, or DMEM supplemented with TGF-α 20 ng/ml or HGF 20 ng/ml (positive controls). Inhibitors of EGFR (0.2 μM AG1478) or c-Met (0.05 μM PF04217903) were added during the full culture period in assigned conditions. At 48 hours, HGF and TGF-α both significantly enhanced wound healing in NCI-H292 cells, which was averted to a level comparable to the negative control by their respective inhibitors. (B) In the presence of the EGFR inhibitor, the wound healing capacity of MSC-CM$^{STIM}$ decreased to values below those observed in the negative control as determined after 48 hours. Inhibition of c-Met alone had no effect on wound healing induced by MSC-CM$^{STIM}$. Box and whiskers represent median, interquartile range and minimum and maximum. n=4-7; (*) $p<0.05$.

## DISCUSSION

This study shows for the first time that stimulation of MSCs with pro-inflammatory cytokines improves their capacity to enhance airway epithelial repair in an *in vitro* repair model using the airway epithelial cell line NCI-H292. We show that conditioned medium from human bone marrow-derived MSCs increases wound healing in airway epithelial cells and that this effect is significantly enhanced when the MSCs are treated with a mixture of pro-inflammatory cytokines, i.e. TNF-α and IL-1β. These cytokines increased the mRNA expression of the growth factors *FGF2, HBEGF*, *HGF*, and of *IL6* in MSCs. We provide evidence for the possible involvement of the following mechanisms in this enhancing effect of MSC-CM$^{STIM}$ on wound repair (Figure 6): first, MSC-CM$^{STIM}$ directly activated the MAP kinase ERK1/2 via EGFR, resulting in wound healing. Second, MSC-CM$^{STIM}$ caused ADAM-mediated transactivation of EGFR, further contributing to wound healing. Third, MSC-CM$^{STIM}$ increased mRNA expression of EGFR ligands *AREG* and *HBEGF* in NCI-H292 airway epithelial cells.

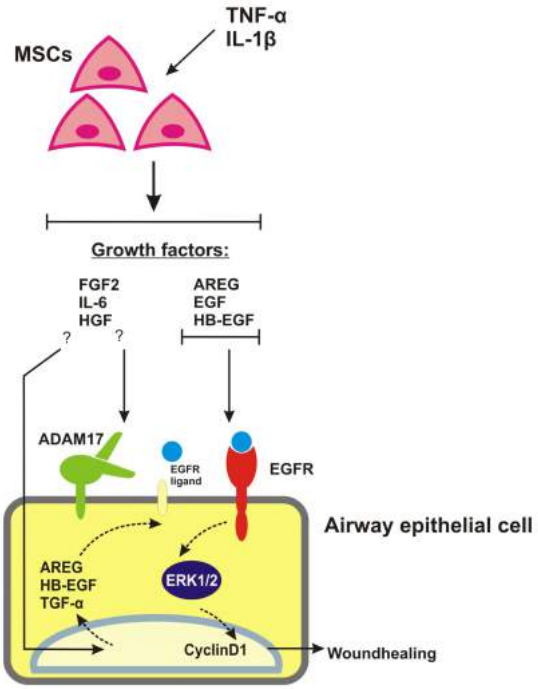


**Figure 6. Proposed model of enhanced wound closure in airway epithelial cells by MSC-CM$^{STIM}$.** In MSCs, exposure to TNF-α and IL-1β increases the mRNA expression of various growth factors. Conditioned medium from these MSCs contributes to wound healing of airway epithelial cells via three mechanisms: direct activation of EGFR; activation of matrix metalloproteases which results in shedding of membrane bound EGFR ligands; induction of mRNA expression of EGFR ligands by the airway epithelium. Activation of the downstream MAP kinase ERK1/2, which is known as a regulator of cell proliferation (a.o. assessed by increased Cyclin D1) and differentiation, subsequently results in increased wound healing.

Previous studies show that MSCs contribute to epithelial repair in wound healing models *in vivo* as well as *in vitro* [8,17,36], and that pro-inflammatory cytokines can attract MSCs to sites of inflammation [37]. Within such an inflammatory environment, MSCs display an anti-inflammatory phenotype that is characterized by an increased expression of *IL6* [38]. In line with this observation, we noted increased expression of *IL6* in MSCs stimulated with TNF-α and IL-1β, suggesting anticipated response to these pro-inflammatory stimuli. Besides increased mRNA expression of *IL6,* we observed increased mRNA expression of several growth factors in TNF-α/IL-1β stimulated MSCs, suggesting that the regenerative potential of these MSCs is enhanced. The increased mRNA expression of several EGFR ligands was not accompanied by increased levels of these ligands as detected by ELISA (data not shown). This may be explained by limitations in the sensitivity of the ELISAs, rapid binding of secreted growth factors to their cellular receptors, and/or by the fact that the observed effects are not explained by detectable levels of single EGFR ligands, but by synergisms between various released mediators. In line with our observations, previous reports also demonstrated that stimulation with pro-inflammatory cytokines induces growth factor expression by MSCs [20,39,40]. However, to our knowledge this is the first study demonstrating that pro-inflammatory cytokine stimulation of MSCs induces wound healing in airway epithelial cells, and our data indicate that this may involve the action of growth factors. Together, this suggests that the capacity of MSCs to enhance wound repair may be increased upon recruitment to areas of inflammation where they are exposed to pro-inflammatory cytokines [25]. This is relevant for a disease such as COPD, where both inflammation and tissue damage are present.

Amongst the growth factors induced in TNF-α/IL-1β-stimulated MSCs, EGFR ligands and HGF are involved in airway epithelial wound repair [41-44]. By using inhibitors for EGFR and c-Met, we show that the effect of MSC-CM$^{STIM}$ on both ERK1/2 activation as well as on wound healing was mediated by EGFR activation, without an apparent effect of c-Met inhibition. As it has been shown that HGF can promote airway epithelial wound repair [43,44], we speculate that in our model HGF has a more subtle contribution to wound healing that is masked by the major role of EGFR signaling in airway epithelial repair. This is supported by a study from Curley *et al,* who showed that the addition of HGF-neutralizing antibodies to MSC-CM in a scratch wound assay using A549 alveolar epithelial cells did not affect wound repair [45]. Besides HGF and EGFR ligands, other MSC-CM$^{STIM}$ constituents can contribute to wound healing. One example of a possibly involved MSC-derived mediator is IL-6, which was previously found to contribute to MSC-mediated epithelial wound repair [36,46]. Although in our model blocking of EGFR fully inhibited the stimulatory effect of MSC-CM$^{STIM}$, we cannot exclude a contribution of MSC-derived IL-6.

In addition to direct EGFR activation, we observe that MSC-CM$^{STIM}$ activates ERK1/2 via transactivation of EGFR. EGFR-transactivation results from activation of G-protein coupled receptors (GPCRs) that activate proteases of the ADAM family, such as TACE/ADAM17. These proteases cleave cell surface-bound EGFR ligands, resulting in autocrine EGFR activation [34].

Numerous factors are able to activate GPCRs, and our study focussed on the role of a modest selection of growth factors and chemokines. It was beyond the scope of this study to investigate other ligands potentially released by MSCs, but this will be an interesting point for future investigations.

In our model, we have used the airway epithelial cell line NCI-H292. This cell line has been shown to respond in a similar fashion as primary epithelial cells of the lung and is frequently used to study effects mediated by the EGFR axis [47-50]. The use of a cell line limits translation to the *in vivo* situation, where in addition to the epithelium also immune and endothelial cells interact in the process of wound repair. The benefit on the other hand is that it allows for detailed investigation of MSC-CM$^{STIM}$ effects on the ERK1/2 signaling pathway as well as on cell proliferation.

The circular wounds used in this study are relatively large and NCI-H292 cells require cell proliferation in order to close this type of wound as we have shown previously [32]. This adds information about effects on cell proliferation that cannot be obtained when using primary bronchial epithelial cells (PBEC) using wounds of a similar size, as migratory mechanisms appear to suffice for the closure of this type of wounds [51]. For the same reasons, the wound repair model also provides additional information to the more commonly used scratch wound assays, as these scratch wounds close quickly, and primarily via cell migratory mechanisms [17]. Using ALI-PBEC, we observed effects of MSC-CM$^{STIM}$ on epithelial wound healing, but unlike the observation in NCI-H292, this effect was not significantly different compared to control medium (DMEM$^{STIM}$). We speculate that higher intrinsic rate of wound closure in ALI-PBEC and donor variability limited the experimental window to observe beneficial effects of MSC-CM. Besides, differences in intrinsic wound healing characteristics (e.g. primarily via migration rather than proliferation in ALI-PBEC), as well as direct effects of TNF-$\alpha$ and IL-1$\beta$ on migratory processes might further explain differences in effects of MSC-CM$^{STIM}$ on ALI-PBEC versus NCI-H292.

Our data provide evidence for the concept of a cell-based therapy with cells that specifically interact with an inflammatory environment to enhance their beneficial properties. Safety concerns regarding tumorigenesis or fibrosis might arise when using cell-based therapies, but based on current data obtained from clinical trials the use of MSCs in patients is considered to be safe [52]. Moreover, it has been shown in multiple studies that MSCs can induce apoptosis of cancer (but not healthy) cells via tumor necrosis factor-related apoptosis-inducing ligand [53,54].

MSCs are currently considered as treatment for COPD and the first patient safety and feasibility study has recently been published [55]. Interestingly, the results from the present study suggest that exposure of MSCs to pro-inflammatory cytokines increases their ability to repair damaged tissue. This may imply that MSCs are more effective at sites of inflammation and that *in vitro* stimulation of MSCs with cytokines before infusion in patients may potentially result in a larger therapeutic effect. Future investigations should be directed at further exploring the mechanisms involved in the enhancing effect of MSCs on epithelial wound healing, using e.g. primary airway and alveolar epithelial cells, preferably using co-cultures of MSCs with epithelial cells.

## CONCLUSIONS

We have found that MSC conditioned medium obtained from MSCs stimulated with a mixture of cytokines potently enhances wound repair in injured airway epithelial cells. This effect is predominantly mediated by (trans)activation of EGFR and subsequent activation of the ERK1/2 signaling cascade. This observation implies that in areas of tissue damage where inflammatory mediators are present (such as in the lungs of COPD patients), MSCs may display increased regenerative properties via the secretion of growth factors. This supports the concept that MSCs are a promising candidate for cell-based therapy in inflammatory lung diseases such as COPD.



## ACKNOWLEDGEMENTS

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**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ADAM | a disintegrin and metalloprotease |
| ALI | air liquid interface |
| AREG | amphiregulin |
| BSA | bovine serum albumin |
| BPE | bovine pituitary extract |
| CCDN1 | cyclin D1 |
| CM | conditioned medium |
| COPD | chronic obstructive pulmonary disease |
| EGF(R) | epidermal growth factor (receptor) |
| ERK | extracellular signal-regulated kinase |
| FGF2 | fibroblast growth factor 2 |
| GPCR | G-protein coupled receptor |
| HB-EGF | heparin binding EGF-like growth factor |
| HC | hydrocortisone |
| HGF | hepatocyte growth factor |
| IL-1β | interleukin-1β |
| IL-6 | interleukin-6 |
| MAPK | mitogen activated protein kinase |
| MMP | matrix metalloproteinase |
| MSC | mesenchymal stromal cell |
| MSC-CM$^{CTRL}$ | conditioned medium obtained from unstimulated MSCs |
| MSC-CM$^{STIM}$ | conditioned medium obtained from TNF-α/IL-1β stimulated MSCs |
| PBEC | primary bronchial epithelial cells |
| PDGFA | platelet derived growth factor a |
| RA | retinoic acid |
| SEM | standard error of the mean |
| TACE | TNF-α converting enzyme |
| TGF-α | transforming growth factor-α |
| TNF-α | tumor necrosis factor-α |
| VEGF(R) | vascular endothelial growth factor (receptor) |

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# FUNCTIONAL CHARACTERIZATION OF BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS FROM COPD PATIENTS

*CHAPTER 4*

**ERJ OPEN RESEARCH, 2016 Jun 28; 2 (2)**

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**ABSTRACT**

Autologous bone marrow-derived mesenchymal stromal cells (BM-MSCs) are evaluated for clinical use in COPD patients, but it is unclear whether COPD affects BM-MSCs.

To investigate this, BM-MSCs from 9 COPD patients and 9 non-COPD age-matched controls were compared in immunophenotype, growth and differentiation potential, and migration capacity. Other functional assays included the response to proinflammatory stimuli and inducers of the Nrf2 antioxidant response element (Nrf2-ARE) pathway, and effects on NCI-H292 airway epithelial cells.

No significant differences were observed in morphology, proliferation and migration, except for increased adipocyte differentiation potential in the COPD group. Both groups were comparable regarding mRNA expression of growth factors and inflammatory mediators, and in their potential to induce mRNA expression of EGFR ligands in NCI-H292. MSCs from COPD patients secreted more IL-6 in response to proinflammatory stimuli. Activation of the Nrf2-ARE pathway resulted in a comparable induction of mRNA expression of 4 target genes, but the expression *of NQO1* was lower in MSCs from COPD patients.

The observation that MSCs from COPD patients are phenotypically and functionally comparable to those from non-COPD controls implies that autologous MSCs can be considered for use in the setting of clinical trials as a treatment for COPD.

## INTRODUCTION

Mesenchymal stromal cells (MSCs) are defined as plastic adherent cells with the capacity to self-renew and differentiate into multiple lineages of the mesenchyme [1]. Importantly, it has been shown that MSCs stimulate recovery of damaged tissue via paracrine effects, potentiation of cell growth and wound healing, suppression of apoptosis and possibly induction of endogenous progenitor cell potential [2]. In addition to their regenerative potential, MSCs have pleiotropic effects on several immune cells and can contribute to immune responses by modifying the inflammatory environment [3].

In light of these regenerative and immunomodulatory properties of MSCs, interest has arisen in the clinical application of MSCs as a treatment for chronic obstructive pulmonary disease (COPD). COPD is a heterogeneous disease, defined by persistent airflow limitation that is usually progressive. Mechanisms that contribute to COPD development include exaggerated inflammatory responses to inhaled noxious gases, imbalance between proteinases and proteinase inhibitors, and excessive oxidative stress [4]. On a cellular level, an imbalance of cell death and replenishment of structural cells ultimately results in tissue damage.

In COPD, MSC-based treatments have already been used in the context of clinical trials investigating both allogeneic [5] and autologous MSCs (ClinicalTrials.gov NCT01306513). A concern when using MSCs is that donor-related factors might affect the therapeutic potential of MSCs. This is conceivable in MSCs from COPD patients, since COPD is considered to have a systemic component [6]. Indeed, in a preliminary report by Jahn *et al*, functional differences between MSCs from COPD and control patients were observed [7]. Alternatively, it has been hypothesized that altered MSC function contributes to the development of COPD [8].

Since autologous MSCs are currently used in clinical trials to evaluate their ability to induce (favourable) responses in lung tissue, it is relevant to know whether MSCs from COPD patients have similar properties and potential as MSCs from healthy donors. In this study this was investigated using *in vitro* cultures of bone marrow-derived MSCs (BM-MSCs) from COPD patients and age-matched non-COPD controls. We compared the MSC (immuno)phenotype and several functional parameters, including differentiation and migration, response to proinflammatory stimuli and inducers of the Nuclear factor (erythroid derived 2)-like 2 antioxidant response element (Nrf2-ARE) pathway, and regenerative effects on airway epithelial cells.

## MATERIALS AND METHODS

A more detailed description of the methods used for this study is provided in an online supplement, available via ERJ Open Research.

*Patients and ethical considerations*

BM-MSCs were obtained from COPD patients participating in a clinical trial to evaluate BM-MSCs for severe emphysema (ClinicalTrials.gov NCT01306513), and from non-COPD controls [9].

Groups were matched for age (COPD 53.1 ± 6.4 years [mean ± SD] versus non-COPD 48.8 ± 4.5; p=0.12) and sex (both groups 3 males out of 9). The control group included 1 Asian donor, all others were Caucasian. COPD donors had $FEV_1$ < 40% predicted, emphysema on CT-scan, and had quit smoking at least 6 months prior to bone marrow aspiration. Controls had no known pulmonary disease, and were never smokers (the smoking history of three controls could not be traced). The use of MSCs for preclinical research was approved by the Medical Ethical Committee of the Leiden University Medical Center, and donors consented to the possible use of their MSCs for this purpose.

*MSC cultures and characterization*

MSC isolation from bone marrow and expansion *in vitro* was done following a previously published protocol [10], monitoring expansion rates during initial cultures. MSC immunophenotype was confirmed by FACS analysis, differentiation potential was quantified by measuring adipocyte, osteoblast and calcium staining intensity of differentiated MSCs, and migration was assessed using Electric Cell-substrate Impedance Sensing (ECIS). MSCs at passage three or four were used for experiments, after overnight incubation in serum-free (SF) medium.

*Stimuli*

Cigarette smoke extract (CSE) and sulforaphane were used to induce the Nrf2-ARE pathway [11-13]. Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) (Peprotech, Rocky Hill, NJ, USA) at 20 ng/ml were used as proinflammatory stimuli to evaluate immune modulatory properties and growth factor induction. MSC-conditioned medium was generated by culturing MSCs during 24 hours in SF medium, or in SF medium supplemented with TNF-α/IL-1β and was used to stimulate NCI-H292 airway epithelial cells.

*qPCR and ELISA*

MSCs were stimulated during 6 hours for quantitative real-time PCR (qPCR) analysis, or during 24 hours for ELISAs. NCI-H292 airway epithelial cells were incubated during 9 hours with MSC-CM to assess induction of mRNA expression of EGFR-ligands.

Following RNA extraction and cDNA synthesis, qPCR was performed in triplicate using primers for target genes (supplementary Table S1), and relative gene expression compared to reference genes was calculated according to the standard curve method, with housekeeping genes selected based on Genorm software [14], i.e. *B2M* and *RPS29* for MSCs and *ATP5B* and *RPL13A* for NCI-H292.

IL-6 (Sanquin, Amsterdam, the Netherlands) and IL-8 (R&D Systems, Minneapolis, MN, USA respectively) secretion was measured using ELISA, following the manufacturer's protocol. Optical density values were measured with a microplate reader.

*Statistical analysis*

Data analysis and composition of the figures was done using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS 20 (IBM SPSS Statistics, Armonk, NY, USA). Statistical analysis was performed using Mann-Whitney U-test for analysis of differences between subject groups or Wilcoxon matched pairs signed rank test for paired observations to compare conditions within groups. Differences were considered statistically significant at $p<0.05$.

## RESULTS

*BM-MSCs from COPD patients have the same (immune)phenotype as non-COPD controls, apart from a stronger potential towards adipocyte differentiation*

BM-MSCs from COPD patients and non-COPD controls were morphologically similar, displaying the characteristic spindle-shaped appearance (Figure 1A). No differences were observed in immunophenotype (Figure 1B) and all MSCs could be differentiated into adipocytes and osteoblasts, confirming their MSC phenotype. MSCs from COPD donors showed a significantly higher potential to differentiate into adipocytes than MSCs from non-COPD donors (Figure 1C). No significant differences were observed in osteoblast differentiation and mineralization potential. In 6 patients (4 in the non-COPD group, 2 in the COPD group), no data were obtained for osteoblast differentiation, mineralization or both, due to ruptures and subsequent detachment of the monolayer, caused by contractility of cells at the periphery of the well during differentiation.

*Proliferation and migration potential of MSCs is not affected in MSCs from COPD patients*

Proliferation was assessed during expansion following initial isolation from bone marrow. No differences were observed between groups regarding the time between passages, and the number of MSCs harvested per passage (Figure 2A). Migration was assessed as the potential of MSCs to cover the surface area of an electrode using ECIS analysis to measure resistance and capacitance. This showed that the migration potential of MSCs from COPD patients was similar to that of non-COPD controls (Figure 2B).

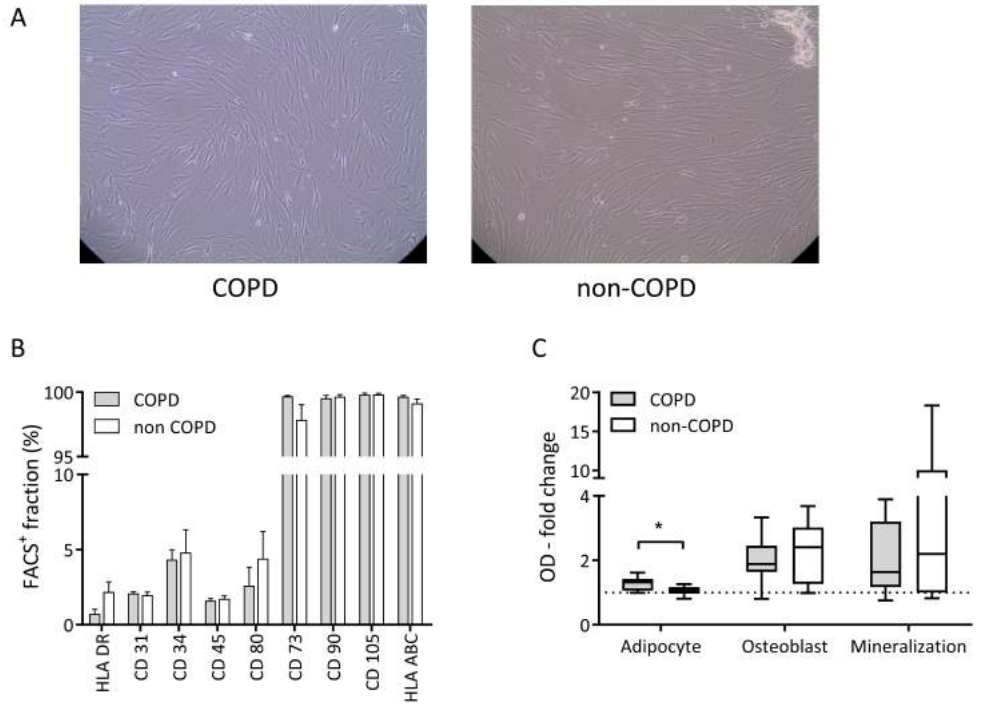


**Figure 1. MSCs phenotype from COPD and non-COPD patients.** Bone marrow-derived MSCs from COPD and non-COPD patients cultured *in vitro* were characterized by FACS analysis and by their potential to differentiate into mesenchymal lineages. (A) Morphology of MSCs from COPD and non-COPD donors: example of MSC culture. (B) FACS data. Per antibody, data are shown as mean ± SEM (n=9 per group). (C) Differentiation into adipocytes and osteoblasts, and mineralization potential. Staining intensity of oil red O, alkaline phosphatase and alizarin red (calcium) was quantified on a microplate reader, and per donor OD-values were calculated as fold change compared to its control (co-cultured undifferentiated MSCs). Box whiskers represent median, interquartile range and minimum and maximum values. * $p < 0.05$ for n = 9 per group (for osteoblast/mineralization 6-8 donors/group).

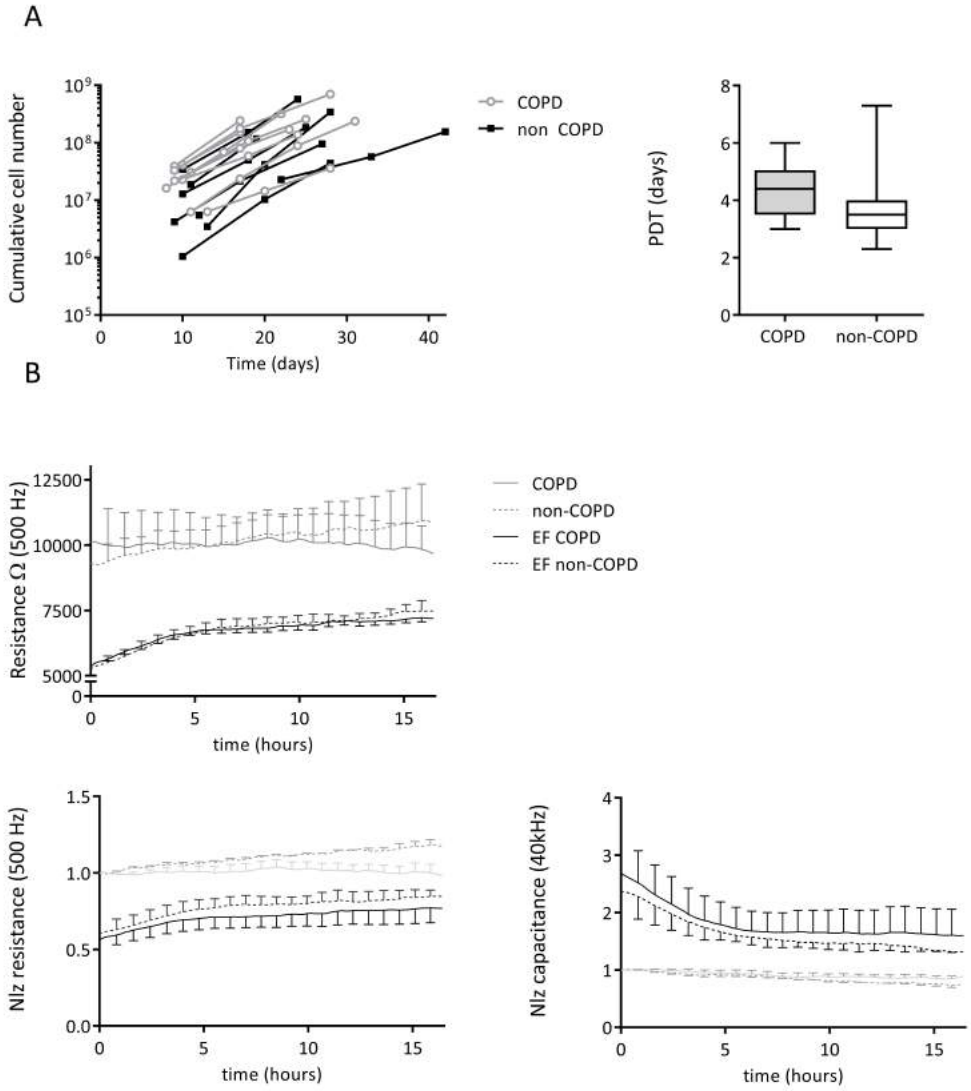


**Figure 2. MSC proliferation and migration capacity.** (A) *In vitro* proliferation of MSCs from COPD and non-COPD patients was followed over time. Time needed for cultures to become near-confluent and the number of MSCs that were obtained per passage were assessed. Population doubling time (PDT) in days was calculated by dividing the natural logarithm of 2 by the exponent of growth. Box whiskers represent median and interquartile range, minimum and maximum values. (B) Migration as assessed using ECIS. MSCs were cultured in ECIS arrays in the presence of an electrical fence (EF), which prevented cell adherence across the electrode area. Resistance (at 500 Hz) and capacitance (at 40 kHz) were measured continuously and followed up to 15 hours after removal of the electrical fence (at time = 0). Restoration of the resistance and capacitance to control values (corresponding to full coverage of the electrode) was used as a measure for migration capacity of MSCs. Normalized (nlz) data were obtained by correcting for the resistance/capacitance values obtained in control wells without an electric fence, at the time the electric fence was removed. Data are shown as mean ± SEM. n = 5-8 per group.

*BM-MSCs' response to proinflammatory stimuli is similar between groups, except for higher IL-6 secretion in MSCs from COPD patients*

Previously, MSCs were shown to express growth factors and immune mediators upon exposure to proinflammatory stimuli [15,16]. We therefore investigated whether this response was preserved in MSCs from COPD patients. In MSCs from COPD patients as well as non-COPD controls, incubation with TNF-α and IL-1β resulted in a significant induction of gene expression of Amphiregulin (*AREG*), Heparin-binding Epidermal Growth Factor like Growth Factor (*HBEGF*), Fibroblast Growth factor 2 (*FGF2*), Chemokine (C-C motif) ligand 20 (*CCL20*), Interleukin 6 (*IL6*), Chemokine (C-X-C motif) ligand 8 (*CXCL8*) and Tumor necrosis factor-Stimulated Gene 6 (*TSG6*) in both the COPD and non-COPD group (Figure 3A). Furthermore, it increased Transforming growth factor-α (*TGFA*) in the non-COPD group, and hepatocyte growth factor (*HGF*) in the COPD group (data not shown), and lowered expression of Adrenomedullin (*ADM1*) in both groups (Figure 3A). Between groups, no significant differences were observed. No significant induction was observed for other genes investigated (Supplementary Figure S1).

IL-6 and IL-8 protein secretion was measured in medium obtained from TNF-α and IL-1β exposed MSCs. In unstimulated MSCs, IL-8 levels were below the detection limit, and IL-6 secretion was comparable between groups (164 ± 27.7 pg/ml [mean ± SEM] for COPD, versus 174 ± 31.6 pg/ml in non-COPD, p=0.34). Upon stimulation with proinflammatory cytokines, IL-8 and IL-6 secretion significantly increased in MSCs from both COPD and non-COPD donors. Differences between groups were observed, as MSCs from COPD patients secreted significantly higher amounts of IL-6, and showed a trend towards higher IL-8 secretion (p=0.12) (Figure 3B).

*Oxidative stress response in MSCs from COPD patients is comparable to non-COPD MSCs*

In COPD, insufficient antioxidant response has been implicated in disease pathogenesis [17]. Therefore, we evaluated the antioxidant response of MSCs from COPD patients, using CSE and sulforaphane to activate the Nrf2-ARE pathway and assessed induction of several downstream target genes involved in antioxidant response [11-13]. In both groups, incubation of MSCs with CSE resulted in a significant induction of heme oxygenase 1 (*HMOX1*), NAD(P)H:quinone reductase 1 (*NQO1*) and smoke and cancer-associated lncRNA-1 (*SCAL1*). For *HMOX1* and *SCAL1*, this induction was dose dependent. Glutathion peroxidase 2 (*GPX2*) expression decreased in CSE exposed MSCs. Induced gene expression of *NQO1* and *SCAL1* was generally lower in MSCs from COPD patients, resulting in significant differences between groups in *NQO1* expression at higher concentrations of CSE (Figure 4A).

Sulforaphane significantly increased all analysed Nrf2-ARE regulated target genes in non-COPD patients, whereas it only significantly induced *HMOX1* and *SCAL1* gene expression in MSCs from COPD patients (Figure 4B). Despite this observation, groups did not significantly differ from each other in their response to sulforaphane.

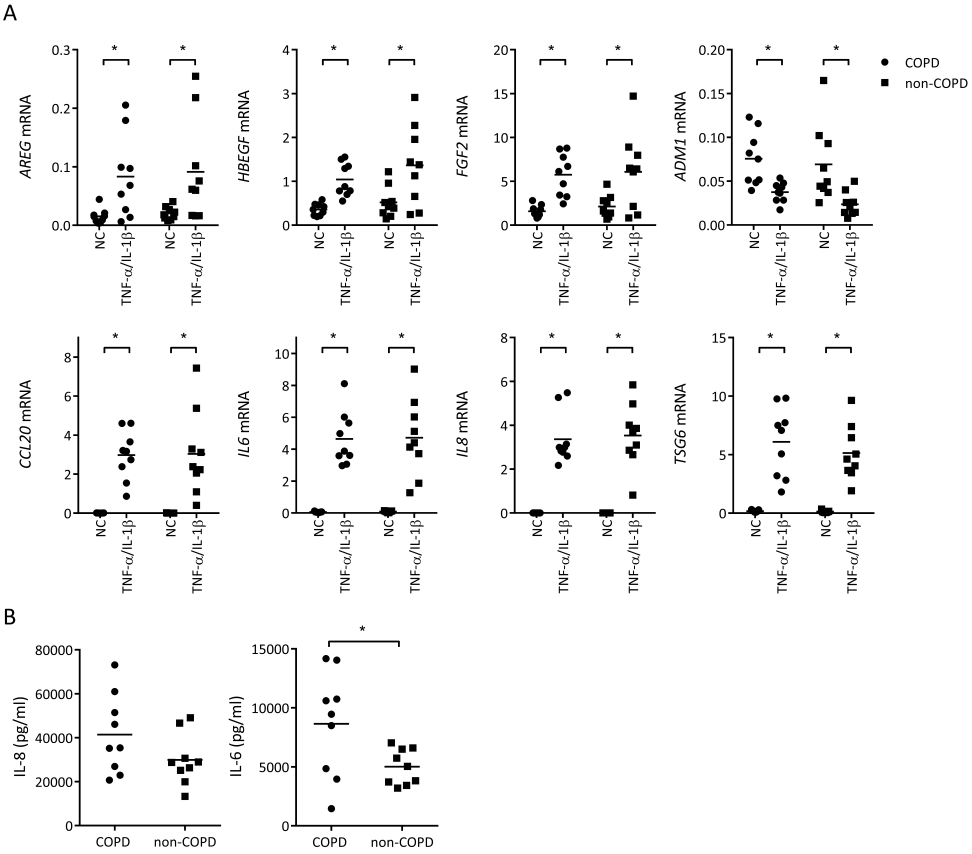


**Figure 3. Induction of immune mediators and growth factors upon stimulation with proinflammatory cytokines.** MSCs were stimulated with TNF-α and IL-1β (20 ng/ml each) or control medium (NC) during 6 hours. (A) mRNA expression of immune mediators and growth factors, normalized for housekeeping genes, shown for COPD (circles) and non-COPD donors (squares). Individual data are shown in graphs, horizontal bars represents mean. * $p < 0.05$ for n = 9 per group. (B) IL-6 and IL-8 protein secretion by MSCs from COPD and non-COPD donors, assessed 24 hours after addition of TNF-α and IL-1β. Individual data points are shown, horizontal bars represent mean. * = $p < 0.05$ for n = 9 per group.

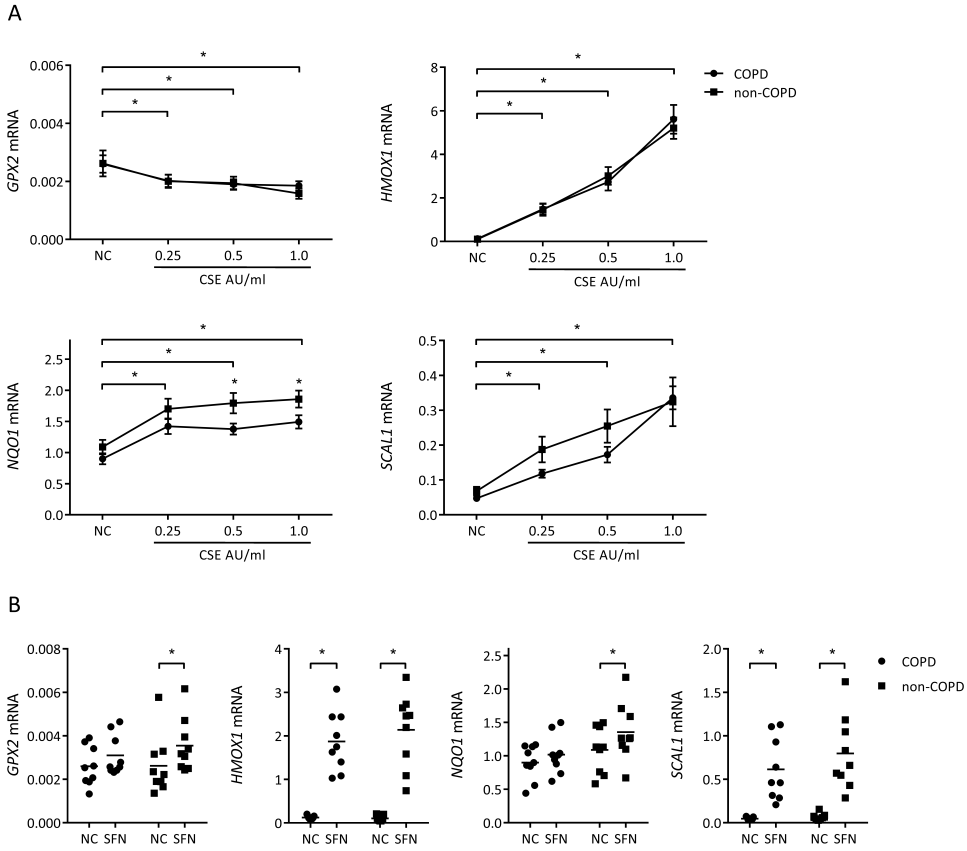


**Figure 4. Induction of Nrf2-ARE target genes in MSCs from COPD and non-COPD donors.** Gene expression of *GPX2*, *HMOX1*, *NQO1* and *SCAL1* was assessed in MSCs from COPD and non-COPD donors 6 hours after addition of cigarette smoke extract (CSE) or sulforaphane (SFN), which are both inducers of the Nrf2-ARE pathway, or plain culture medium as a negative control (NC). (A) Normalized mRNA expression in response to increasing concentrations of CSE. Data are presented as mean ± SEM. * $p < 0.05$ for n = 9 per group (except CSE 1.0 AU/ml: n = 7 in COPD group). (B) Normalized mRNA expression in response to 25 μM SFN, compared to housekeeping genes. Individual data are shown, with mean represented by the horizontal bar. * $p < 0.05$ for n = 9 per group.

*The regenerative potential of MSCs from COPD patients is comparable to non-COPD MSCs*

MSC conditioned medium (MSC-CM) from healthy donors induced EGFR ligand expression in NCI-H292 airway epithelial cells [15]. We assessed if MSC-CM from COPD patients has the same regenerative potential by investigating induction of mRNA expression of EGFR ligands in NCI-H292 airway epithelial cells, following incubation with MSC-CM$^{CTRL}$ (from MSCs cultured in SF medium) or MSC-CM$^{STIM}$ (from MSCs cultured in SF medium supplemented with TNF-α/IL-1β). Compared to control culture medium, both MSC-CM$^{CTRL}$ as well as MSC-CM$^{STIM}$ induced gene expression of the EGFR ligands *AREG* and *HBEGF* and to a lesser extent of *TGFA* (data not shown)

in NCI-H292. In addition, both MSC-CM$^{CTRL}$ and MSC-CM$^{STIM}$ induced mRNA expression of the cell cycle regulator Cyclin D1 (*CCDN1*), that is considered as a marker for cell proliferation. Compared to MSC-CM$^{CTRL}$ no significant differences were observed between the COPD and non-COPD groups in the potential of MSC-CM$^{STIM}$ to induce EGFR ligands in NCI-H292 airway epithelial cells, although there were differences in the significance of effects of MSC-CM$^{STIM}$ from non-COPD and COPD patients (Figure 5).

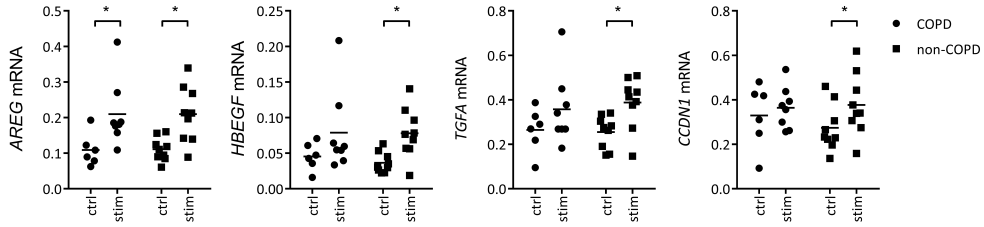


**Figure 5. Paracrine effects of MSCs on H292 airway epithelial cells.** NCI-H292 cells were incubated with conditioned medium obtained from MSCs. mRNA expression of EGFR ligands after 9 hours incubation with conditioned medium from MSCs cultured in serum free LG-DMEM (ctrl, from MSC-CM$^{CTRL}$) or in SF LG-DMEM supplemented with TNF-α and IL-1β (stim, from MSC-CM$^{STIM}$). Normalized values compared to housekeeping genes are shown. Box whiskers represent median, interquartile range and minimum and maximum values. * $p < 0.05$ for $n = 9$ per group. For all genes investigated, the mRNA expression was lower in cells cultured in control medium (LG-DMEM without additional stimulation) compared to mRNA expression in cells cultured in MSC-CM (data not shown).

## DISCUSSION

In this study we show that MSCs from patients with COPD are largely comparable in phenotype and function to MSCs from non-COPD controls. MSCs from COPD patients responded similarly to those from non-COPD controls for the majority of the investigated parameters, including (immuno) phenotype, proliferation and migration potential, response to proinflammatory stimuli and effects on airway epithelial cells. However, we did observe differences in adipocyte differentiation, IL-6 secretion in response to proinflammatory stimuli, and induction of gene expression of *NQO1* upon stimulation of the Nrf2-ARE pathway. These observations have important implications for the use of autologous MSCs as a potential new therapy to treat COPD.

This is the first study in humans that investigates whether MSCs from COPD patients are comparable to those from healthy controls. Our observation that underlying COPD does not appear to affect MSC function is in line with previous observations made in chronic systemic diseases [18-21]. In contrast, altered MSC function was observed in MSCs from patients with disorders of the central nervous system [22,23], and altered MSCs growth and differentiation has been observed in hematologic diseases [24,25]. Possibly, these differences might relate to the affected organ of the underlying disease, which is especially conceivable for diseases originating from the bone marrow.

MSCs secrete a spectrum of soluble factors, including cytokines, chemokines and growth factors [2,3], and it was shown that this secretion differs depending on for instance site of origin of MSCs, or systemic conditions such as hypoxemia [26,27]. Likewise, a proinflammatory environment can 'activate' MSCs, as proinflammatory cytokines were shown to induce expression of immune mediators and trophic factors in MSCs (reviewed in [16]). We used the proinflammatory cytokines TNF-$\alpha$ and IL-1$\beta$ that are implicated in COPD pathogenesis [28,29] and likewise observed induction of immune mediators and growth factors, which was unaffected by COPD status. Along with the observation that MSC-CM-induced EGFR ligand expression in airway epithelial cells was comparable between groups, these data provide evidence that MSCs from COPD patients respond in a similar fashion to inflammation. This suggests that their ability to modify inflammation and induce repair upon recruitment to inflammatory sites, such as the lung in COPD, is preserved.

MSCs from COPD and non-COPD donors were phenotypically similar and all had trilineage differentiation potential, confirming their status as progenitor cells. We found significantly higher adipocyte differentiation in MSCs from COPD patients compared to their healthy counterparts and a trend towards lower osteogenic differentiation. Our data might have even underestimated the osteogenic differentiation potential of MSCs from the non-COPD group, due to loss of the differentiated monolayer due to strong contractility of differentiated cells at the wells periphery, in a substantial subgroup of COPD and non-COPD subjects.

Similar to MSCs from COPD patients, increased adipocyte differentiation has been observed in aged MSCs and this appears to occur at the expense of osteoblast differentiation [30-32]. A definite statement about this so called "adipogenic switch" in aged MSCs has not yet been made due to inconsistencies between studies [33]. However, if cellular ageing affects the differentiation potential of MSCs, then based on the current data we propose that increased cellular ageing may underly the "adipogenic-switch" we observed in MSCs from COPD patients. Interestingly, accelerated cellular ageing has been thought to underlie COPD [34]. Since oxidative stress contributes to cellular ageing [35] as well as to COPD pathogenesis [17], we speculate that oxidative stress might explain the shared features of altered differentiation between MSCs from COPD patients and aged MSCs. This hypothesis is supported by observations in aged mice MSCs, that display decreased antioxidant power despite increases in proteins involved in antioxidant defence [36], which resembles observations made in COPD patients who have decreased antioxidant capacity [37], despite increased Nrf2 expression [38]. Concordantly, we observed lower expression of NQO1 and SCAL1 in MSCs from COPD patients, suggesting a lower antioxidant response upon induction of the Nrf2-ARE pathway. We found no differences in HMOX1 and GPX2 expression between groups, potentially explained by the role of other signalling pathways (c-Met and p63, resp) [39,40] in induction of these genes, whereas NQO1 and SCAL1 are thought to be more selective representatives of the Nrf2-ARE pathway [41,42]. Whether decreased mRNA expression of Nrf2-ARE target genes also results in decreased antioxidant activity needs further

elucidation before making more definite statements on this issue.

We have used MSCs from patients with severe to very severe COPD, and compared these to MSCs from non-COPD controls. MSCs from the control group were obtained from donors from whom sufficient MSCs were expanded during initial cultures, thus allowing storage of excess MSCs. We cannot rule out that this created a selection bias in the control group, in favour of better MSC proliferation and potentially even function, however such differences were not observed. We successfully prioritized on age-matching of both groups, but were unable to correct for smoking history, as all COPD patients were ex-smokers versus a majority of non- or never smokers in the control group. Whereas our data are unlikely affected by acute effects of CS on BM-MSCs (all COPD donors had quit smoking for at least six months before enrolment), we cannot exclude that we assessed effects on MSCs due to chronic CS exposure in the past. Effects of chronic CS exposure on MSCs has only been investigated to a limited extend in animal models, and to our knowledge this has not yet been investigated in human MSCs. Our study was not designed to address this question and therefore we cannot formally draw conclusions in this respect. However, taking into account the differences between the COPD and non-COPD group regarding smoking history, our data do not confirm previous results showing for instance detrimental effects of nicotine on proliferation and migration of MSCs [43].

Since Le Blanc's report in 2004 describing remission of severe graft versus host disease after MSC infusion [44], much attention has been paid to the clinical application of MSCs in a variety of diseases, including lung diseases. Besides promising effects of MSCs on inflammation and regeneration, the interest in MSC-based cell therapy is attributable to the relatively easy method to obtain and expand MSCs and to their low-immunogenic status. These properties make MSCs interesting candidates for (commercial) cell-therapy programs. However, although allogeneic MSCs did not appear to induce immune responses in immunocompromised recipients, their application in immune competent recipients might not be ideal, as allogeneic MSCs were shown to elicit alloimmune responses in immune-competent recipients [45], which moreover appeared to have detrimental effects on MSC function [46]. This underlines the importance of evaluating the treatment potential of autologous MSCs. The data provided by the current study support the implementation of autologous MSCs as a cell-based therapy for patients with chronic lung disease in future clinical trials.

In conclusion, we observed that the (immuno)phenotype and function of MSCs from patients with COPD is preserved, compared to their healthy counterparts. This finding is important in light of the potential clinical use of autologous MSCs to treat COPD patients. However, we did observe differences in differentiation potential and in activation of the Nrf2-antioxidant response element pathway that might link systemic manifestations of COPD to increased cellular ageing of MSCs.

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The online supplementary information can be accessed via: http://openres.ersjournals.com/content/erjor/suppl/2016/06/28/2.2.00045-2015.DC2/ERJOR-00045-2015_supplementary_data.pdf

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ADM1 | adrenomedullin |
| AREG | amphiregulin |
| BM-MSCs | bone marrow-derived MSC |
| CCDN1 | cyclin D1 |
| CCL20 | chemokine (C-C motif) ligand 20 |
| CD | cluster of differentiation |
| CM | conditioned medium |
| COPD | chronic obstructive pulmonary disease |
| CSE | cigarette smoke extract |
| CXCL8 | chemokine (C-X-C motif) ligand 8 |
| ECIS | electric cell-substrate impedance sensing |
| EF | electric fence |
| EGF(R) | epidermal growth factor (receptor) |
| $FEV_1$ | forced expiratory volume in 1 second |
| FGF | fibroblast growth factor |
| GPX2 | glutathion peroxidase 2 |
| HBEGF | heparin binding EGF-like growth factor |
| HGF | hepatocyte growth factor |
| HMOX1 | heme oxygenase 1 |
| IL | interleukin |
| LG-DMEM | low glucose Dulbecco's modified eagle medium |
| MSC | mesenchymal stromal cell |
| $MSC\text{-}CM^{CTRL}$ | MSC-conditioned medium from unstimulated MSCs |
| $MSC\text{-}CM^{STIM}$ | MSC-conditioned medium from TNF-α/IL-1β stimulated MSCs |
| Nrf2-ARE | nuclear factor (erythroid derived 2)-like 2 antioxidant response element |
| NQO1 | NAD(P)H:quinone reductase 1 |
| PDGFA | platelet derived growth factor subunit A |
| PDT | population doubling time |
| SCAL1 | smoke and cancer-associated lncRNA-1 (*SCAL1*) |
| SF | serum free |
| SFN | sulforaphane |
| TGFA | transforming growth factor-α |
| TNF-α | tumor necrosis factor-α |
| TSG6 | tumor necrosis factor-inducible gene 6 |
| VEGF | vascular endothelial growth factor |

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**SUPPLEMENTARY TABLE S1. qPCR primer sequences**

| Gene | Primer sequence forward | Primer sequence reverse |
|---|---|---|
| *ATP5B* | 5'-TCACCCAGGCTGGTTCAGA-3' | 5'-AGTGGCCAGGGTAGGCTGAT-3' |
| *B2M* | 5-'GATCGAGACATGTAAGCAGC-3' | 5'-TCAAACATGGAGACAGCAC-3' |
| *RPL13A* | 5'-AAGGTGGTGGTCGTACGCTGTG-3' | 5'-CGGGAAGGGTTGGTGTTCATCC-3' |
| *RPS29* | 5'-GCACTGCTGAGAGCAAGATG-3' | 5'-ATAGGCAGTGCCAAGGAAGA-3' |
| *ADM1* | 5'-ATGAAGCTGGTTTCCGTCG-3' | 5'-GACATCCGCAGTTCCCTCTT-3' |
| *AREG* | 5'-GGTGGTGCTGTCGCTCTT G-3' | 5'-AGGTGTCATTGAGGTCCAATCC-3' |
| *CCDN1* | 5'-CAATGACCCCGCACGATTTC-3' | 5'-CATGGAGGGCGGATTGGAA-3' |
| *CCL20* | 5'-GCAAGCAACTTTGACTGCTG-3' | 5'-TGGGCTATGTCCAATTCCAT-3' |
| *CXCL12 -1* | 5'-CTACAGATGCCCATGCCGAT-3' | 5'-GTGGGTCTAGCGGAAAGTCC-3' |
| *CXCL12 -2* | 5'-GTAGCCCGGCTGAAGAACAA-3' | 5'-GCGTCTGACCCTCTCACATC-3' |
| *EGF* | 5'-TGCAGAGGGATACGCCCTAA-3' | 5'-CAAGAGTACAGCCATGATTCCAAA-3' |
| *FGF2* | 5'-TGGCTATGAAGGAAGATGGAAGA-3' | 5'-TCCAATCGTTCAAAAAAGAAACAC-3' |
| *FGF7* | 5'-TCCTGCCAACTTTGCTCTACA-3' | 5'-CAGGGCTGGAACAGTTCACAT-3' |
| *GPX2* | 5'-GAATGGGCAGAACGAGCATC-3' | 5'-CCGGCCCTATGAGGAACTTC-3' |
| *HBEGF* | 5'-TGGACCTTTTGAGAGTCACTTTATCC-3' | 5'-CGTGCTCCTCCTTGTTTGGT-3' |
| *HGF* | 5'-TCCAGAGGTACGCTACGAAGTCT-3' | 5'-CCCATTGCAGGTCATGCAT-3' |
| *HMOX1* | 5'-AACCCTGAACAACGTAGTCTGCGA-3' | 5'-ATGGTCAACAGCGTGGACACAAA-3' |
| *IL6* | 5'-CAGAGCTGTGCAGATGAGTACA-3' | 5'-GATGAGTTGTCATGTCCTGCAG-3' |
| *CXCL8* | 5'-CAGCCTTCCTGATTTCTGC-3' | 5'-ACTTCTCCACAACCCTCTGC-3' |
| *NQO1* | 5'-GAAGAGCACTGATCGTACTGGC-3' | 5'-GGATACTGAAAGTTCGCAGGG-3' |
| *PDGFA* | 5'-CACCACCGCAGCGTCAA-3' | 5'-CCTCACCTGGACTTCTTTTAATTTTG-3' |
| *SCAL1* | 5'-GGCATTTACCAGCTGAGGGA-3' | 5'-TACCCCTACCTAGCACAGCA-3' |
| *TGFA* | 5'-AGGTCCGAAAACACTGTGAGT-3' | 5'-AGCAAGCGGTTCTTCCCTTC-3' |
| *TSG6* | 5'-AGAATTTGTGAGCAGCCCCT-3' | 5'-TGTATTTGCCAGACCGTGCT-3' |
| *VEGF* | 5'-CGAGGGCCTGGAGTGTGT-3' | 5'-TGGTGAGGTTTGATCCGCATA-3' |

**SUPPLEMENTARY FIGURE**

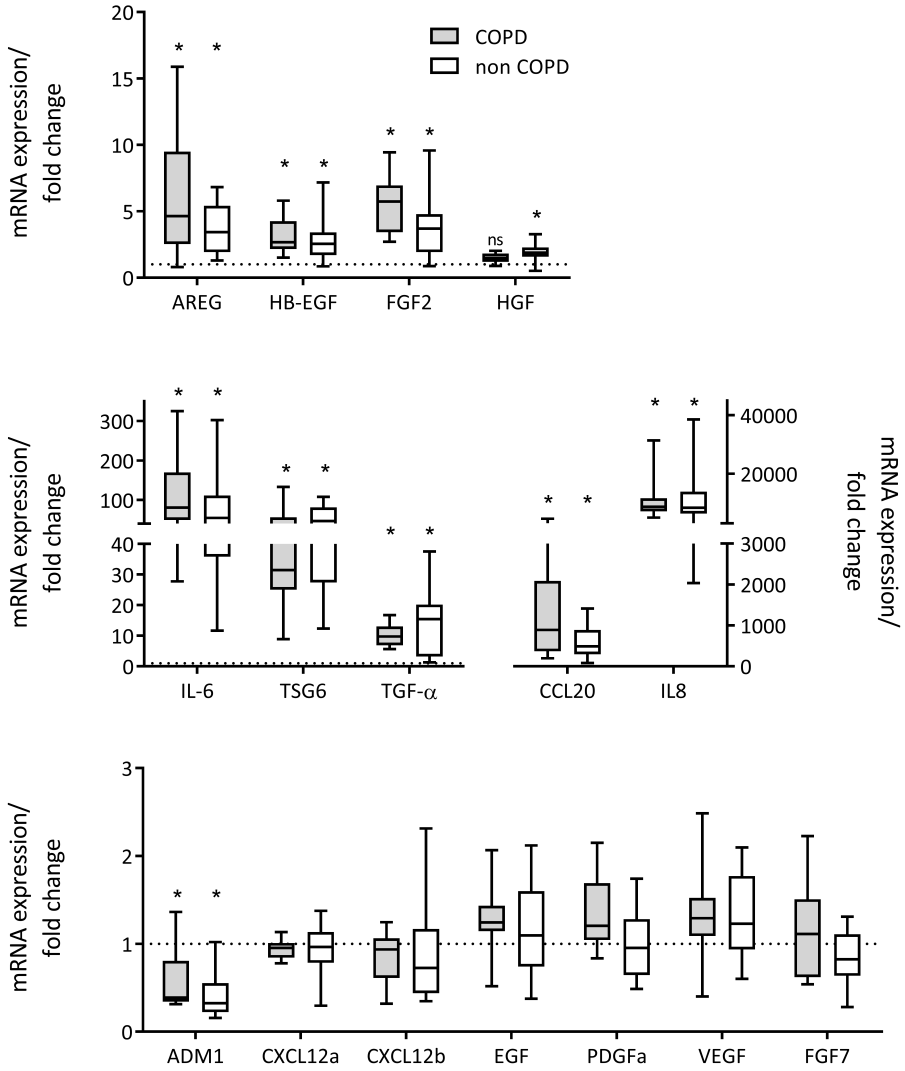


**Figure S1. mRNA expression of immune mediators and growth factors in response to proinflammatory cytokines.** mRNA expression upon stimulation of MSCs with TNF-α and IL-1β, calculated as fold change compared to mRNA expression in unstimulated MSCs from the same donor (at t=6 hours). Box whiskers represent median, interquartile range and minimum and maximum values. * = $p < 0.05$ for n = 9 per group.

# A PHASE I STUDY FOR INTRAVENOUS AUTOLOGOUS MESENCHYMAL STROMAL CELL ADMINISTRATION TO PATIENTS WITH SEVERE EMPHYSEMA

*CHAPTER 5*

**QUARTERLY JOURNAL OF MEDICINE, 2016 May 109 (5): 331-6**

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[1] both authors contributed equally to this work

**ABSTRACT**

**Background:** Mesenchymal stromal cells (MSCs) may reduce inflammation and promote tissue repair in pulmonary emphysema.

**Aim:** To study the safety and feasibility of autologous bone marrow-derived (BM-) MSCs intravenous administration to patients with severe emphysema.

**Design:** A phase I, prospective open-label study registered at ClinicalTrials.gov as NCT01306513. Eligible patients had lung volume reduction surgery (LVRS) on two separate occasions. During the first LVRS bone marrow was collected, from which MSCs were isolated and expanded *ex vivo*. After 8 weeks, patients received two autologous MSC infusions 1 week apart, followed by the second LVRS procedure at 3 weeks after the second BM-MSC infusion.

**Methods:** Up to 3 weeks after the last MSC infusion adverse events were recorded. Using immunohistochemistry and qPCR for analysis of cell and proliferation markers, emphysematous lung tissue obtained during the first surgery was compared with lung tissue obtained during the second surgical session to assess BM-MSC effects.

**Results:** From ten included patients three were excluded: two did not receive MSCs due to insufficient cultures expansion, and one had no second surgery. No adverse events related to MSC infusions occurred and lung tissue showed no fibrotic responses. After LVRS and MSC infusions alveolar septa showed a 3-fold increased expression of endothelial marker CD31 ($p = 0.016$).

**Conclusion:** Autologous MSC treatment in severe emphysema is feasible and safe. The increase in CD31 expression after LVRS and MSC treatment suggests responsiveness of microvascular endothelial cells in the most severely affected parts of the lung.

## INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent cells that can differentiate into several cell types, including fibroblasts, osteoblasts, adipocytes and chondrocyte progenitors [1]. In addition to their differentiation capacity, it has been shown that bone marrow-derived MSCs (BM-MSCs) release a variety of soluble factors implicated in anti-apoptotic signalling, cell growth and wound healing, potentially facilitating the endogenous regenerative potential [2,3]. Moreover, it has become evident that BM-MSCs have potent immunomodulatory effects *in vitro* and in animal models of chronic inflammation *in vivo* [2]. Importantly, encouraging results have been obtained with the treatment of severe steroid resistant Graft vs Host Disease in response to treatment with BM-MSCs and the treatment of refractory perianal fistulas in patients with Crohn's disease [4,5]. The combination of immunosuppressive, growth-potentiating, angiogenic and anti-apoptotic properties suggests that MSCs might induce lung repair, and this hypothesis is supported by animal models of emphysema [6,7]. In a rat model of elastase-induced pulmonary emphysema, placement of biodegradable sheets coated with adipose tissue-derived MSCs on the cut surface of the remaining lung after lung volume reduction surgery (LVRS) resulted in significant regeneration of alveolar and endothelial cells in lung tissue when compared with control animals treated by LVRS and a sheet without MSCs [6]. In another study in rats, infusion of BM-MSCs via the tail vein ameliorated papain-induced pulmonary emphysema, which was accompanied by suppression of alveolar cell apoptosis [7]. However, in a large phase II study in patients with chronic obstructive pulmonary disease (COPD) / emphysema, allogeneic MSCs showed no effect on forced expiratory volume in one second ($FEV_1$), the primary endpoint.

It remains to be established whether emphysematous tissue is repairable in humans with cigarette smoke-induced emphysema. To develop a clinical experimental treatment program using autologous BM-MSC administration for patients with severe emphysema, we started with a phase I study to assess the safety and feasibility of intravenous administration of BM-MSCs to subjects with severe emphysema. We evaluated standard WHO criteria of safety as well as lung tissue responses possibly induced by BM-MSCs.

## METHODS

*Patients*

Patient characteristics are shown in Table 1. In- and exclusion criteria for eligible patients are reported in the supplement that can be found at QJM online. In brief, patients (aged >40 years) had emphysema in both upper lung lobes with an equal distribution pattern in both lungs, as assessed by lung densitometry [8]. Additionally, $FEV_1$ was 40% predicted or lower. Patients had stopped smoking more than 6 months prior to screening.

**TABLE 1. Patient baseline characteristics**

| Patient no. | Sex F/M | Age (years) | $FEV_1$ in L (% pred) | $K_{CO}$ (% pred) | Perc15 (HU) | BMI (kg/m²) |
|---|---|---|---|---|---|---|
| 1 | F | 48 | 0.62 (22) | 32 | -945 | 27.3 |
| 2 | F | 43 | 0.82 (35) | 41 | -970 | 22.1 |
| 3 | F | 49 | 1.21 (38) | 37 | -970 | 21.2 |
| 4 | M | 65 | 1.21 (40) | 42 | -953 | 29.1 |
| 5 | M | 58 | 1.20 (38) | 39 | -962 | 21.5 |
| 6 | F | 56 | 0.94 (30) | 36 | -967 | 28.6 |
| 7 | F | 61 | 0.45 (20) | - | -987 | 20.3 |
| 8 | M | 53 | 1.22 (40) | 41 | -973 | 25.3 |
| 9 | F | 51 | 0.96 (39) | 57 | -933 | 22.8 |
| 10 | M | 55 | 0.58 (16) | - | -988 | 20.5 |

Patient 5 was of Asian race, all others were Caucasian. Perc15 is CT-based lung density value of both lungs at the 15th percentile point, expressed as Hounsefield Units (HU). During the screening of patients who volunteered for the study we noticed that the inclusion of subjects who had emphysema equally distributed in the upper lung lobes was rather rare. For the histopathological analysis, we reasoned that equal distribution was of prime importance. This was the reason why the ethical board allowed higher $K_{CO}$ values coinciding with equally distributed emphysema calculated by CT scan-derived lung densitometry.

*Study design*

The study is a phase I, open-label prospective study. Eligible patients had bilateral LVRS by video-assisted thoracoscopy in two separate sessions. The side for the first surgical procedure was randomly assigned. During this session bone marrow was aspirated from the posterior iliac crest. The second surgical procedure on the contralateral lung was preceded by two intravenous infusions of BM-MSCs (dosage 1-2x$10^6$ BM-MSCs/kg), at passage 1-3 (see Table 2), 4 and 3 weeks prior to the second surgery. Time between first and second LVRS was 12 ± 2 weeks (Figure 1). The occurrence of adverse events was monitored during the first 3 weeks after infusion, using WHO toxicity criteria. At baseline and at 12 months follow-up, we measured spirometry, gas transfer, lung volumes and computer tomography (CT)-derived lung densitometry [8]. Since emphysema is defined by histopathological criteria, we also investigated possible deleterious effects of BM-MSC treatment on lung tissue.

The study was approved by the Central Committee on Research Involving Human Subjects (CCMO) of The Netherlands and all study participants gave written consent. The study was registered at ClinicalTrials.gov as NCT01306513. The CCMO limited the total number of participants to 10 for safety reasons.

**TABLE 2. Overview of bone marrow aspirates and number of MSCs for infusions**

| Patient no. | Bone marrow volume (ml) | Expansion cycles | No. of BM-MSCs (per kg bodyweight) | No. of BM-MSCs (total) |
|---|---|---|---|---|
| 1 | 140 | P2 | $1.7 \times 10^6$ | $130 \times 10^6$ |
| 2 | 183 | P1 | $2.1 \times 10^6$ | $108 \times 10^6$ |
| 3 | 127 | P1 | $1.2 \times 10^6$ | $70 \times 10^6$ |
| 4 | 290 | P2 | $0.9 \times 10^6$ | $75 \times 10^6$ |
| 5 | 85 | | None | None |
| 6 | 225 | P2 | $1.9 \times 10^6$ | $140 \times 10^6$ |
| 7 | 109 | P2 | $1.4 \times 10^6$ | $80 \times 10^6$ |
| 8 | 0 | | None | None |
| 9 | 128 | P2 | $1.0 \times 10^6$ | $60 \times 10^6$ |
| 10 | 139 | P3 | $1.6 \times 10^6$ | $105 \times 10^6$ |

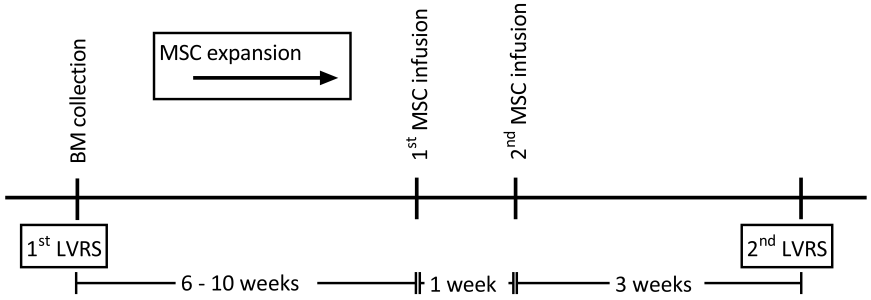


**Figure 1. Schematic overview of the study design.** Eligible patients had LVRS on two separate occasions. During the first LVRS bone marrow (BM) was collected, from which MSCs were isolated and expanded *ex vivo*. After clinical recovery, patients received two autologous MSC infusions, followed by the second LVRS procedure. The expansion of MSCs is described in the supplementary methods available at QJM online.

*MSC expansion*

Bone marrow aspiration, isolation and *ex vivo* expansion of BM-MSCs was performed as described previously (see online supplement at QJM online for details on MSC isolation, expansion and immunophenotyping) [9]. The number of expansion cycles and expanded BM-MSCs required to reach the target dose was assessed (see Table 2). MSCs were cryopreserved in 10% DMSO. After thawing, the MSC product was diluted with 50% (v/v) 0.9% saline and administered to the patient within 30 minutes.

*Immunohistochemistry*

Sections of emphysematous peripheral lung tissue were encoded for blinded analysis and stained with antibodies directed against CD3, CD4, CD8 (T-lymphocytes), CD20 (B-lymphocytes), CD31 (endothelial cells), CD68 (macrophages), Ki67 (cellular proliferation) or Surfactant Protein-C (SP-C, alveolar type II cells). Per section, alveolar septa were analysed in 15 randomly selected

fields, summing up to 1000 µm total septum length per field. The number of positive cells was calculated per length of alveolar septa. CD31 and CD20 stainings were quantified calculating the density and area fraction of positively stained area within the alveolar septa. A detailed protocol is provided in the supplement available at QJM online. Following analysis of tissue from the patient cohort, we retrieved archived paraffine-embedded resected lung tissue of three patients who had bilateral LVRS for emphysema in the past (1996-2010), with a similar time interval between the two surgical sessions as in this study [8]. This tissue was stained and analysed for CD31 following the protocol as described.

*PCR analysis of lung tissue*

Peripheral lung tissue (5x5x5 mm) was frozen in RNA-later. RNA extraction and cDNA synthesis was performed following the protocol described in the supplement available at QJM online. Gene expression of markers for proliferation, fibrosis, epithelial and vascular cells, growth factors and immune mediators was assessed by qPCR as described in the online supplement. The relative expression compared to housekeeping genes was calculated.

*Statistical analysis*

Results are described as mean ± SD unless depicted otherwise; data in graphs are presented as mean with individual data points. Paired data analysis was performed using Wilcoxon signed rank test (GraphPad Prism 6; GraphPad Software Inc., La Jolla, CA). Subjects with missing data are shown in the graphs and tables, but excluded from statistical analysis. Differences at p-values below 0.05 were considered statistically significant.

## RESULTS

*Feasibility and safety*

Seven patients completed the study protocol. Bone marrow could be aspirated from nine patients with a mean volume of 158 ml (± 64 ml), and in eight patients the targeted total MSC number was obtained after three expansion cycles (Table 2). No bone marrow could be aspirated from patient number 8 and very poor expansion of MSCs occurred in cultures from patient number 5, resulting in withdrawal from the protocol. Patient number 7 could not be evaluated at the histological level, because a second surgical procedure was not possible due to persistent air leak after the first LVRS (see below). In the first 48 hours after both BM-MSC infusions vital functions remained stable in all patients and WHO-toxicity criteria showed no change. At 3 weeks after the second BM-MSC infusion, the day before the second LVRS, no symptoms were reported that could be related to the infusions.

*Clinical parameters and follow-up*

Post-surgical air leak in days was similar between the initial and the second procedure (8.3 ± 3.4 versus 8.9 ± 3.1 days). Patient number 7 had persistent air leak and we decided to administer autologous BM-MSCs at Week 5 and 6 after the first LVRS with the opportunity to induce beneficial effects on wound healing. However, air leak persisted and mandated second surgery (pleural rubbing) on the initial side, resulting in withdrawal from the study protocol. At Week 14 the patient was successfully treated with endobronchial valves, followed by hospital discharge at Week 17.

At 12-months follow-up, the $FEV_1$ was increased by 390 ml ± 240 ml (p = 0.03) compared with baseline (Figure 2). Residual volume decreased by 540 ± 145 ml (p = 0.053) and gas transfer was not significantly different. The difference in CT-derived lung density at 15$^{th}$ percentile point (Perc15) obtained from a chest CT-scan prior to the first LVRS and 1-year after the second LVRS was 7.3 ± 4.3 g/L (p = 0.013). These results are comparable to our results reported previously [8]. The weight of all patients increased significantly, on average with 4.6 kg (range 1 – 10 kg; p = 0.016) (Figure 2).



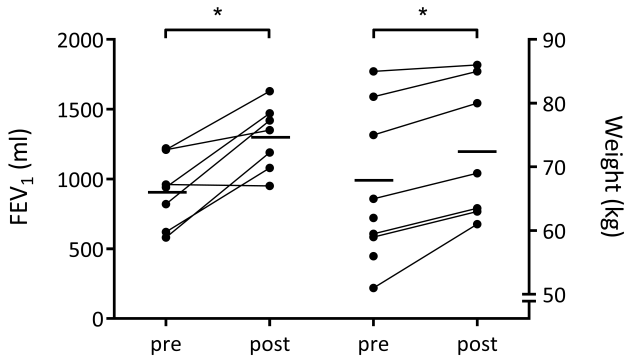


**Figure 2. $FEV_1$ and weight measurements at baseline and follow-up.** $FEV_1$ (left) and weight (right) were measured in patients with severe emphysema at baseline prior to the first LVRS procedure (pre), and at 1-year follow-up (post). Data in graph represent mean (horizontal bar) and individual data points. Paired data n = 7, * $p < 0.05$.

*Immunohistochemical analysis of lung tissue*

Immunohistochemical (IHC) stainings for cell markers and proliferation markers were analysed in seven paired samples of resected peripheral lung tissue, obtained during initial and second surgery (pre- and post BM-MSC infusion). The $CD31^+$ (endothelial cells) area within the alveolar septa was 3-fold higher post LVRS + BM-MSC infusion ($p = 0.016$), corrected for alveolar septum length as well as for total alveolar area (Figure 3A and B and Supplementary Figure S1). CD31 expression was not significantly changed in alveolar septa of the historic controls (Figure S2). No changes were observed for SP-C (alveolar type II cells) (Figure 3C).

The number of $CD3^+$ T cells in alveolar septa was significantly higher after LVRS + BM-MSC infusion compared to before ($p = 0.016$) (Figure 4A). This was accompanied by increased numbers of $CD4^+$ T cells in alveolar septa post LVRS + BM-MSC infusion in all patients, except one ($p = 0.30$; calculated as fold change $p = 0.047$) (Figure 4B, Figure S3). The number of $CD8^+$ T cells did not change ($p = 0.22$) (Figure 4C). No change was observed in the number of $CD68^+$ cells (macrophages) (Figure S3). The number of $CD20^+$ B cell aggregates varied substantially between patients, but there were no significant differences before and after LVRS + BM-MSC infusions. In one patient no aggregates were observed in either of the tissue samples obtained, therefore this patient was excluded from statistical analysis for CD20. The total $CD20^+$ area did not change significantly ($p = 0.16$) (Figure S3).

Since only very few $Ki67^+$ cells were observed, this staining was not further quantified.

*Gene expression analysis of lung tissue*

From six patients mRNA expression data of paired lung tissue samples (pre- and post BM-MSC infusion) were obtained. mRNA isolation from one sample failed because of tissue degradation. Quantitative real time PCR was used to analyse growth factors, immune mediators, proliferation markers and lung cell markers.

mRNA expression of SP-C was not altered after LVRS + BM-MSC infusion (Figure 3D). No changes were observed in the proliferation markers Cyclin D1 (CCDN1) and Cyclin Dependent Kinase Inhibitor 1A (CDKN1A) (Figure S4). Genes encoding epithelial cell markers and fibrosis markers (Figure S5A), growth factors including angiogenic growth factors (Figure S5B) and immune mediators (Figure S5C) did not change significantly. Interestingly, a trend towards higher mRNA expression of IL10 and TSG6 in tissue obtained after LVRS + BM-MSC infusion was observed, but this difference did not reach statistical significance ($p = 0.06$). mRNA expression of Platelet Endothelial Cell Adhesion Molecule 1 (PECAM1, the gene encoding for CD31) was not increased following LVRS + BM-MSCs (Figure S6A). Other vascular markers (i.e. Von Willebrand Factor (VWF) and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)) were also not significantly changed (Figure S6B and C).

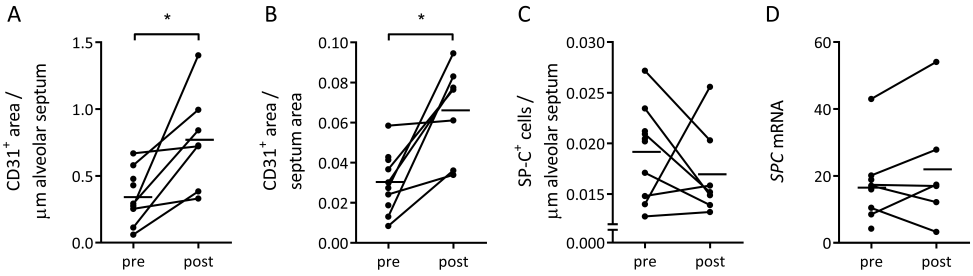


**Figure 3. CD31 and SP-C expression analysis in lung tissue before and after MSC infusion.** Immunohistochemistry and mRNA expression performed on lung tissue obtained during first LVRS (pre) and a second LVRS procedure that was preceded by two MSC infusions (post). Quantification of CD31 IHC staining with (A) CD31 density, normalized by length of alveolar septa; and (B) CD31 area fraction, normalized by area of alveolar septa. (C) Quantification of IHC staining of SP-C (alveolar type II cell marker) normalized by length of alveolar septa. (D) Normalized mRNA expression of SP-C. Data in graph represent mean (horizontal bar) and individual data points. Paired data n = 7 for IHC and n = 6 for mRNA, * $p < 0.05$.

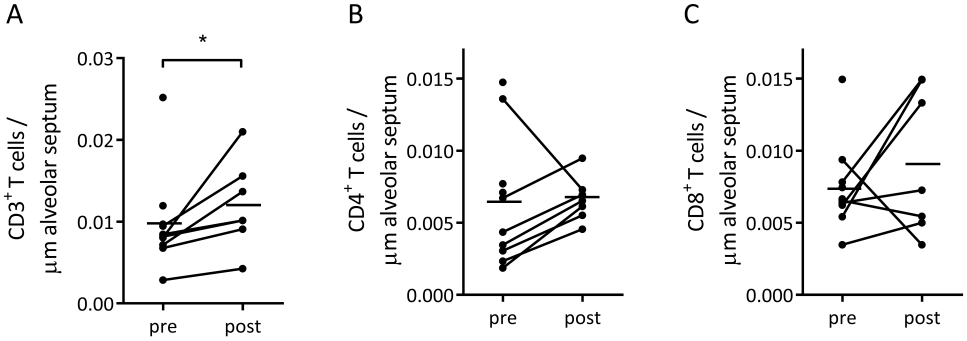


**Figure 4. IHC analysis of T cell markers pre- and post- MSC infusion.** The number of $CD3^+$, $CD4^+$ and $CD8^+$ T cells was assessed in surgical specimen obtained before and after MSC infusion. Quantification of (A) CD3, (B) CD4 and (C) CD8, expressed per length of alveolar septa. Data in graph represent mean (horizontal bar) and individual data points. Paired data n = 7, * $p < 0.05$.

## DISCUSSION

In this study we show that infusion of autologous BM-MSCs 3 and 4 weeks prior to LVRS in patients with severe emphysema is feasible and safe. By histological analysis of lung tissue surgically removed from the most severely affected emphysematous areas we found no evidence of induction of fibrotic responses in the lung by MSCs. There was neither an increase in α-SMA or Connective Tissue Growth Factor (CTGF) mRNA expression, nor in the number of Ki67 positive cells or in mRNA expression of proliferation markers in lung homogenates obtained after LVRS + BM-MSC infusion. We observed no increase in pulmonary fibrosis by CT-derived parenchymal analysis at 1-year follow-up. Treatment by LVRS and BM-MSCs was accompanied by a 3-fold increased expression of the endothelial cell marker CD31 in the alveolar septa of emphysematous

lung tissue. This increased tissue expression of CD31 was not accompanied by an increase in mRNA expression of CD31/PECAM1 or other vascular markers. Following LVRS and BM-MSC treatment, $CD3^+$ T cells were increased almost 2-fold in lung tissue sections, but mRNA expression of various immune mediators in lung tissue homogenates were not changed.

The BM-MSC target dosage of 1-2 x $10^6$ cells/kg bodyweight used in this study has been selected on the basis of clinical experience reported in the literature, including our own [2,4]. In this study, autologous BM-MSC administration at this dosage to patients with severe to very severe COPD appeared to be safe and well tolerated, which is in line with a prior clinical trial conducted using allogeneic MSCs in moderate and severe COPD patients [10]. We noted no adverse events and the observed changes in $FEV_1$ and lung densitometry after 1 year follow-up are comparable with a historic cohort of lung volume reduction surgery patients [8]. We observed a significant increase in body weight in our patients. Such an increase after BM-MSC treatment has not been reported before, and it is more likely that it resulted from the effects of LVRS than from the BM-MSC infusions [11].

The hallmark of pulmonary emphysema is the destruction of alveoli and therefore we focussed our analysis on changes in alveolar septa [12]. Three and four weeks after MSC infusion we obtained no clinical, microscopic or molecular evidence for repair of emphysematous lesions. Although no changes were observed in alveolar type II cells as identified by staining for SP-C, we did observe an effect on CD31 in the alveolar septa after BM-MSC infusions, despite the low number of study participants. Our study protocol did not include a control group due to its design as a phase I safety and feasibility study. We cannot exclude a possible carry-over effect by the first LVRS on CD31 responsiveness in the blood vessels around alveoli. Studies addressing this issue in humans are lacking, but a study in rodents analysing stromal cells in emphysema did not reveal surgery-induced effects on vascularization, as expressed by the vascular density [6]. Our posthoc analysis using archived tissue specimen of three patients who had bilateral LVRS with no specific treatments in between the two surgical sessions showed no significant change in CD31 expression in alveolar septa. This suggests a role for MSC-induced effects on CD31 expression.

CD31 (or PECAM1) is widely used as a marker for endothelial cells and endothelial damage is a prominent characteristic of emphysema. This has been demonstrated by the presence of increased levels of circulating endothelial micro-particles reflecting endothelial damage in COPD [13,14]. In mice, the contribution of endothelial damage to the development of emphysema has also been established, as impairment of endothelial cell function by inhibition of the VEGF receptor or administration of anti-endothelial antibodies resulted in emphysema development [15]. Conversely, whether the increased expression of CD31 in alveolar septa reflects the number of endothelial cells or has functional effects in alveolar septa remains to be investigated [16]. Since CD31 plays a central role in endothelial cell migration and angiogenesis [17], endothelial cell-cell interaction as well as in downstream anti-apoptotic effects [18], the increased expression of CD31 as seen in our patients may indicate a protective and/or repair response against further tissue destruction. Furthermore, whereas CD31 is generally considered and used as an endothelial cell

marker, its expression is not restricted to endothelial cells alone. Therefore, further randomized, placebo-controlled studies in a larger cohort and using additional markers are needed to explore this finding.

In addition to the increase in CD31, we also observed a significant increase of $CD3^+$ and $CD4^+$ T cells in randomly selected parenchymal tissue sections. The role of T-cells in the pathogenesis of emphysema is not fully clear, and in view of the before mentioned limitations of our study design we decided to not further investigate this observation in the historic cohort.

LVRS itself has a substantial effect on e.g. $FEV_1$, which likely could not be further enhanced by the BM-MSC treatment [8]. A randomized placebo-controlled clinical trial by Weiss *et al* showed no statistically significant differences in $FEV_1$ or $FEV_1$ % predicted during 2 years follow-up after 4 monthly administrations of allogeneic BM-MSCs, nor did they observe differences between the groups in FVC, FVC % predicted, total lung capacity, or carbon monoxide diffusing capacity from baseline to 1 year or 2 years. The authors questioned the treatment regimen, which was adopted from other clinical trials [10].

Since we observed an increase in CD31 expression and the number of $CD3^+$ cells in a relatively short period after MSC infusions, it appears reasonable to further investigate MSCs as a treatment for emphysema, exploring other MSC treatment regimens in a placebo-controlled study.

In conclusion, we demonstrated that infusion of autologous BM-MSCs to patients with severe emphysema is safe and we showed no signs of development of fibrosis in lung tissue 3-4 weeks after MSC infusion, nor in CT-derived parenchymal analysis at 1-year follow-up.



**ACKNOWLEDGEMENTS**

The authors would like to thank Vera van Rijn for technical assistance with the preparation of tissue sections, Annemarie van Schadewijk for assistance with the IHC stainings, Yvonne de Mooij-Eijk for staining the sections and performing most of the immunohistochemical analysis, Jasmijn Schrumpf for technical assistance in tissue mRNA extraction and running qPCRs (all from department of Pulmonology, Leiden University Medical Center, the Netherlands), and Ruy Pires-Neto for his advice regarding the IHC analysis (from department of Pathology, São Paulo University Medical School, São Paulo, Brazil).

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The online supplementary information can be accessed via:
https://academic.oup.com/qjmed/article-lookup/doi/10.1093/qjmed/hcw001#36769975

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| α-SMA | alpha-smooth muscle actin |
| BM-MSC | bone marrow-derived mesenchymal stromal cells |
| CCDN1 | cyclin D1 |
| CDKN1A | cyclin dependent kinase inhibitor 1A |
| CD | cluster of differentiation |
| COPD | chronic obstructive pulmonary disease |
| CT | computed tomography |
| CTGF | connective tissue growth factor |
| DMSO | dimethylsulfoxide |
| $FEV_1$ | forced expiratory volume in 1 second |
| FVC | forced vital capacity |
| IHC | immunohistochemistry |
| IL | interleukin |
| LVRS | lung volume reduction surgery |
| $K_{CO}$ | carbon monoxide transfer coefficient |
| PECAM1 | platelet endothelial cell adhesion molecule 1 |
| Perc15 | $15^{th}$ percentile point |
| SPC | surfactant protein-C |
| TSG6 | tumor necrosis factor-inducible gene 6 |
| VEGF(R2) | vascular endothelial growth factor (receptor 2) |
| VWF | von Willebrand factor |

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## SUPPLEMENTARY FIGURES

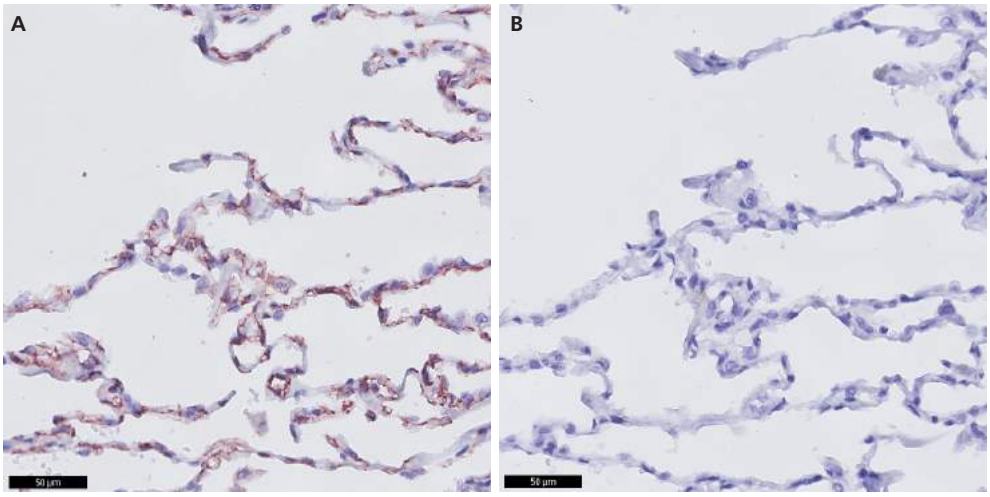


**Figure S1. Example of CD31 staining.** Immunohistochemical staining with (A) CD31 antibody directed at endothelium in alveolar septa and (B) isotype control; scale bar, 50 µm.

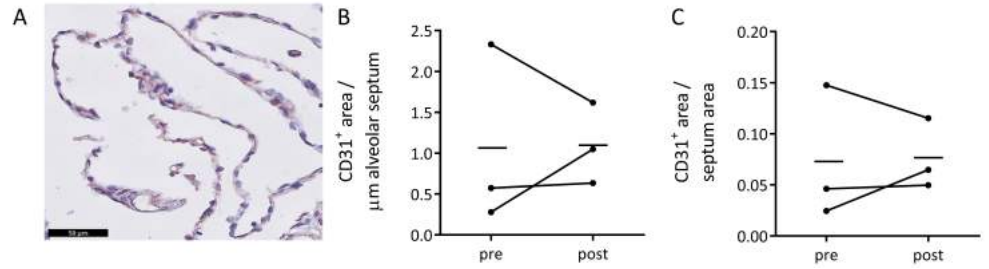


**Figure S2. CD31 expression in historic patient cohort.** Quantification of CD31 staining of lung tissue obtained from historic controls, who had lung volume reduction surgery for severe emphysema between 1996 and 2010, without intervention between the two surgical procedures. (A) example of CD31 immunohistochemical staining in historic cohort patient; (B) CD31 density, normalized by length of alveolar septa; (C) CD31 area fraction, normalized by area of alveolar septa. Data in graph represent mean and individual data points. Paired data n = 3.

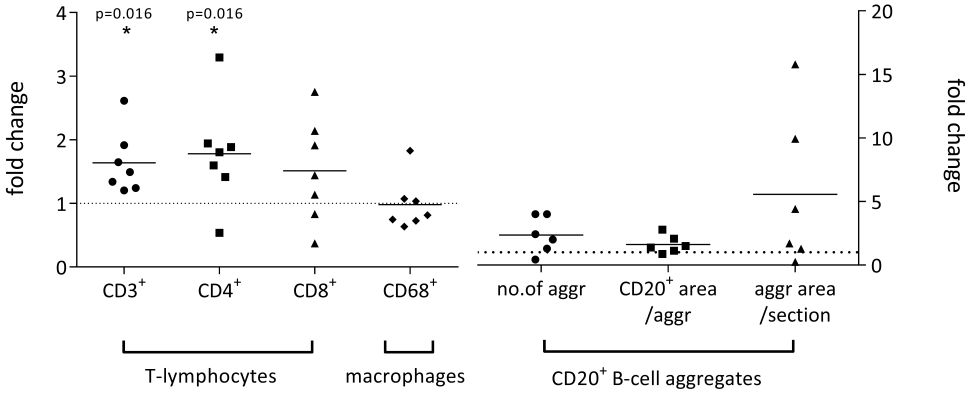


**Figure S3. Immunohistochemical analysis of immune cells after MSC infusion.** IHC data from the first LVRS procedure was compared to data from the second LVRS procedure that was preceded by two MSC infusions. Data from second surgery (post-MSC) were calculated as fold change compared to data from initial surgery. Individual data points and mean are shown; paired data n = 7, * $p < 0.05$.



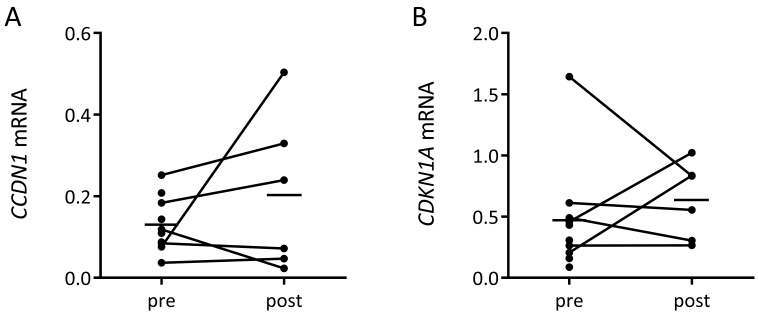


**Figure S4. mRNA expression of proliferation markers CCDN1 and CDKN1A after MSC infusions.** Expression of genes encoding the proliferation markers Cyclin D1 (CCDN1) and Cyclin Dependent Kinase Inhibitor 1A (CDKN1A) was compared in lung tissue obtained from LVRS before (pre) and after (post) infusion of MSCs. mRNA normalized expression of (A) CCDN1 and (B) CDKN1A. Individual data points and mean are shown; paired data n = 6.

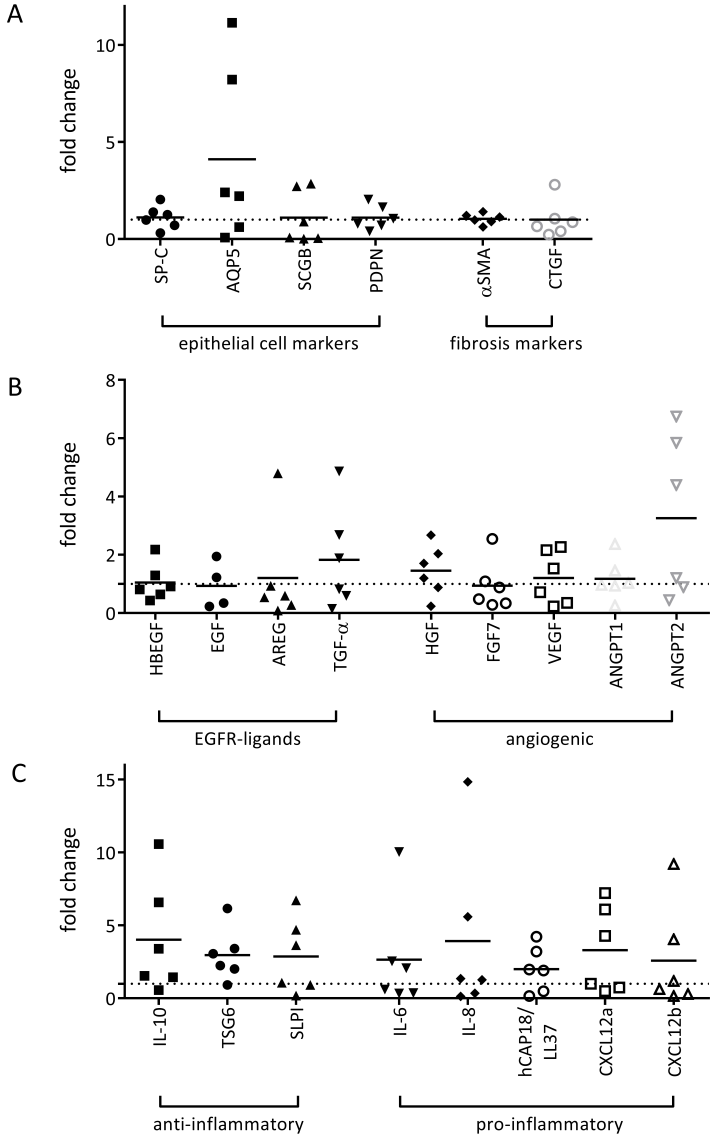


**Figure S5. Gene expression of cell markers, growth factors and immune-mediators following MSC treatment.** mRNA expression of several genes was assessed in surgical specimen obtained before and after MSC infusion. Data in graphs are depicted as fold change compared to mRNA normalized values measured in the first LVRS lung tissue sample. Fold change compared to baseline of mRNA expression of (A) cell markers, (B) growth factors, and (C) immune-mediators. Individual data points and mean are depicted in the graphs; paired data n = 6.

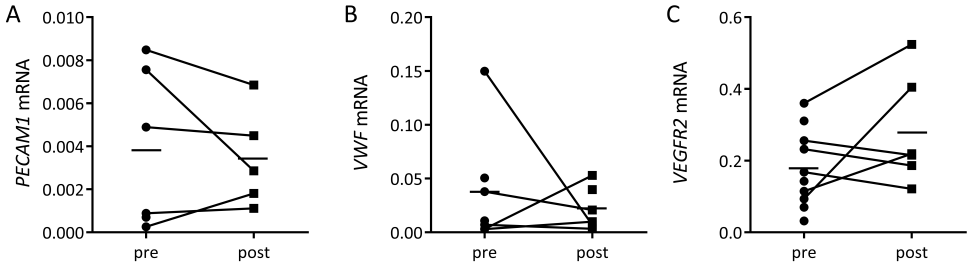


**Figure S6. Expression of vascular markers following MSC infusion.** mRNA expression of Platelet Endothelial Cell Adhesion Molecule 1 (PECAM1), Von Willebrand Factor (VWF) and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) was assessed in lung tissue before (pre) and after (post) MSC infusion. mRNA normalized expression of (A) PECAM1, (B) VWF and (C) VEGFR2. Graphs represent individual data points and mean; paired data n = 6.

# MESENCHYMAL STROMAL CELLS: A NOVEL THERAPY FOR THE TREATMENT OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE?

*CHAPTER 6*

**UNDER REVIEW**

WINIFRED BROEKMAN, P. PADMINI S.J. KHEDOE, KOEN SCHEPERS, HELENE ROELOFS, JAN STOLK, PIETER S. HIEMSTRA

**ABSTRACT**

Cell culture and animal studies have demonstrated the capacity of mesenchymal stromal cells (MSCs) to modify immune responses and to enhance tissue repair. These properties of MSCs provided a rationale to investigate their potential for treatment of a variety of diseases that are characterized by tissue destruction and inflammation, including chronic obstructive pulmonary disease (COPD). New treatments for COPD are highly relevant, given the lack of curative treatments and the progressive nature of the disease. Based on preclinical data, MSCs offer such a potential new treatment as they were found to restore damaged lung tissue and exert anti-inflammatory effects. Although promising results have been obtained in various immune related disorders, clinical trials investigating MSC efficacy in COPD have thus far only demonstrated the safety of this treatment, but have not yet demonstrated clinically relevant effects.

In this review, we discuss the rationale for MSC-based cell therapy in COPD, the main findings from *in vitro* and *in vivo* COPD model studies, clinical trials in COPD patients, and directions for further research.

## INTRODUCTION

Mesenchymal stromal cells (MSCs) are cells of non-hematopoietic origin, with the capacity to differentiate into multiple lineages of the mesenchyme, i.e. chondrocytes, osteoblasts and adipocytes. By definition, isolated MSCs are plastic adherent, and express CD73, CD90 and CD105 on their cell surface, but lack the expression of several hematopoietic and endothelial markers (i.e. CD45, CD34, CD11b or CD14, CD79 or CD19 and HLA-DR in human MSCs) [1]. Unique MSC-specific markers have not yet been identified, and MSCs constitute a heterogeneous cell population, including both multipotent (stem) cells and progenitor cells and might even contain pluripotent cell fractions [2]. MSCs were first described in the bone marrow where they constitute a small fraction of cells (0.001-0.01%) that closely interact with hematopoietic cells to support hematopoiesis and skeletal homeostasis [3,4]. Since then, it has become evident that MSCs reside in many tissues, including mesenchymal tissues (bone, adipose tissue, connective tissue), umbilical cord, and several organs including the liver, spleen and lung (reviewed in [5]). Functional *in vitro* assays indicate different physiological roles of MSCs related to their heterogeneity and tissue location of origin [6-8].

Upon infusion, culture-expanded MSCs regulate inflammatory and immune responses and tissue repair following injury. Indeed, following early observations that MSCs inhibit T-cell proliferation [9], MSCs were found to interact with the majority of innate and adaptive immune cells [10]. Through these interactions and in concert with local inflammatory mediators, intravenously infused MSCs are able to migrate to sites of injury where they 'sense' their environment and depending on local needs functionally mature towards either a pro-inflammatory or anti-inflammatory phenotype to regulate inflammation [11-13]. MSCs furthermore contribute to tissue homeostasis through anti-apoptotic and regenerative properties [14]. These various effects cannot only be mediated via cell-to-cell interactions and secretion of soluble factors including growth factors, matrix proteins and cytokines, but also through mitochondrial transfer and secretion of exosomes [15,16]. Finally, transdifferentiation and engraftment of MSCs into local tissue have been described [17,18], but it is unclear to which extent this contributes to putative repair-enhancing activities of infused MSCs. These findings suggest that MSCs exert a wide range of activities that may be beneficial clinically, but the extent to which these largely preclinical observations (both *in vitro* and *in vivo*) relate to MSC activity in humans is incompletely understood. The first clinical trials in the late 90's [19] assessed safety of MSCs in non-hematopoietic diseases. The clinical potential of MSCs was put in the spotlight by a high-impact case report by le Blanc *et al.* in 2004, indicating MSC efficacy on immune restoration in a pediatric patient with refractory graft versus host disease [20]. This boosted the interest in MSC-based cell therapy for a variety of diseases characterized by dysregulated immune responses (inflammation) and/or by tissue damage (e.g. ischemic heart disease, spinal cord injury, osteogenesis imperfecta). Thus far, clinical trials have indicated that

MSC administration is safe and have shown promising results in immune-related disorders but mixed results regarding the clinical benefit in other diseases [21,22]. The field is steadily advancing towards phase III placebo controlled trials to further evaluate the efficacy of MSCs and research is ongoing to improve treatment efficacy and extend to treating other patient groups.

With respect to MSC use for the treatment of respiratory diseases, preclinical data indicate effectiveness of MSCs in pulmonary fibrosis, acute lung injury and bronchopulmonary dysplasia (reviewed in [23-25]) and clinical trials have already demonstrated safety of MSC administration (intravenous and intratracheal) in limited numbers of patients with these disorders [26-28]. Remarkably, a significant increase in forced expiratory volume in 1 second ($FEV_1$) was observed following MSC administration in patients with recent myocardial infarction and normal lung function, indicating responsiveness of a pulmonary function parameter that is relevant in chronic obstructive pulmonary disease (COPD) [29]. Because COPD is characterized by inflammation, airway remodelling and destruction of lung architecture [30,31], the clinical potential of a cell product that creates an anti-inflammatory, regenerative environment seems obvious. Indeed, supported by preclinical studies and based on promising results in immune diseases, MSCs have already been investigated in patients with COPD. Here, the data from these (pre)clinical studies will be summarized, subdivided by data from *in vitro*, *in vivo* and clinical studies.

## EFFECTS OF MSCs IN LUNG INJURY MODELS *IN VITRO*

This section will provide a non-exhaustive overview of *in vitro* studies focussing on effects of MSCs on inflammation and repair using lung epithelial or endothelial cell injury models. For a broader perspective on the anti-inflammatory, regenerative and paracrine effects of MSCs we refer to the following reviews ([10,14,16]).

### *Anti-inflammatory effects*

Relevant to COPD, evidence of anti-inflammatory effects of MSCs *in vitro* include the observation that MSCs induce expression of the protease inhibitor secretory leukocyte protease inhibitor (SLPI) in elastase-treated lung epithelial cells via MSC-secreted Epidermal Growth Factor (EGF) and Hepatocyte Growth Factor (HGF) [32]. This response is likely beneficial, as protease inhibitors counteract protease-mediated tissue injury and degradation of protective mediators [33]. In co-cultures with cigarette smoke extract (CSE)-stimulated macrophages, MSCs increased the viability of macrophages and decreased their expression of the pro-inflammatory mediators Cyclo-Oxygenase 2 (*COX2*), Interleukin (*IL*) 6 and cytokine-inducible Nitric Oxyide Synthase (*iNOS*), whereas secretion of the anti-inflammatory cytokine IL-10 was induced [34]. These effects potentially contribute to dampening of inflammation in COPD.

*Antimicrobial effects*

In addition to the anti-inflammatory effects, antibacterial effects are also ascribed to MSCs. Murine studies showed that tissue-resident MSCs produced various mediators, including IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage migration inhibitory factor (MIF). Secretion of these factors leads to recruitment of neutrophils and enhances their pro-inflammatory and anti-bacterial activity that may contribute to host defence [35]. Furthermore, Toll-like receptor (TLR)-3 activation of human BM-MSCs, inducing a MSC-2 phenotype, enhances neutrophil survival through secretion of factors such as IL-6, Interferon-β and GM-CSF [36]. Production of these factors can lead to enhanced Indoleamine 2,3-dioxygenase (IDO) expression in human BM-MSCs and thereby mediate antibacterial, antiprotozoal and antiviral effects [37]. In addition to these indirect antimicrobial effects, MSCs and its conditioned medium may inhibit bacterial growth directly via the secretion of antimicrobial peptides such as lipocalin 2, the cathelicidin hCAP-18/LL-37 and β-defensin 2 [38-40].

*Lung epithelial and endothelial repair*

*In vitro* models of lung epithelial and endothelial injury have demonstrated that MSCs can restore damaged monolayers. These models included scratch wound assays and electroporation of monolayers to assess effects of MSCs on wound closure and barrier function, or the addition of stimuli relevant in COPD pathogenesis, such as CSE, elastase, papain or pro-inflammatory mediators (cytokines, lipopolysaccharide (LPS)). Using these models, MSCs as well as MSC-conditioned medium (MSC-CM) were shown to induce repair and to protect against airway epithelial cell damage. MSCs and MSC-CM enhanced wound closure in scratch wounds in A549 alveolar epithelial cell (AECs) lines and primary small airway epithelial cells. Increased migration and proliferation of target cells are thought to underlie this effect [41-43]. Increased proliferation was also observed in AECs incubated with CSE, pro-inflammatory cytokines or LPS when co-cultured with MSCs or MSC-CM and this was considered to be a protective response [43-45]. Similar results were obtained in NCI-H292 airway epithelial cells [46] and in endothelial cells [47-49], including observations that adipose-tissue derived stromal cell (AT-MSC) conditioned medium restored endothelial barrier function following CSE-exposure [48]. Furthermore, MSCs reduced apoptosis in pulmonary cell cultures derived from papain-treated mice and in CSE-stimulated endothelial cells [49,50]. The potential mechanisms that underlie these effects are partially attributed to MSC-secreted factors: secretion of IL-6, IL-8 and Chemokine (C-X-C motif) ligand 1 (CXCL1) by MSCs was found to enhance A549 (AEC) migration [42], Keratinocyte Growth Factor (KGF) secretion induced epithelial cell proliferation [45] and reduction of the number of apoptotic cells was linked to Vascular Endothelial Growth Factor (VEGF)-A [49,50] and HGF [51]. It was furthermore suggested that MSCs support epithelial cell attachment and spreading via secretion of extracellular matrix proteins [41,52]. Finally, the observation that mitochondrial transfer from MSCs to airway epithelial cells may protect against cigarette smoke induced injury is of special

interest in view of the increasing number of reports on mitochondrial dysfunction in COPD [53]. This non-exhaustive list of factors constitutes only a small fraction of the factors secreted by MSCs, as 720 different proteins were detected in the conditioned medium of MSCs [43], and future investigations are expected to further elucidate mechanisms involved in MSC-mediated wound repair *in vitro*.

*Preconditioning of MSCs*

Preconditioning of MSCs e.g. with pro-inflammatory cytokines or hypoxic culture conditions was found to polarize MSCs towards an anti-inflammatory profile (referred to as MSC2) and to enhance their therapeutic potential in various disease models (reviewed in [54,55]. In line with this, preconditioning of MSCs had favourable effects on lung epithelial repair. Our own data show that preconditioning of MSCs with TNF-$\alpha$ and IL-1$\beta$ induced the expression of several growth factors and enhanced wound closure in NCI-H292 airway epithelial cells [46]. Similarly, TNF-$\alpha$, IL-6 and IL-1$\beta$-stimulated MSCs induced A549 (AEC) proliferation via increased KGF secretion [45]. Increased secretion of other growth factors, i.e. VEGF, Fibroblast Growth Factor 2 (FGF2), Insulin-like Growth Factor 1 (IGF1) and HGF, in response to stimulation with TNF-$\alpha$, LPS or hypoxia was also shown, but functional effects were not assessed [56]. Furthermore, mediators that are released by damaged AECs increased the migration of MSCs and amniotic fluid-derived stem cells [41,57]. Overall, these data indicate that an inflammatory environment can alter the secretome of MSCs in a way that promotes wound repair.

In summary, *in vitro* studies show that MSCs exert anti-inflammatory effects relevant to COPD, including improved protease / protease inhibitor balances, interactions with macrophages and anti-microbial effects. Besides, MSCs enhance wound healing in lung epithelial and endothelial cells *in vitro* by increasing proliferation and migration of target cells, and reducing apoptosis. The observation that MSC-CM exerts similar effects as MSCs supports the paracrine actions of MSCs, but many of the active factors still need to be elucidated. Furthermore, future investigations should focus on pre-conditioning of MSCs to enhance their regenerative and migratory potential.

## EFFECTS OF MSCs ON COPD MODELS *IN VIVO*

The first animal study assessing the effects of cell therapy in COPD showed promising results. Shigemura *et al.* used porcine pancreatic elastase (PPE) to induce emphysema in rats, followed by intravenous administration of AT-MSCs (plastic adherent, $CD44^+/CD90^+/CD45^-$) on day 7. After 14 days, MSC treatment resulted in restoration of both alveolar and endothelial structures in AT-MSC-treated rats compared to control rats as shown by immunohistochemical analysis. A significant increase in proliferating cells and significantly lower numbers of apoptotic cells were

observed in the treatment group. Additionally, improved gas exchange and exercise tolerance was observed [58].

Following this initial encouraging observation, several studies have investigated *in vivo* effects of MSCs in experimental models of COPD and emphysema, mainly in rat and mice. The protocols to induce COPD-like features are established [59], and encompassed instillation of proteolytic enzymes (PPE or papain) or chronic passive exposure to cigarette smoke with or without additional LPS. Administered MSCs were usually species-related allogeneic MSCs from the bone-marrow or adipose tissue, but other sources of MSCs (amniotic fluid, lung or human) were also investigated. They were either administered systemically or locally via intratracheal instillation, with notable variation in frequency, dosage and timing of administration as well as in the period allowed to assess effects (see table 1 for details on study protocols).

The initial observation by Shigemura *et al*. showing that MSC-based cell therapy improves lung architecture, decreases apoptosis and increases cell proliferation was confirmed by several subsequent *in vivo* studies [44,49,53,60-63]. The exact mechanisms responsible for this repair have not yet been fully elucidated. The large body of circumstantial evidence is summarized in the following section, with a particular focus on effects of MSCs on inflammation and repair (see Figure 1 for a schematic overview and see Table 1 for details on study protocols).



*Anti-inflammatory effects*

COPD is characterized by an enhanced inflammatory response [31], and assessment of MSC-mediated effects on inflammation is therefore relevant. Assessment of the effect of MSC treatment on inflammation in *in vivo* studies mostly included immunohistochemical evaluation of pulmonary inflammatory infiltrates, bronchoalveolar lavage fluid (BALF) analysis of inflammatory cells and cytokines, and analysis of mRNA expression of inflammatory cytokines in lung tissue.

MSC treatment reduced inflammatory cell infiltrates in peribronchiolar, perivascular and alveolar septa in lung tissue compared to control [34,49,60,64], and a relative increase in anti-inflammatory (or M2) macrophages was observed [34,60]. This increased abundance of macrophages with an anti-inflammatory phenotype may contribute to reducing inflammation and enhancing repair responses [65]. In BALF, the total number of inflammatory cells and its subsets, i.e. macrophages, neutrophils and lymphocytes, were lower in MSC-treated animals [62,64,66], whereas there was a relative increase in type 2 macrophages [34]. BALF analysis of inflammatory cytokines involved in COPD pathogenesis showed a significant reduction of IL-1$\beta$, TNF-$\alpha$ and KC (murine IL-8 homologue) concentrations following MSC treatment [32,66], although one study did not observe effects on IL-1$\beta$ and KC in BALF [67]. In line with decreased BALF-cytokine concentration, decreased mRNA expression of these cytokines were observed in emphysematous lung tissue following MSC treatment [32,49,51,60,64], but results for Monocyte Chemo-attractant Protein 1 (MCP1) were conflicting [49,64]. Besides, treatment with MSCs decreased concentrations of several Matrix Metalloproteinases (MMPs), i.e. MMP2, MMP9 and MMP12 [49].

**TABLE 1. Animal models investigating MSCs in COPD: methods and main outcomes**

| Author (year) | Model | Cell source, number, route | Timing / frequency of cell therapy (from start) | Assessment of effects (from last cell therapy) | Main findings |
|---|---|---|---|---|---|
| Antunes (2014) | C57BL/6 mice<br>IT PPE weekly<br>4 weeks | AT-MSC, BM-MSC & LR-MSC<br>0.1 x $10^6$ IV or IT | Week 4<br>Once | 7 days | All sources improved MLI, reduced inflammation and apoptosis. AT-MSC and BM-MSC improved mPAP and increased VEGF. Change of macrophages from M1 to M2 profile in BM-MSC group. |
| Gu (2015) | SD-rat<br>CS exposure<br>12 weeks | BM-MSC<br>6 x $10^6$ IT | Week 8-12, twice-weekly<br>10 times | 28 days | Improved MLI and reduced inflammation (including increased M2 macrophages in BALF) through down-regulation of COX-2 and PGE2, possibly via alveolar macrophages. |
| Guan (2013) | SD-rat<br>CS exposure<br>11 weeks | BM-MSC<br>6 x $10^6$ IT | Week 7<br>Once | 9 weeks | Improved MLI and PFT, reduction of pro-inflammatory mediators and proteases, reduced apoptosis. Increased VEGF, VEGF-receptor and TGF-β. |
| Hoffman (2011) | C57BL/6J mice<br>IT PPE once | BM-MSC & LR-MSC<br>0.5&1.0 x $10^6$ IV (1),<br>0.33 x $10^6$ IV (2) | Week 6 or 7, once (1)<br>2-weekly, thrice (2) | 22 (1) or 28 (2) days | Both sources improved MLI and increased IL-6 levels. No evidence of transdifferentiation. LR-MSC showed higher survival and retention in the lung compared to BM-MSCs. |
| Huh (2011) | Lewis rat<br>CS exposure<br>6 months | BMC / BM-MSC<br>0.6 x $10^6$ / 6 x $10^6$<br>RB or MSC-CM | Month 6<br>Once | 1, 7, 14, 28 days (BMC) and 8 weeks | Improved MLI and vascular parameters (mPAP, numbers of small pulmonary vessels), increased proliferation and reduced apoptosis. Paracrine effect rather than engraftment. |
| Ingenito (2012) | Sheep<br>EB PPE monthly<br>5 months | Autol. LR-MSC<br>5-10 x $10^6$<br>EB on scaffold | Week 8<br>Once | 28 days | Increased tissue mass on CT with increased lung perfusion and ECM content. Only a fraction of LR-MSCs appeared to engraft. Proposed mechanism: promoted outgrowth of epithelial and endothelial cells through secretion of ECM components. |
| Katsha (2011) | C57BL/6 mice<br>IT PPE once | BM-MSC<br>0.5 x $10^6$ IT | Day 14<br>Once | 7, 14 and 21 days | Improved MLI, increased levels of HGF, EGF and SLPI. Proposed mechanism via paracrine factors; infrequent engraftment or differentiation into epithelial cells. |
| Kennely (2016) | NOD/SCID/IL-2Rγ$^{null}$ mice<br>IN PPE 6 times<br>2 weeks | BM-MSC (human)<br>0.5 x $10^6$ IV or MSC-CM | Day 0 (1), 7 (2) or 12 (3) or day 0 (CM)<br>Once | 14 (1), 7 (2) or 16 (3) days or 14 days (CM) | Dose-dependent, protective effects of MSCs: decreased inflammation, less apoptosis and fibrosis. CM is protective but less effective. Proposed mechanism via HGF secretion. |
| Kim (2015) | C57BL/6J mice<br>IT PPE once | UC-MSC (human)<br>0.01-0.1 x $10^6$ IV | Day 7<br>Once | 7 days | Dose finding: improved MLI and increased VEGF with 0.05x$10^6$ MSCs. No effects on apoptosis, MMPs, SLPI, TIMP1, HFG and FGF2. |
| Li (2014) | SD-rat<br>CS exposure<br>56 days | BM-MSC & iPSC-MSC (human)<br>3 x $10^6$ IV | Day 29 and 43<br>Twice | 14 days | Both sources improved MLI, but iPSC-MSCs were more effective which is ascribed to higher mitochondrial transfer capacity of iPSC-MSCs. |
| Li (2014) | SD-rat<br>CS exposure + LPS twice<br>12 weeks | AF-MSC<br>4 x $10^6$ IT | Week 12<br>Once | 20 and 40 days | Improved MLI, less apoptosis of AT2 cells, increased expression of SPA, SPC and TTF1. Proposed mechanism: integration into lung tissue and differentiation into AT2-like cells. |

| | | | | | |
|---|---|---|---|---|---|
| Liu (2015) | C57BL/6 mice<br>CS exposure<br>12 weeks | BM-MSC<br>4 x $10^6$ IV | Week 5-12, once-weekly<br>8 times | 14 days | Improved MLI, decreased apoptosis and inflammation, increased proliferation. No effects on PFT. Significant increase in numbers of BASCs. |
| Peron (2015) | C57BL/6 mice<br>CS exposure<br>75 days<br>+/- laser | T-MSC (human)<br>1 x $10^6$ IN or IP | Day 60 and 67<br>Twice | 9 days | Laser irradiated MSCs resulted in less inflammation, mucus production, collagen accumulation and tissue damage. Proposed mechanism: reduced NF-kB and NF-AT activation and increased IL-10. |
| Schweitzer (2011) | DBA/2J and C57BL/6 mice<br>CS exposure 2 w (1), 24 weeks (2) or VEGFR-inh (3) | AT-MSC (human)<br>0.3 x $10^6$ IV | Day 14 once (1), month 2-4 2-weekly, 4 times (2) or day 3 once (3) | 1, 7, 21 days (1); 1 day (2) or 3 and 25 days (3) | Reduced inflammatory infiltration, decreased lung cell death and airspace enlargement. Effects on bone marrow and weight loss. |
| Shigemura (2006) | Lewis rat<br>IT PPE once | AT-MSC<br>50 x $10^6$ IV | Day 7<br>Once | 7, 14, 21 and 28 days | Increased HGF. Inhibition of alveolar cell apoptosis, enhancement of epithelial cell proliferation and promotion of angiogenesis. Restored PFT. |
| Song (2014) | SD-rat<br>CS exposure<br>7 weeks | BM-MSC<br>6 x $10^6$ IT | Week 8<br>Once | 28 days | Less pro-inflammatory cytokines and inflammatory cells in BALF, improved histopathology and airflow obstruction. Proposed mechanism via induction of TGF-β1. |
| Tibboel (2014) | C57BL/6 mice<br>IT PPE once | BM-MSC<br>0.5 x $10^6$ IT (1) or 0.1 x $10^6$ IV (2) | 1 day prior, day 1 or day 21 (1); 30 min prior (2) once | 19, 20 and 21 days | MSCs iv inhibited deterioration of lung function, without effects on histology. IT administration of MSCs had no effects. |
| Zhang (2014) | SD-rat<br>CS exposure + IT LPS twice 8 weeks<br>+/- SPA (d 61) +/- irr (d 90) | BM-MSC<br>4 x $10^6$ IV | Day 90<br>Once | 31 days | Following SPA suicide gene system infusion: increased recruitment of MSCs with induction of pulmonary fibrosis, proposed mechanism: due to vacant AT2 cell niches. Decreased IL-6 in BALF. |
| Zhen (2008) | Lewis rat<br>IT papain once<br>+/- irr | BM-MSC<br>4 x $10^6$ IV | Day 0<br>Once | 28 days | Amelioration of emphysematous changes. MSC engraftment in recipient lungs and differentiation into AT2 cells. Suppression of alveolar cell apoptosis. |
| Zhen (2010) | Lewis rat<br>IT papain once | BM-MSC<br>4 x $10^6$ IV | Day 0 (2h)<br>Once | 28 days | Improved MLI, restoration of reduced VEGFA expression. |

Abbreviations: AF = amniotic fluid-derived; AT-MSC = adipose tissue-derived stromal cell; AT2 = alveolar type 2 cell; Autol. = autologous; BALF = broncho-alveolar lavage fluid; BASCs = bronchoalveolar stem cells; BM = bone marrow-derived; BMC = bone marrow cells; COX2 = cyclo-oxygenase 2; CS = cigarette smoke; CT = computed tomography; EB = endobronchial; ECM = extracellular matrix; EGF = epidermal growth factor; HGF = hepatocyte growth factor; IL = interleukin; IN = intranasal; inh = inhibition; IP = intraperitoneal; iPSC = induced pluripotent stem cell; irr = irradiation; IT = intratracheal; IV = intravenous; LPS = lipopolysaccharide; LR = lung resident (lung-derived); MLI = mean linear intercept; MMPs = matrix metalloproteases; mPAP = mean pulmonary artery pressure; MSC = mesenchymal stromal cell; MSC-CM = MSC-conditioned medium; NF-AT = nuclear factor of activated T-cells; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; NOD/SCID/IL-2R$\gamma^{null}$ = non obese diabetic/severe combined immunodeficiency IL-2 receptor gamma knockout; PFT = pulmonary function test; PGE2 = prostaglandin E2; PPE = porcine pancreatic elastase; RB = retrobulbar; SD = Sprague Dawley; SLPI = secretory leukocyte protease inhibitor; SPA = surfactant protein A; SPC = surfactant protein C; T = tubal-derived; TGF-β = transforming growth factor beta; TIMP = tissue inhibitor of metalloproteinases; TTF1 = thyroid transcription factor 1; UC = umbilical cord; VEF(R) = vascular growth factor (receptor).

Although MMPs are important regulators of extra-cellular matrix homeostasis, abundance of MMPs has been linked to tissue destruction in emphysema [68] suggesting that decreased levels may contribute to tissue homeostasis.
Only limited *in vivo* data are available concerning potential mechanisms of action of MSCs. MSCs are thought to attenuate inflammation via reduction of *COX2* expression and Prostaglandin E2 (PGE2) synthesis by macrophages [34] and decreased expression of TNF-α is attributed in part to an MSC-mediated increase in Transforming Growth Factor-β (TGF-β) secretion by macrophages [64]. It is furthermore hypothesized that induction of TGF-β signaling by MSCs inhibits MMP9 and MMP12 expression in alveolar macrophages [49].
In conclusion, administration of MSCs appears to dampen inflammation in animal models of emphysema, reflected by a decrease in cytokine concentrations, inflammatory cells and infiltrates in lung tissue. There appears to be a role of MSC-mediated changes in macrophage polarization towards anti-inflammatory type 2 macrophages, likely contributing to dampening of inflammation, but effects of MSCs on other immune cells were not systematically investigated. Furthermore, the precise mechanisms of action of MSCs *in vivo* are yet to be investigated.

*Lung tissue repair*
Tissue destruction in emphysema is characterized by a loss of alveolar attachments, and MSC treatment was found to restore damaged alveolar structures in animal models of emphysema, reflected by a decrease in the mean linear intercept (a measure that describes the mean free distance in air spaces) [32,34,44,48-51,53,60-63,69]. This is likely related to a decrease in numbers of apoptotic cells, usually assessed using TUNEL assays or by measuring Caspase 3 concentrations [44,48,49,51,58,60-63] and to increased numbers of proliferating cells, i.e. $Ki67^+$ or $PCNA^+$ cells [44,58,62]. Besides, an MSC-induced reduction in collagen deposition was observed in elastase-induced emphysema, suggesting anti-fibrotic effects that may contribute to inhibition of airway remodelling in COPD [51]. Factors that contribute to MSC-mediated tissue repair are described in the following section.

*Paracrine effects.* Administration of MSC-CM induced protective effects on lung tissue architecture [44,51], in line with the concept that MSCs exert their effects in part via paracrine signaling, including secretion of growth factors. Indeed, following MSC administration, mRNA expression of HGF [32,58], EGF [32], VEGF [48-50,58,60] and KGF [44] was increased in emphysematous lung tissue compared to control. These growth factors are thought to contribute to restoration of tissue architecture in the lung [70], and HGF in specific was linked to anti-apoptotic effects of MSCs [51]. The increased concentrations of HGF appeared to result from a combination of secretion by MSCs and induced secretion by local cells [58], whereas for the other growth factors this was undetermined. Conflicting data were obtained for the effect of MSCs on TGF-β secretion [49,60]. However, the relevance and contribution of TGF-β in the context of

COPD is unclear, as TGF-β has been linked both to small airway fibrosis in COPD [71] as well as to dampening of immune responses [64].

*Effects on endothelium.* Endothelial integrity is essential for maintenance of the alveolar-capillary unit, with a pivotal role for VEGF signalling [72]. VEGF-receptor blocking can induce apoptosis of endothelial cells and emphysema, and treatment with human AT-MSCs can abrogate this effect [48]. Others have also demonstrated a lowering in destruction and apoptosis of endothelial cells following MSC treatment in cigarette or PPE-induced emphysema [49,60]. Functional effects include higher numbers of pulmonary capillaries corresponding with increased perfusion of the lung [58], and reduced pulmonary artery pressure [44,60].

*Engraftment and transdifferentiation.* Engraftment and transdifferentiation of MSCs in epithelial cells have been proposed to contribute to the reconstruction of destructed lung architecture in emphysema. To address this, a number of animal studies have used green-fluorescent labelling of MSCs or administration of MSCs from male donors to female recipients, allowing detection of the Y-chromosome. MSCs were thus found to engraft into the lung tissue within 24 hours after administration, but their numbers appear to be low and decrease in a time-dependent fashion [32,34,44,58]. Although MSC engraftment and retention time in the lung can be increased following radiation [63,67] or by using lung-resident MSCs [69], indications of functional benefits are lacking. Some studies provide evidence for transdifferentiation of MSCs into structural cells of the alveolar unit [61,63], but these data could not be reproduced by others [32,69]. The initial results have been attributed to misinterpretation of data due to technical difficulties, and although transdifferentiation is still a matter of debate, evidence for its contribution to architectural reconstruction of destructed lung tissue is limited [73].

Collectively these studies show that administration of MSCs restores lung architecture, decreases apoptosis and increases cell proliferation in animal models of emphysema. Several indicators of inflammatory responses are affected by MSCs, apparently in favour of dampening inflammation. Besides, indirect evidence suggests that a regenerative environment is created via paracrine effects of MSCs and MSC-induced secretion of growth factors by local cells, resulting in higher concentrations of soluble factors that are relevant for tissue repair and that prevent apoptosis of endothelial cells. MSC engraftment and differentiation on the other hand are unlikely to deliver a relevant contribution to tissue repair (see figure 1). However, it should be taken into account that these studies were designed to detect maximum effects of MSCs, and predominantly used "acute" models of emphysema. Moreover, the numbers of cells per kg body weight that are administered in mice were about 10-25 times higher in comparison to what is reported for humans. Thus far, the clinical relevance of these promising results still needs to be established, as will be discussed in the next section.

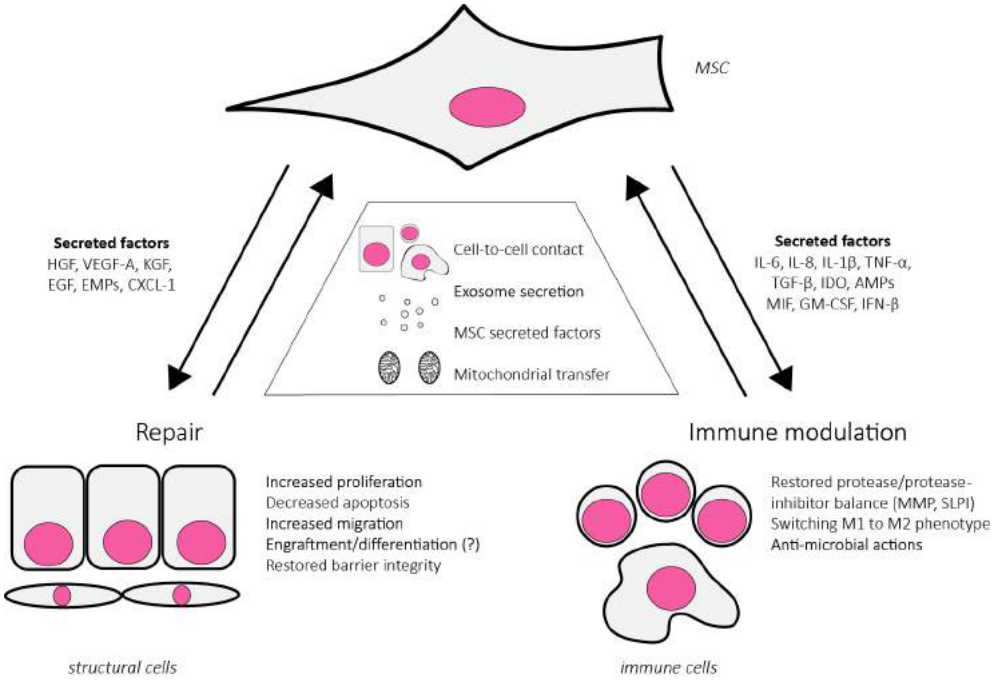


**Figure 1. Mechanisms underlying the modulation of inflammation and lung tissue repair by MSC in COPD.** MSC potentially act through cell-to-cell contact, mitochondrial transfer and secretion of soluble factors (either directly secreted or in exosomes), including growth factors, (anti)-inflammatory cytokines and chemokines (as indicated), thereby improving tissue homeostasis by favouring repair and dampening inflammatory responses.

## CLINICAL TRIALS

The interest in using MSCs for the treatment of COPD or emphysema has translated into clinical trials. This section describes the main observations from these clinical trials, including an overview of ongoing clinical trials (Table 2). For more details we refer to a recently published review on this topic [74].

The first trial in patients with moderate to severe COPD (GOLD II-III) was conducted by Weiss *et al.*: the safety and efficacy of treatment with 4 intravenous infusions of allogeneic bone marrow-derived MSCs (BM-MSCs) from a pool of non HLA-matched donors *(Prochymal®)* was compared to placebo in 62 patients, in a double-blind study. Infusions (100 x $10^6$ cells/infusion) were well tolerated in all patients, and no clinically relevant adverse events related to the cell therapy were reported. Treatment with MSCs had no effect on clinical parameters, including pulmonary function and quality of life. There was a significant decrease in C-reactive protein (CRP) levels up to 1 month after the first infusion. In the discussion, the authors suggest that effects of MSCs

may have been missed due to the dosage and treatment regimen, sample size or due to the chronic nature of COPD which might be a less effective target for MSCs compared to more acute inflammatory disorders, such as acute respiratory distress syndrome [75].

The next clinical trial that investigated the safety of MSC administration in patients with severe to very severe COPD (GOLD III-VI) was conducted by our own group. The study protocol was designed around patients who were eligible for bilateral lung volume reduction surgery. Autologous MSCs (1-2 x $10^6$ cells per kg bodyweight) were administered twice intravenously in between the two surgical interventions, which thus allowed comparison of lung tissue obtained before and after MSCs administration. Seven patients completed the study protocol, without occurrence of therapy-related adverse events, indicating that MSC treatment was safe. Changes in $FEV_1$ and body weight were attributed to the surgical intervention. The majority of analysed tissue parameters were unchanged in post-MSC tissue, except for increased CD3, CD4 (T-cell markers) and CD31 (endothelial cell marker) expression. Although we cannot formally exclude surgery-related effects underlying these changes, especially the observed increase in CD31 may be indicative of a reparative response. The increase in the endothelial marker CD31 is especially relevant in view of the observation that loss of endothelial integrity contributes to development of emphysema [76].

Finally, data from a clinical trial in patients scheduled for endobronchial valve (EBV) placement for severe to very severe COPD (GOLD III-IV) demonstrated the safety of endobronchial instillation of allogeneic BM-MSCs (100 x $10^6$ cells) prior to EBV placement, compared to saline-treated controls (5 patients per group). In the treatment group, serum CRP concentrations significantly improved up to 90 days follow-up [77].

At present, several trials evaluating cell therapy for the treatment of COPD are still ongoing or their results are awaited. An overview of these trials is provided in table 2. In view of the outcomes of the conducted clinical trials, it seems reasonable to optimize treatment protocols and identify relevant measurable outcome parameters for future clinical trials

**TABLE 2. Clinical trials investigating cell therapy for COPD treatment**

| NCT number | Study design | No. | Cell type | Route | FU | Primary outcome | Study completion | Remarks |
|---|---|---|---|---|---|---|---|---|
| NCT00683722 (USA) [75] | Placebo-ctrl Randomized Double-blind | 62 | Allog. BM-MSC | IV | 2 y | Safety/ efficacy (phase 2) | 2010 Dec | |
| NCT01306513 (Netherlands) [76] | Single group Open label | 10 | Autol. BM-MSC | IV | 1 y | Safety (phase 1) | 2012 Nov | With LVRS |
| NCT01758055 (Iran) [80] | Single group Open label | 12 | Autol. BM-MSC | EB | n.s. | Safety (phase 1) | 2014 Jan | |
| NCT01872624 (Brazil) [77] | Placebo-ctrl Non-randomized Open label | 10 | Allog. BM-MSC | EB | 4 mo | Safety (phase 1) | 2015 Mrch | With EB-valves |
| NCT02645305 (Vietnam) [80] | Single group Open label | 20 | Autol. AT-MSC | IV | 1 y | Safety/ efficacy (phase 2) | 2016 Dec | With APRP |
| NCT02041000 (USA) [80] | Single group Open label | 100 | Autol. AT-MSC | IV | 6 mo | Safety/ efficacy (phase 2) | 2017 Jan | Commercial (Bioheart) |
| NCT02412332 (Brazil) [80] | Placebo-ctrl Randomized Open label | 20 | Autol. AT-MSC, BMMC or both | IV | 1 y | Safety/ efficacy (phase 2) | 2017 April | |
| NCT01849159 (Russia) [80] | Placebo-ctrl Randomized Open label | 30 | Allog. BM-MSC | IV | 2 y | Safety/ efficacy (phase 2) | 2017 Jun | Hypoxia-cultured |
| NCT02216630 (USA) [80] | Single group Open label | 200 | Autol. AT-MSC | IV | 1 y | Safety/ efficacy (phase 2) | 2017 Aug | Commercial (Kimera) |
| NCT02161744 (USA) [80] | Single group Open label | 60 | Autol. AT-MSC | IV | 1 y | Safety/ efficacy (phase 1) | 2017 Aug | |
| NCT01559051 (USA) [80] | Single group Open label | 100 | Autol. AT-MSC | IV/EB | 6 mo | Safety/ efficacy (phase 2) | 2017 Nov | Commercial (Ageless Regenerative institute) |
| NCT02348060 (USA) [80] | Single group Open label | 75 | Autol. AT-MSC | n.s. | 1 y | Quality of life | 2018 Feb | Commercial (StemGenex) |

Abbreviations: Allog. = allogeneic; APRP = activated platelet rich plasma (from peripheral blood); AT-MSC = adipose tissue-derived stem cells; Autol. = autologous; BMMCs = bone marrow-derived mononuclear cells; BM-MSC = bone marrow-derived mesenchymal stromal cells; Ctrl = controlled; d = day; EB = endobronchial; FU = follow-up; IV = intravenous; LVRS = lung volume reduction surgery; mo = month; NCT = ClinicalTrials.gov Identifier number; No. = number of participants enrolled; n.s. = not specified; route = route of administration; y = year.

## FUTURE DIRECTIONS

There are several possible explanations for the lack of translation of the promising preclinical data of MSC treatment to clinically relevant effects in patients with COPD. The animal models were optimized to detect maximum effects, and used higher cell numbers per kilogram bodyweight and more 'acute' models of COPD or COPD-like inflammation which can enhance MSC efficacy. Contrary to most clinical trials that predominantly assessed effects on minimally invasive clinical parameters, such as pulmonary function testing or quality of life assessment, the available preclinical *in vivo* studies applied invasive methods such as tissue resection and BALF for analysis. Although relevant, these clinical read-outs might not be responsive to MSC-therapy upon short-term treatment. It is therefore important to find parameters that might precede clinical improvement. Potentially, these include induction of CD31 expression in lung tissue, as indicated by the data from our own institution [76], or alterations in the composition of inflammatory cells in sputum, BALF and lung tissue. Likewise, timing, duration, dosage and frequency of administration, as well as the route of administration need to be optimized in humans. Regarding route of administration, it is conceivable that endobronchial administration might be more effective in COPD patients with symptoms related to airway obstruction and chronic bronchitis, versus intravenous administration when emphysema characterizes the disease. Furthermore, in light of the observed effect of pre-treatment of MSCs by pro-inflammatory cytokines *in vitro*, the clinical efficacy of MSCs might be inducible by pre-conditioning or by administration during COPD-exacerbations. In line with this, there is some evidence linking heterogeneity of MSCs to efficacy *in vivo*, and further studies are needed to identify 'superior' cell products to enhance the clinical efficacy of MSCs [7]. Finally, administration of MSCs engineered to overexpress mediators that increase their therapeutic potential, as for instance shown for MSCs overexpressing Angiopoietin-1 or IL-10 which prevent acute respiratory distress syndrome in mice [78,79], may hold promise for future applications.
It is evident that despite the preclinical data, a cure for COPD is not yet at hand. It will take time and effort to elucidate the precise mode of action of MSCs. Identification of inducible targets or biomarkers in the lungs of patients with COPD that can serve as an early indicator that the progressive course of COPD is amended will be highly relevant. To reduce costs and limit the number of patients required to answer the unresolved questions there is an urgent need for preclinical models that accurately reflect the human pathophysiology, e.g. *ex-vivo* lung perfusion, organoids, microfluidic lung-on-a-chip and lung tissue slices.

## CONCLUSION

Current studies suggest that cell therapy using MSCs is a promising new treatment strategy for COPD. Indeed, both *in vitro* and *in vivo* studies have demonstrated the regenerative potential of MSCs, which is reflected by their ability to induce airway epithelial and endothelial repair, and restore lung tissue architecture in emphysematous lung in animal models. These effects relate to increased proliferation and migration of target cells and reduction of apoptosis. Besides, MSCs dampen inflammatory responses in COPD-models and affect protease/protease inhibitor balances favouring tissue homeostasis. The precise mechanisms are not fully unravelled, although the involvement of a number of secreted factors including cytokines and growth factors has been suggested. Whereas initial studies have revealed that MSC treatment of COPD patients is safe, further clinical studies are needed to demonstrate that MSC-based treatments are of clinical relevance to COPD patients. Important challenges need to be addressed, including optimizing the MSC treatment regimens and identification of responsive outcome parameters e.g. in lung tissue. Such information may guide us in the choice of clinical outcome parameters for MSC treatment in COPD patients. The lack of effective interventions to restore lung function in COPD will be an important driver for these and other innovative approaches to the treatment of this highly prevalent disease.

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| AEC | alveolar epithelial cells |
| AMP | anti-microbial peptide |
| ASC | adipose tissue-derived stem cells |
| BALF | bronchoalveolar lavage fluid |
| BM-MSC | bone marrow-derived MSCs |
| CXCL1 | chemokin C-X-C motif ligand 1 |
| CD | cluster of differentiation |
| COPD | chronic obstructive pulmonary disease |
| COX2 | cyclooxygenase 2 |
| CSE | cigarette smoke extract |
| EGF | epidermal growth factor |
| EMP | extracellular matrix proteins |
| $FEV_1$ | forced expiratory volume in 1 second |
| FGF2 | fibroblast growth factor |
| GM-CSF | granulocyte macrophage colony-stimulating factor |
| hCAP-18/LL-37 | human cathelicidin 18 / LL-37 |
| HGF | hepatocyte growth factor |
| IDO | indoleamine 2,3 dioxygenase |
| IFN-β | interferon-β |
| IGF1 | insulin-like growth factor |
| iNOS | inducible nitric oxide synthase |
| KC | keratinocyte-derived protein chemokine |
| KGF | keratinocyte growth factor |
| LPS | lipopolysaccharide |
| MCP1 | monocyte chemotactic protein 1 |
| MIF | migration inhibitory factor |
| MMP | matrix metalloproteinase |
| MSC | mesenchymal stromal cell |
| P63 | tumor protein 63 |
| PCNA | proliferating cell nuclear antigen |
| PGE2 | prostaglandin E2 |
| PPE | porcine pancreatic elastase |
| SLPI | secretory leukocyte protease inhibitor |
| TGF- β | transforming growth factor-β |
| TNF- α | tumor necrosis factor-α |
| VEGFA | vascular endothelial growth factor-A |

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# SUMMARY AND GENERAL DISCUSSION

*CHAPTER 7*

## SUMMARY AND GENERAL DISCUSSION

The aim of this thesis was to investigate airway epithelial injury and potential repair mechanisms by mesenchymal stromal cells (MSCs), in the context of chronic obstructive pulmonary disease (COPD). COPD can develop in susceptible individuals following chronic exposure to inhaled noxious compounds, such as cigarette smoke, which is the main risk factor for COPD. The presenting symptoms of chronic cough and dyspnea relate to the pathogenic processes underlying the disease: enhanced inflammatory responses, airway epithelial remodelling, mucus hypersecretion and impaired clearance, and destruction of lung parenchyma [1]. COPD significantly contributes to disease burden and health care costs, and has a high impact on an individual patients' quality of life. It has remained the third leading cause of death worldwide over the past decade [2], and despite widespread availability of therapies that relieve COPD-related symptoms, there are no treatments that halt disease progression or moreover: cure COPD.

In search for such a curative treatment for COPD, disease mechanisms need to be unravelled to find potential modifiable targets. Based on current knowledge, obvious directions for novel therapies include those targeting the major denominators of COPD: inflammation and tissue destruction. In this light, cell therapy is a candidate new treatment, as cell-based therapies may modify inflammation and regenerate destructed tissue [3,4]. Cell therapy refers to administration of living cells, and its clinical application has shown promising results in a variety of diseases, including Graft-versus-host-Disease (GvHD) [5]. As a cell source, particular interest has arisen in the use of MSCs. The popularity of MSCs is partly explained by the fact that these cells can be relatively easily obtained from e.g. the bone marrow or adipose tissue, their high expansion rates *ex vivo*, as well as the lack of HLA-DR expression which helps to prevent recipient (host) immune responses upon administration of allogeneic cells. MSCs constitute a heterogeneous population of cells with properties of both stem and progenitor cells, and immunomodulatory and regenerative properties as established in preclinical studies *in vitro* and *in vivo* [6]. MSCs were shown to reduce inflammation, apoptosis and restore alveolar damage in animal models of emphysema [7-9]. However, it remains unclear whether similar effects on repair and inflammation can be obtained in humans and if so, how MSCs exert these effects.

This thesis' aim was therefore to investigate the effects of MSCs and MSC-secreted factors on airway epithelial repair following injury, in the context of COPD. The presented studies describe a novel *in vitro* model of airway epithelial injury, and assess the effect of MSCs both *in vitro* as well as in patients with COPD. The main findings are briefly summarized, followed by a discussion of the presented studies in a broader perspective.

## SUMMARY OF MAIN FINDINGS

The first part of this thesis investigated airway epithelial repair, focussing on the effects of cigarette smoke and MSC-secreted factors on wound healing *in vitro*.

Cigarette smoke (CS), the major risk factor associated with COPD was found to delay epithelial wound closure and modulate innate immune responses in primary bronchial epithelial cells cultured at the air-liquid interface (ALI-PBEC). Effects of CS on basal cells (BCs) included selectively induced expression of the BC-derived antimicrobial protein *RNase 7* following CS exposure, and impairment of migration and spreading of BCs into the wounded area. The effects of CS on wound repair and immunity were found to be mediated in part by oxidative stress, and via EGFR and ERK1/2 activation **(Chapter 2)**.

We next demonstrated that airway epithelial repair can be enhanced by MSC-secreted factors, in particular when MSCs are pre-conditioned with pro-inflammatory cytokines, which significantly induced the expression of several growth factors in MSCs. The underlying mechanisms include activation of ERK1/2 signaling in airway epithelial cells, predominantly via direct activation of EGFR and transactivation, and induction of airway epithelial EGFR-ligand expression (**Chapter 3)**.

In the second part of this thesis we focussed on clinical implementation of autologous MSCs in patients with COPD.

We first demonstrated that autologous bone marrow-derived MSCs (BM-MSCs) from patients with severe to very severe COPD are phenotypically and functionally comparable to MSCs from age-matched controls. Functional assays included response to pro-inflammatory stimuli and effects on airway epithelial cells. Based on minor differences with MSCs from controls, it is speculated that MSCs from COPD-donors share characteristics with aged MSCs, including decreased adipocyte differentiation and altered oxidative stress responses **(Chapter 4)**.

These studies supported the role of autologous MSCs for clinical use in COPD. In the clinical trial, we demonstrated the safety of autologous BM-MSCs administration in patients with severe to very severe COPD, without MSC-related effects on clinical parameters and on most of the analysed tissue parameters. However, increased expression of the endothelial cell marker CD31 and increased numbers of $CD3^+$ T-cells in alveolar walls were observed. It is tempting to ascribe these exciting observations to MSC-related effects, but the study design did not allow for such conclusions as a control group was lacking **(Chapter 5)**.

Although MSC administration in patients with COPD appears to be safe, the results from three independently conducted clinical trials have not been able to demonstrate clinically relevant effects. This is in contrast with the results from *in vitro* and *in vivo* models of lung epithelial and endothelial injury, where MSCs were shown to restore lung architecture and decrease inflammation. The discrepancy between these preclinical data and clinical results might relate to differences in optimization of treatment regimens and to a lack of proper outcome parameters (**Chapter 6)**.

The discussion will start by highlighting models to study lung development and repair, including our perspective on the importance of primary epithelial cultures to investigate repair in the human lung in the context of COPD. Next, our view on the directions of future studies with MSCs and how to improve our understanding of MSC efficacy and behaviour in humans will be discussed. Finally, perspectives to optimize MSC-based cell products and alternative strategies using MSCs in regenerative medicine are evaluated, and a general conclusion and outlook into future COPD management in light of regenerative medicine is presented.

## SECTION I MODELS OF AIRWAY EPITHELIAL INJURY

There is an urgent need to better understand repair mechanisms in the lung, to find new treatments and ultimately hopefully a cure for patients with COPD. In this regard, increased knowledge of lung development and physiological maintenance of the airway epithelium will be of great importance, as well as insight in airway epithelial responses to injury relevant to the topic of COPD. Appreciation of physiological processes will elucidate whether its dysregulation evolves into pathology, and will potentially provide useful tools for new intervention strategies to prevent or even reverse the development of lung diseases.



Insight in lung development and physiological maintenance of the airway epithelium has increased considerably over the past decade. Several studies have elucidated the role of transcription factors, growth factors and other signaling molecules, including Wnt and retinoic acid, during lung organ maturation, for instance via reconstructing the process of branching morphogenesis combined with transgenic mouse technologies *in vivo* or using matrigel cultures of lung endoderm *in vitro* (as reviewed in [10]). Information about stem and progenitor populations, their location and differentiation during lung development in the murine lung was obtained using "lineage tracing", a technique in which a transgenic (labelled) construct is inserted in the cell of interest, allowing tracing of these cells and their progeny [11]. This technique has also proven useful for investigating airway epithelial responses following injury *in vivo*, and thus contributed to our understanding of the progenitor function of basal cells in airway epithelial repair [12]. Since targeted genetic

manipulations are not feasible in humans, in humans mutations in mitochondrial DNA were used to identify and trace cells of interest and their progeny [13]. This way, clonal expansion of single airway epithelial progenitor cells was studied and progenitor cells were identified that maintain the upper airways in humans, and effects of aging and smoking on the heterogeneity of progenitor cell populations were demonstrated. For future research, this model can be used to compare the composition of epithelial progenitor cell populations between healthy subjects, healthy smokers and smokers with COPD and this may shed light on the hypothesis that COPD originates from alterations in basal cell biology [14], advance our understanding why not all smokers develop COPD and provide targets for early treatment interventions.

To gain insight in COPD pathogenesis and potential new treatment targets, it is common to use animal models of emphysema. Induction of emphysema is achieved by exposure to cigarette smoke (either alone or combined with lipopolysaccharide) or intratracheal instillation of proteolytic enzymes like elastase to induce pulmonary emphysema [15]. Alternatives include pharmacological induction of emphysema by treatment with vascular endothelial growth factor (VEGF)-receptor blockers or the use of transgenic mice [16,17]. Using animal models, therapeutic effects of several agents, including e.g. retinoic acid, growth factors (hepatocyte growth factor (HGF), VEGF) and MSC-based cell therapies have been investigated [18-20]. However, it is clear that the rodent lung does not accurately represent human biology, reflected also by the so far limited clinical success of therapies with retinoic acid [21] or MSCs [22], which underlines the importance of models more representative of the human situation.

In this light, the use of *in vitro* cell cultures of human cells obtained from the airway (primary bronchial epithelial cells (PBEC), peripheral lung (alveolar type 2 (AT2) cells) or pulmonary endothelium (lung-derived human primary microvascular endothelial cells (HPMVEC)) are increasingly relevant, preferably using cultures of primary cells. Primary cells represent a cell population that is present in the human body and more accurately reflect *in vivo* conditions compared to cell lines that are immortalized or tumor-derived. The benefit of cell culture studies relates to the possibility of controlled manipulation of specific cellular functions and processes, and simplification of complex interactions that are difficult to study *in vivo*. Using this model, processes essential to wound repair were elucidated, including spreading, migration and proliferation, as well as the interactions with extracellular matrix (ECM) and role of signaling molecules (reviewed in [23]). Frequently used *in vitro* injury and repair models include chemical, mechanical (e.g. scratch wounds) or electrical disruption of epithelial layers. By combining these models with exposure to a variety of airborne substances, such as allergens, air pollution, microbial pathogens, the effect of these environmental exposures on the repair process can be studied [24-26]. Apart from investigating barrier responses to injury, *in vitro* models have been used to detect differences between COPD patients and controls regarding wound closure rates, immunologic responses following injury,

tight junction formation and levels of Wnt expression [27-31], demonstrating its value to detect potential mechanisms involved in COPD pathogenesis.

Given the causative role of airborne toxicants to COPD development, it is essential to investigate airway epithelial cell responses to airborne toxicants, particularly to cigarette smoke (CS). Commonly, CS effects are tested using CS-extract (CSE): a filtered solution containing the noxious compounds from CS in suspension. CSE was shown to cause a loss of barrier integrity and differential effects on wound closure depending on the CSE-concentration were shown [29,32], but standardization of CSE preparation is difficult and the application to the cells does not reflect the *in vivo* situation. In **Chapter 2** we presented a more physiological model: PBEC cultured at the air-liquid interface were exposed to whole CS at the air-exposed side. Our laboratory has gained long-term experience using PBEC cultured at the air-liquid interface [33,34], reflecting *in vivo* conditions of the airway epithelium [35]. As demonstrated, this model allows combining CS-exposure with mechanical and chemical injury, enabling detailed investigation of repair and inflammatory responses. Options to extend the information derived from this model are numerous. For example: a detailed study of differentiation of cells, restoration of cell junctions and tracking of cells of interest using time lapse movies. This adds to knowledge derived using CSE or non-primary cells [29,36,37]. Furthermore, CRISPR-Cas9 based gene editing tools can be used to identify key mediators in repair. Besides, it would be highly relevant to set-up co-cultures of AT2 with HPMVEC to mimic the gas-exchange unit to increase our understanding of emphysema development and obtain new targets for therapy. Apart from the simplicity of *in vitro* models, it also offers a physiologically relevant alternative to animal studies, in compliance with a universal aim to reduce the number of animals needed for biomedical research. This also accounts for more novel *in vitro* culture systems, including organoids, microfluidic lung-on-a-chip and lung tissue slices. It is desirable that future studies on repair will increasingly make use of such *in vitro* systems.



## SECTION II OPTIMIZATION OF MSC-BASED TREATMENT REGIMENS

Prior to publication of data derived from the clinical trial described in chapter 5, one clinical trial investigating efficacy of intravenous administration of MSCs in patients with COPD had been published. This phase II trial conducted in 62 patients with moderate to severe COPD demonstrated safety of MSC administration, but was unable to show clinical improvement of pulmonary function parameters or quality of life [22]. The lack of clinical effects of MSC treatment has not discouraged research in this field, and the results presented in our clinical trial (**Chapter 5**), i.e. an increase of CD31 expression and changes in inflammatory cell numbers in alveolar walls, in fact support efforts to further explore the potential of MSCs to induce airway epithelial repair.

To do this, there are some obvious questions that require attention, for instance clarification of the optimal cell source, dosage, timing and route of administration during the course of the disease, as also argued by others [39]. Thus far, clinical trials that were conducted to investigate MSC efficacy have used heterogeneous protocols (even within organ systems and diseases), and were predominantly designed as safety studies without a control group [40]. This has hampered optimization of MSC-treatment protocols. As we have come to the point where MSC administration is considered safe [41], further clinical trials with expedient comparisons of the mentioned items using matched controls is a logical first step. This also asks for measurable outcome parameters to assess MSC-related effects, which in case of COPD trials typically include functional responses (e.g. pulmonary function testing, performance, quality of life), laboratory parameters and quantitative imaging. However, these parameters might not be as modifiable as we had hoped, as demonstrated by the negative phase II trial by Weiss *et al* [22]. This is potentially due to delayed effects of MSCs on these parameters, requiring prolonged treatment programs and/or follow-up. To circumvent this, parameters that may precede clinical improvement should be included, such as parameters of pulmonary inflammation and repair on tissue (similar to our approach as described in **Chapter 5**), as well as composition of inflammatory cells in sputum and bronchoalveolar lavage fluid (BALF), including cytokine concentrations. Adding bronchial and peripheral biopsies would allow analysis of potential effects on alveolar and endothelial structures, inflammatory infiltrates and airway epithelial remodelling, but requires invasive procedures to obtain tissue. Ideally, less invasive methods or biomarkers are needed to assess effects, e.g. in blood. It is postulated that although alterations in these parameters might not directly translate in clinical improvement, they might be early indicators that the progressive course of COPD can be amended, and changes in such 'basic' parameters might be more practical endpoints during optimization of MSC-treatment protocols in future clinical trials.

Routes of MSC administration to consider in COPD treatment include intratracheal instillation and intravenous administration. Intratracheal instillation was found to modify pulmonary inflammation and disease severity in bronchopulmonary dysplasia in new-borns [42]. Whether a similar response can still be expected in the adult lung needs to be elucidated, but is suggested by the decreased levels of C-reactive protein following intrabronchial MSC administration in COPD patients [43]. Intravenous administration on the other hand is probably relevant to target the periphery of the lung and the vascular component in particular, as our own data tentatively indicated responsiveness of the endothelium (**Chapter 5**). If either effect is indeed present, it seems reasonable to personalize the optimal route of MSC-administration, implying intravenous administration if COPD is characterized by emphysema and tissue destruction, and intratracheal administration when airway obstruction and chronic bronchitis predominate; or a combination of both.

Other items that need attention to optimize MSC treatment include MSC-host interactions and use of allogeneic versus autologous cells. The efficacy of MSCs appears to depend at least in part on MSC-host interactions, probably related to local inflammation, retention and survival of MSCs within subjects [5]. Increasing our understanding of MSC-host interactions is therefore important but it is also difficult, since MSCs do not express unique markers and labelling of MSCs in humans is restricted, but alternative (non-nuclear) labelling techniques are currently explored and may become available in the near future for use in humans as well [44,45]. Meanwhile, alternative models are needed to assess MSC-host interactions, and the use of *ex vivo* lung perfusion (EVLP) models can be of benefit in this respect. EVLP employs a laboratory set-up to preserve lungs that are unsuitable for lung transplantation, with the aim to maintain these lungs under physiological circumstances (albeit outside the human body) using a perfusion circuit and protective lung ventilation [46]. Its value in MSC-research was shown in a model of acute lung injury [47,48]. Using EVLP, labelled MSCs can be infused and tracked to gain insight in MSC homing, retention and survival in the lung. The information can be extended to assess MSCs' responses to COPD-related tissue destruction or inflammation, following for instance instillation of proteolytic enzymes (e.g. elastase) or even exposure of EVLP to cigarette smoke via the ventilator. It should be taken into account that the clinical translation of this model is limited by a lack of interactions with other organs and systemic responses and the relatively short preservation time of the model (approx. 7 days), which does not reflect the chronic course of COPD. EVLP-donor characteristics should also be considered, notably the fact that the lungs were rejected for transplantation implying some degree of organ dysfunction. However, this could become an advantage if lungs were rejected due to smoking-related disease, which would allow comparisons with 'healthy' donor lungs.



In clinical trials, both allogeneic and autologous MSCs are used. The advantage of allogeneic MSCs relates to their potential use as an 'off-the-shelve' therapeutic making them suitable for acute diseases, but they carry the risk of evoking alloimmune responses [49,50]. Autologous MSCs are unlikely to elicit such immune responses but are thought to display age- and disease related impairments [51], and their application is logistically more challenging and time-consuming. So far, based on the data presented in **Chapter 4** we consider treatment with autologous MSCs suitable in patients with advanced COPD, taking into account that the results should not be generalized to current smokers since acute effects of CS on bone marrow cells were not assessed. As both allogeneic and autologous MSCs have their advantages and disadvantages, it seems reasonable to compare their efficacy in clinical trials, including immune monitoring testing to assess safety of allogeneic MSCs, as has been suggested by others as well [49].

## SECTION III INCREASING THE THERAPEUTIC POTENTIAL OF MSC-BASED CELL THERAPIES

Apart from creating uniformity in study protocols for MSC administration, there is a need to create uniformity of MSC cultures themselves to improve interpretability of (pre-) clinical data. MSC cultures are heterogeneous due to a lack of MSC-specific markers and use of different culture protocols, contributing to functional variation between cell products (discussed in [52,53]). Minimal potency requirements of MSC-based cell products are still undefined, as it is unknown which assays best predict the potency of MSCs [54] and to what extent this potency *in vitro* translates to increased potential *in vivo* [52]. Besides, the desired potency profile of MSCs may differ between diseases. Proposed evaluation of MSCs' potency for use in COPD patients includes proliferation and migration potential, mRNA expression of several growth factors and cytokines in response to COPD-relevant inflammatory mediators, and wound repair potential, as we have presented in **Chapter 4**. For future investigations, interactions with immune cells should also be included, for instance T-cell proliferation assays and MSC-induced polarization of macrophages. Besides, interactions of MSCs with endothelial cells should be further investigated. This approach may contribute to the identification of superior cell products that are more effective at targeting COPD-related inflammation and tissue damage.

Continuing on, the next step should be to determine characteristics of these 'superior' cell products with respect to the composition of its subpopulations (as proposed in [53]). For instance, although still a matter of debate subpopulations of MSCs were shown to differentiate into lineages other than the mesenchyme, implying pluripotency of a fraction of MSCs (reviewed in [55]), and there is some evidence that links heterogeneity of MSCs to efficacy *in vivo* [56]. In potential, identification of 'superior' subpopulations can increase knowledge on their relative contribution to the cell products' potency and could contribute to development of a more purified cell product with increased clinical potential, or identify vectors to treat specific diseases. Such an approach using a subpopulation of MSCs, referred to as "multi-lineage differentiating stress enduring" (MUSE) cells [57], is in fact being developed by a Japanese-based company called Clio (www.clio-inc.com), and clinical trials from this company are awaited to demonstrate whether this approach should be carried forward.

Another approach to increase the therapeutic potential of MSC-based cell products encompasses pre-conditioning of MSCs during culture, for example by using pro-inflammatory mediators, growth factors or hypoxic culture conditions (reviewed in [58]). *In vivo*, compared to control-cultured MSCs, interferon-γ stimulated MSCs ameliorated colitis in mice [59], and MSCs cultured under hypoxic conditions protected against bleomycin-induced pulmonary fibrosis in mice [60], supporting the idea that preconditioning of MSCs increases their therapeutic potential. We observed increased expression of several growth factors and signaling molecules

following stimulation of MSCs with pro-inflammatory cytokines (**Chapter 3**), coinciding with increased regenerative potential of MSCs *in vitro*. However, manipulating MSCs prior to clinical administration warrants caution as potential adverse effects might be underestimated in animal models, and long term effects are unclear. To the best of our knowledge, this approach has thus far not resulted in clinical trials, except for one clinical trial that is currently recruiting patients with COPD to assess effects of hypoxia-cultured MSCs compared to placebo treatment (NCT01849159, to be completed in June 2017).

Similarly, genetic engineering of MSCs may increase their therapeutic potential, as for instance shown for MSCs overexpressing angiopoietin 1 (ANGPT1) or Interleukin (IL)-10 in mice models of acute respiratory distress syndrome [61,62]. However, genetic engineering or targeted gene addition usually involves transfection of cells using viruses as vectors, which limits the applicability in humans but does not make it impossible, as demonstrated by the use of gene therapy in patients with inherited primary immunodeficiency diseases [63]. The potential hazards of this virus-based approach can be bypassed in the future by using other gene editing tools, such as CRISPR-Cas9 based gene editing. Alternatively, MSCs are investigated as delivery vectors of therapeutic agents including nano-particles, suicide gene/enzyme prodrug systems, or oncolytic viruses, predominantly in the field of cancer research [64], but this can be applied to the field of regenerative medicine as well. However, to our knowledge no clinical data on either approach are available to date.

## SECTION IV ALTERNATIVE STRATEGIES TO TARGET TISSUE REPAIR



Clinical trials in COPD using MSCs have investigated effects of bone marrow-derived MSCs via administration of whole live cells. However, within the field of regenerative medicine other approaches to tissue repair are being developed, including exploration of the potential to activate endogenous lung progenitor cells including lung-resident MSCs, *ex vivo* tissue engineering and the use of induced pluripotent stem cells. These topics will briefly be addressed to generate a sense of the position of MSC-based therapies within this area of research.

MSCs reside in many different tissues other than the bone marrow, including the lung [65,66]. Lung-resident MSCs (LR-MSCs) possess distinct phenotypical and functional characteristics when compared to bone marrow-derived MSCs, including higher expression of lung-related signaling genes such as *FOXF1* and *SFRP1* (involved in Wnt signaling) [67]. LR-MSCs are suggested to form part of the lung stem cell niche, and although their specific contribution to the niche is still largely uncharacterized, animal studies indicate that they support epithelial stem cell growth and differentiation (as reviewed in [39]). As repair mechanisms in the lung are thought to be deficient

in COPD, it would be interesting to investigate endogenous repair by LR-MSCs, including the possibility of activating LR-MSCs to enhance repair, or the potential of LR-MSCs to activate key signaling pathways in other local progenitor cells, including activation of Wnt signaling which was found to attenuate experimental emphysema [68]. A comparison of LR-MSCs from COPD versus non-COPD controls will be relevant in this respect. Besides, lung-derived MSCs may be considered as a source for cell therapy: LR-MSCs can be obtained via bronchoscopy with BAL or peripheral biopsies and display high expansion rates *in vitro*, which are favourable properties for their potential use as cell therapy [65,66], and have a longer retention time in the lung compared to bone marrow-derived MSCs [69]. It is conceivable that increased retention might have beneficial effects on restoration of destructed lung tissue, especially in chronic diseases such as COPD, but this requires further investigation.

Another potential source of cell therapy is formed by induced pluripotent stem cells (iPSCs). iPSCs are derived from adult somatic cells (frequently skin fibroblasts), that are first reprogrammed towards cells with embryonic stem cell properties, and from this state can differentiate into theoretically any cell type [70,71], including MSCs (iMSCs) [72] and airway and alveolar epithelial (progenitor) cells [73]. Research in this field has provided important information on pathways that regulate lung development and epithelial cell differentiation, including bone morphogenetic protein (BMP), Wnt, fibroblast growth factor (FGF) and nodal signaling pathways [10,73,74]. Following exploration of pathways and signaling requirements to reprogram iPSCs towards pulmonary epithelium, it is conceivable that iPSCs can be used to investigate how modifications of reprogramming protocols influences epithelial cell development, which might increase our understanding of development of respiratory diseases. Besides, detailed knowledge on how these pathways are regulated should in theory enable us to selectively activate epithelial progenitor cells and the stem cell niche *in vivo*, with the ultimate aim to induce or enhance activity of endogenous stem cells for tissue repair. However, safety margins of such interventions are probably small, as demonstrated for instance by the relation of Wnt and Notch signaling with occurrence of fibrosis, cystic formation and cancer [75-77].

Apart from induction of endogenous repair or administration of cell therapies, an area of attention in the field of regenerative medicine consists of tissue engineering, i.e. the construction of functioning lungs *ex vivo* using a synthetic structure or decellularized lung as a scaffold, which is subsequently coated with cells that cover the scaffold and differentiate into pulmonary epithelium [78-80]. In potential, combining this approach with iPSCs as a patient-specific cell source will reduce the number of donor lungs needed and simultaneously eliminate problems related with graft rejection [71]. Bone marrow and adipose tissue-derived MSCs were also shown to adhere to scaffolds and differentiate towards lung epithelial phenotypes [81], supportive of their potential use as a stem cell source in tissue engineering, although functionality of this approach still needs

to be demonstrated. As an intermediate step before whole-organ engineering, implantation of smaller scaffolds at sites of severe tissue destruction might improve tissue structure as demonstrated in sheep: endobrochial placement of a scaffold covered with MSCs at sites pre-treated with elastase resulted in local tissue regeneration and improvement of supporting matrix [82]. Similarly, application of sheets coated with adipose tissue-derived cells in rats having had lung volume reduction surgery for emphysema increased alveolar and vascular regeneration and improved gas exchange and exercise tolerance [83]. Although largely unexplored, such alternative approaches to deliver MSCs (or other stem cells) at sites of tissue destruction should be considered in humans as well. In this light, alternative tissue engineering approaches like a 'lung-on-a-chip', a micro-physiological system that replicates the functional gas-exchange unit of the living human lung, also bears the potential to contribute to gas-exchange in destructed parts of lung tissue, provided that such a device can be integrated into local tissue.

## FUTURE DIRECTIONS AND GENERAL CONCLUSION

After reading the discussion, the impression may remain that the field of cell therapy in the context of COPD is characterized by questions rather than answers, and is still in its infancy. However, although we acknowledge the many uncertainties for the future of MSC-based cell therapies for COPD, we feel that this area of research has progressed towards its puberty, with a growing body of evidence on the mode of action and safety of MSC-treatments in several diseases. Nevertheless, it does challenge us with new questions.



It is likely, that different strategies for regenerative medicine are needed at various stages of the disease (see Figure 1). In limited disease, when airway epithelial progenitors and stem cell niches are still responsive to key signaling pathways, these pathways could be activated by a pharmacological approach in order to restart or enhance local tissue maintenance programs. Upon further disease progression, restoration of tissue architecture via exogenously administered MSCs or other stem/progenitor cell populations could be considered. Ideally, these stem cells are integrated within the local stem cell niche and will orchestrate restoration of pulmonary epithelium from here. In severe end-stage disease, when it has become impossible for stem cells to adhere to local tissue due to severe destruction, engineered scaffolds could be implanted in the lung or in more severe cases the whole lungs may be replaced by *ex vivo* engineered lungs to restore pulmonary function. Maturation of this area of research will reveal whether this direction for the future therapy of destructive lung diseases such as COPD is realistic.

In conclusion, the studies described in this thesis have provided novel insight into airway epithelial repair mechanisms and their modulation by cigarette smoke, and insight into mesenchymal stromal cell treatment of COPD. We have shown that inflammatory mediators present in the lungs of patients with COPD increase the regenerative potential of MSCs, and that MSCs from patients with severe COPD can be safely used as a cell-based therapy to treat these patients. Many questions remain regarding route of administration, dosage and timing of MSCs administration in COPD. Useful outcome parameters to assess MSC-mediated effects on lung tissue are largely undetermined, and we propose to include analysis of effects on endothelial and inflammatory cells in future clinical trials. The use of ALI-PBEC and alveolar epithelial cell cultures and *ex vivo* lung perfusion models will help to advance our understanding of the potential of MSCs in pulmonary diseases. Parallel developments in other areas of regenerative medicine, including those related to induced pluripotent stem cells and *ex vivo* organ engineering, will synergistically advance the much awaited therapeutic arsenal that is needed to restore pulmonary function in COPD.

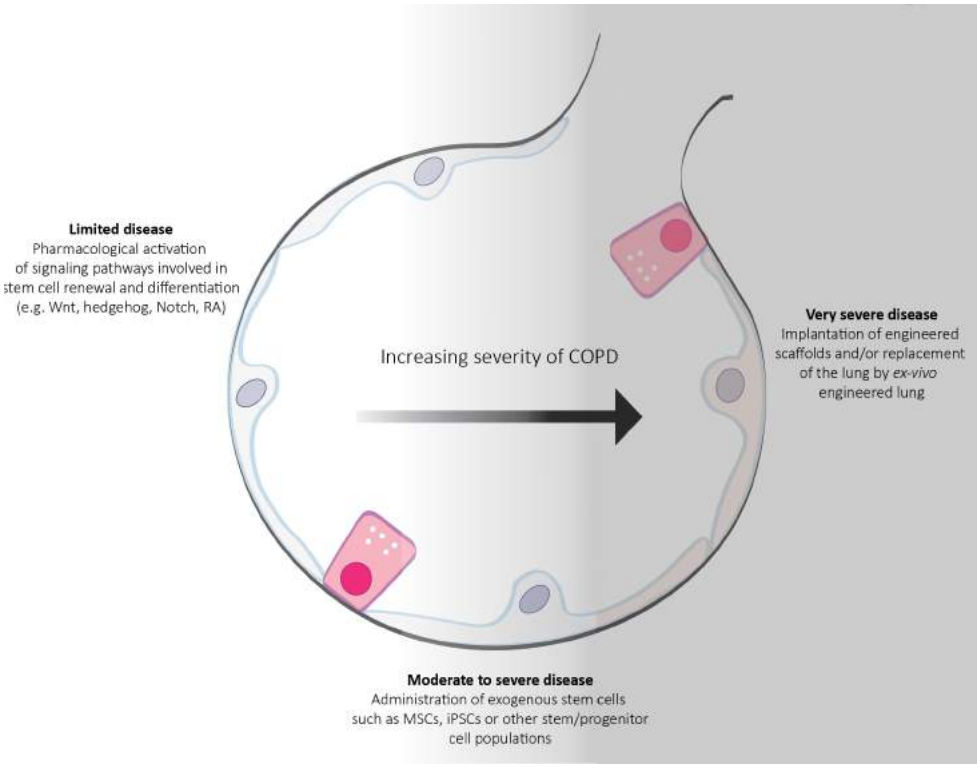


**Figure 1. Proposed approach to implement (future) regenerative strategies to restore destructive lung disease.** Various stages of tissue destruction require different approaches to accomplish tissue repair, related to the presence and responsiveness of endogenous cell populations. In lack of a population of endogenous stem cells that is able to regenerate damaged tissue, exogenous stem or progenitor cells can be administrated to support or even restore the local stem cell niche. When tissue architecture is damaged severely, engineered scaffolds or lungs can be used to restore organ function.

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ALI | air-liquid interface cultured |
| ANGPT1 | angiopoietin 1 |
| AT2 | alveolar type 2 cells |
| BAL(F) | bronchoalveolar lavage (fluid) |
| BC | basal cell |
| BM-MSC | bone marrow-derived MSC |
| BMP | bone morphogenetic protein |
| CS(E) | cigarette smoke (extract) |
| CD | cluster of differentiation |
| COPD | chronic obstructive pulmonary disease |
| CRISPR/Cas 9 | clustered regularly interspaced short palindromic repeats/Cas 9 |
| ECM | extracellular matrix |
| EGFR | epidermal growth factor receptor |
| EVLP | *ex vivo* lung perfusion |
| ERK1/2 | extracellular signal-regulated kinase 1/2 |
| FGF | fibroblast growth factor |
| FOXF1 | forkhead box F1 |
| GvHD | graft vs host disease |
| HGF | hepatocyte growth factor |
| HLA-DR | human leukocyte antigen D related |
| HPMVEC | human primary microvascular endothelial cells |
| IL | interleukin |
| iPSC | induced pluripotent stem cell |
| LR-MSC | lung resident MSC |
| MSC | mesenchymal stromal cell |
| MUSE | multi-lineage differentiating stress enduring |
| PBEC | primary bronchial epithelial cell |
| RNase 7 | ribonuclease 7 |
| SFRP1 | secreted frizzled related protein 1 |

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# NEDERLANDSTALIGE SAMENVATTING
# OVERZICHT PUBLICATIES
# CURRICULUM VITAE
# DANKWOORD

*ADDENDUM*

## NEDERLANDSTALIGE SAMENVATTING

Chronisch obstructieve longziekte (chronic obstructive pulmonary disease; COPD) wordt beschreven als "een gangbare aandoening die kan worden voorkomen en behandeld, waarbij sprake is van klachten van de luchtwegen en bemoeilijkte ademhaling doordat de luchtweg ('bronchitis') of de longblaasjes ('emfyseem') zijn aangetast, meestal als gevolg van relevante blootstelling aan schadelijke partikels en gassen" (bron: richtlijn Global Initiative for Chronic Obstructive Lung Disease 2017). Bij patiënten met benauwdheidsklachten, chronisch hoesten of ophoesten van slijm dient de diagnose COPD te worden overwogen, vooral als zij zijn blootgesteld aan risicofactoren, zoals sigarettenrook. Longfunctie onderzoek is vervolgens nodig, waarbij de verhouding tussen de 1-seconde waarde ($FEV_1$) en de vitale capaciteit (FVC) < 0.7 dient te zijn om de diagnose COPD te stellen.

Wereldwijd lijden meer dan 200 miljoen mensen aan COPD, waarvan er ca. 3 miljoen jaarlijks overlijden ten gevolge van deze ziekte. Hiermee neemt COPD de derde plaats in op de lijst van doodsoorzaken (bron: World Health Organization, Global Health Estimates). De verwachting is dat het aantal mensen dat lijdt aan COPD verder zal toenemen, onder andere door vergrijzing. Daarmee zullen ook de ziektelast en zorgkosten gerelateerd aan COPD stijgen. Voor deze groep patiënten is momenteel geen genezende behandeling beschikbaar. De behandeling van COPD richt zich op het verminderen van ziekte-gerelateerde klachten en het voorkomen van (versnelde) achteruitgang van de longfunctie. Het ontwikkelen van een behandeling die de voortgang van COPD tot staan kan brengen, of zelfs beschadigd longweefsel kan herstellen, is daarom relevant.

Er wordt in dit kader onderzoek verricht naar de werkzaamheid van celtherapie met mesenchymale stromale cellen (MSCs) bij patiënten met COPD. Deze cellen zijn voor het eerst ontdekt in het beenmerg, maar zijn op meerdere plekken in het lichaam aanwezig, zoals in vetweefsel en verschillende organen waaronder de long. MSCs blijken een gunstige invloed te hebben op reacties van het immuunsysteem en op het herstel van beschadigd weefsel. Deze eigenschappen hebben ertoe geleid dat het effect van behandeling met MSCs is onderzocht in proefdieren die COPD hebben ontwikkeld, waarbij toediening resulteerde in herstel van beschadigd longweefsel en vermindering van de ontsteking in de long. Het is de vraag of deze effecten ook bereikt kunnen worden in mensen. Om dit te onderzoeken is het belangrijk te weten welke cellulaire processen betrokken zijn bij het herstel van longweefsel en of er aangrijpingspunten zijn om zulk herstel te bevorderen.

Om te beginnen richt **hoofdstuk 2** zich daarom op het herstellende vermogen van luchtweg-epitheel (het epitheel is de bekleding van de luchtweg) en op hoe dit herstel beïnvloed wordt door de belangrijkste risicofactor voor het ontwikkelen van COPD: sigarettenrook. Epitheelcellen

die werden geïsoleerd uit de bronchi (centrale luchtwegen) werden mechanisch beschadigd met een plastic tip, of chemisch beschadigd door ze tijdelijk te kweken in een calcium-arm medium, waardoor de onderlinge celverbindingen en daarmee de epitheliale barrière werd verbroken. Hierna werd het epitheel blootgesteld aan sigarettenrook (interventie) of schone lucht (controle). Als uitkomst werd de snelheid van wondsluiting gemeten in het mechanische model, en de elektrische weerstand van de epitheliale barrière in het chemische model. Bronchusepitheelcellen bleken uit zichzelf in staat tot volledig herstel van de schade. Blootstelling aan rook gedurende slechts enkele minuten leidde echter tot een significante vertraging in dit herstelproces gedurende de eerste zes uren na blootstelling. Daarnaast zorgde rookblootstelling in beide schademodellen voor meer aanmaak van "RNase 7" (een eiwit met een afweerfunctie tegen microben) en in het chemische schademodel voor een toename van het eiwit IL-8 (dat zorgt voor meer ontsteking). Aangezien RNase 7 ook wordt beschouwd als een marker voor basale cellen (BCs), die op hun beurt worden gezien als voorlopercellen van bronchusepitheel, werd de bijdrage van BCs aan wondherstel verder onderzocht in het mechanische model. Aan de wondrand bleek het merendeel van de cellen BCs te betreffen (ca. 80% ten opzichte van 35% in het niet-beschadigde deel van de epitheellaag). Het absolute aantal BCs per oppervlakte wondrand was groter in rook-blootgestelde cellen ten opzichte van de controle-behandelde cellen. Deze data suggereren dat rookblootstelling de spreiding en migratiemogelijkheden van BCs aantast, wat vermoedelijk het wondherstel vertraagt. Verder werd aangetoond dat oxidatieve stress (een situatie waarbij een overmaat aan zuurstofradicalen in de cel aanwezig is) bijdraagt aan zowel herstel van epitheel na schade als aan de toename van RNase 7 en IL-8. Evenzo werd de rol van "extracellulair signaal-gereguleerde kinase (ERK) 1/2" en van de "epidermale groeifactor (EGF)-receptor" aangetoond, waarbij ook bleek dat oxidatieve stress leidt tot activatie van ERK1/2-signalering, onafhankelijk van de activatie van de EGF-receptor door sigarettenrook.

Vervolgens werd in **hoofdstuk 3** onderzocht of het herstelvermogen van luchtwegepitheel bevorderd kan worden door MSCs. Eerst werd het effect van ontsteking op MSCs onderzocht, omdat ontstekingsbevorderende stoffen (cytokines), zoals TNF-$\alpha$ en IL-1$\beta$, in verhoogde concentraties aanwezig zijn in luchtwegen van patiënten met COPD en het bekend is dat zulke cytokines de werkzaamheid van MSCs beïnvloeden. Hieruit bleek dat MSCs na stimulatie met TNF-$\alpha$ en IL-1$\beta$ meer groeifactoren aanmaken. Vervolgens is met behulp van celkweken van de cellijn NCI-H292 (luchtwegepitheel) onderzocht welk effect 'geconditioneerd medium' (kweekmedium dat in contact heeft gestaan met MSCs en dus door MSCs uitgescheiden moleculen bevat) heeft op de sluiting van mechanische wonden. Hierbij werd aangetoond dat het MSC-geconditioneerde medium wondsluiting versnelde ten opzichte van het controle medium (kweekmedium dat niet in contact was geweest met MSCs). Deze wondsluiting werd significant verder versneld als het geconditioneerde medium werd gebruikt van MSCs die tevoren gestimuleerd waren met TNF-$\alpha$/IL-1$\beta$. Het mechanisme bleek te berusten op versterkte activatie van de ERK1/2-signaleringsroute

in het luchtwegepitheel. Deze activatie werd voornamelijk veroorzaakt door de activatie van de epidermale groeifactor receptor (EGFR). De EGF-receptor werd na toediening van MSC-geconditioneerd medium op drie manieren geactiveerd: 1. door directe binding van moleculen aanwezig in het MSC-geconditioneerde medium aan de EGF-receptor; 2. door het vrijkomen van EGF-receptor activerende moleculen die onder normale omstandigheden vastgebonden zitten aan het epitheel en 3. door de verhoogde aanmaak van nieuwe EGF-receptor activerende moleculen door het luchtwegepitheel. Hoewel ook de hepatocyt groei factor (HGF) via de bijbehorende c-Met-receptor een bijdrage levert aan ERK1/2-activatie en wondherstel, bleek deze ondergeschikt aan de bijdrage van EGF-receptor activatie.

De behandeling met MSCs kan bestaan uit toediening van autologe (patiënt-eigen) of allogene (van een donor verkregen) cellen. De toediening van autologe MSCs heeft mogelijk de voorkeur, omdat de werking van allogene MSCs negatief kan worden beïnvloed door afweerreacties in de ontvanger. Het vaststellen dat autologe MSCs niet verschillen van allogene MSCs is relevant, omdat niet kan worden uitgesloten dat de werking van MSCs van COPD patiënten door de ziekte is aangetast. In **hoofdstuk 4** werden de MSCs van 9 ex-rokers met ernstig tot zeer ernstig COPD gekenmerkt door emfyseem (kapotte longblaasjes) vergeleken met die van 9 leeftijd-gematchte controles zonder longziekten en overwegend nooit-rokers. MSCs van beide groepen brachten dezelfde kenmerkende set oppervlaktemarkers tot uiting en waren in staat zich te ontwikkelen tot vet- en botcellen. Wel bleek dat MSCs van patiënten met COPD zich sterker tot vetcellen kunnen ontwikkelen in vergelijking tot MSCs van gezonde controles. De verdubbelingstijd van celpopulaties en het aantal MSCs dat per donor gekweekt kon worden verschilde niet, evenmin als de spreidingscapaciteit van de cellen. MSCs werden vervolgens gestimuleerd met TNF-$\alpha$ en IL-1$\beta$. Er werd aangetoond dat dit de genen activeert van verscheidene groeifactoren en ontstekingsmediatoren, zoals ook werd gezien in hoofdstuk 3, maar dat er tussen de twee groepen hierin geen verschillen aantoonbaar zijn. Aangezien oxidatieve stress een rol speelt in het ontstaan van COPD werd ook naar de reactie op oxidatieve stress (de antioxidant respons) van MSCs gekeken. Zowel sigarettenrook als sulforafaan (een antioxidant) activeren de antioxidant respons, waarbij er geen verschillen werden aangetoond tussen de respons in MSCs van COPD-patiënten versus gezonde controles, behoudens voor activatie van het gen *NQO1*: deze werd sterker geactiveerd in MSCs van de gezonde controles. Daarnaast werd de cellijn NCI-H292 gestimuleerd met geconditioneerd medium verkregen van MSCs al dan niet gestimuleerd met TNF-$\alpha$/IL-1$\beta$. Er werd geen verschil gevonden wat betreft het activeren van groeifactorgenen in deze cellijn. Hieruit werd geconcludeerd dat de kenmerken en de functie van MSCs van COPD patiënten vergelijkbaar is met die van gezonde, leeftijds-gematchte controles behoudens dat ze makkelijker differentiëren naar vetcellen en minder sterk *NQO1* activeren. Dit zou een uiting kunnen zijn van versnelde celveroudering, als systemisch verschijnsel van COPD.

In studieverband zijn MSCs op dit moment slechts beperkt toegepast in patiënten met COPD. In **hoofdstuk 5** werd onderzocht of de toediening van autologe MSCs veilig en technisch haalbaar is in patiënten met ernstig tot zeer ernstig COPD gekenmerkt door emfyseem (verlies van longblaasjes, uitrekken van de longen). De opzet van de studie maakte het tegelijkertijd mogelijk om effecten van MSCs op niveau van het longweefsel te beoordelen. Dit maakte deze studie uniek. Voor deze studie werden patiënten benaderd die in aanmerking kwamen voor longvolumereductiechirurgie, een chirurgische behandeling waarbij de long kleiner gemaakt wordt door de meest aangetaste longdelen te verwijderen. Deze operatie zou plaatsvinden aan beide longen, uitgevoerd in twee gescheiden operaties. Bij de deelnemende patiënten werd tijdens de eerste operatie ook beenmerg afgenomen, waaruit MSCs werden geïsoleerd en opgekweekt. In de periode tussen beide operaties kregen patiënten hun eigen MSCs toegediend via een infuus. Dit gebeurde tweemaal, 4 en 3 weken voorafgaand aan de operatie aan de andere long. Tijdens beide operaties werd longweefsel verkregen, en hierin werden eventuele effecten van MSCs onderzocht waarbij gebruik werd gemaakt van weefselkleuringen en analyse van genexpressie. Van de 10 patiënten die deelnamen, doorliepen er 7 het volledige studieprotocol. Gemiddeld zaten er 12 weken tussen de eerste en tweede operatie. Er werden geen bijwerkingen gezien ten gevolge van de behandeling met MSCs en bij alle patiënten werd een significante toename gezien van de $FEV_1$ en het lichaamsgewicht, gerelateerd aan de operatie zelf. Verscheidene groeifactoren en markers voor celdeling, ontsteking en voor longcellen bleven voor en na MSC-toediening onveranderd. Waar wel een verschil in werd gevonden was CD31, een marker voor endotheel (bekleding van de bloedvaten). Deze marker kwam verhoogd tot expressie in longweefsel verkregen tijdens de tweede operatie, dus na toediening van MSCs. Daarnaast was het aantal $CD3^+$ T-lymfocyten (een bepaald type witte bloedcel, betrokken in ontstekingsreacties) hoger in het weefsel dat was verkregen tijdens de tweede operatie. Wat deze bevindingen betekenen is onduidelijk, maar mogelijk kan het wijzen op een reactie van de bloedvatwand van bloedvaten rond de longblaasjes. Dit legitimeert verder onderzoek naar de werkzaamheid van MSCs bij ernstig emfyseem.

**Hoofdstuk 6** geeft een overzicht van de studies die de toepassing van MSCs in COPD onderzochten, zowel preklinische studies (celkweken, diermodellen) als klinische studies. Vanuit preklinische studies is gebleken dat MSCs een anti-inflammatoire, ontstekingsremmende werking hebben. Zo verbeteren MSCs de balans tussen proteases, die de long kunnen afbreken, en proteaseremmers en beïnvloeden ze de werking van macrofagen, die hierdoor een anti-inflammatoir profiel krijgen. Daarnaast scheiden MSCs eiwitten uit die remmend werken op de groei van bacteriën. In modellen die wondsluiting of weefselherstel onderzoeken werd gezien dat MSCs celdeling en migratie stimuleren en de "geprogrammeerde celdood" afremmen. Hierdoor sluiten wondjes sneller en treedt er herstel op van de architectuur van de long in proefdieren met emfyseem. Pre-stimulatie met pro-inflammatoire cytokines doet de potentie van MSCs verder

toenemen. Het werkingsmechanisme van MSCs lijkt onder meer te berusten op de uitscheiding van verscheidene signaaleiwitten en -moleculen, zoals cytokines en groeifactoren, en de directe aanzet van epitheel- en endotheelcellen tot het produceren van gelijksoortige moleculen. Er is gesteld dat MSCs kunnen innestelen in het epitheel van de ontvanger en zo kunnen bijdragen aan weefselherstel, maar hiervoor zijn te weinig bewijzen. Qua klinische studies zijn nu drie publicaties beschikbaar over de toediening van MSCs in patiënten met COPD, waaronder onze eigen studie uit hoofdstuk 5. Het betreft in totaal een kleine groep patiënten die is behandeld (38 mensen met matig tot zeer ernstig COPD). Tot nu toe lijkt de toediening van MSCs (autoloog of allogeen) veilig te zijn, maar er werd geen effect gezien op klinisch relevante parameters. Een verklaring voor het verschil ten opzichte van de diermodellen kan liggen in het feit dat de diermodellen zijn geoptimaliseerd voor het vinden van effecten, door een nadruk op het voorkomen van de ontwikkeling van ziekte in plaats van behandeling van bestaande ziekte, en het gebruik van relatief hoge doseringen MSCs. Daarnaast werden in proefdierstudies uitkomstmaten gebruikt die in mensen lastiger te verkrijgen zijn. Het is daarom belangrijk om ook in mensen de behandelprotocollen verder te optimaliseren en op zoek te gaan naar bruikbaardere uitkomstmaten, zoals CD31 of de mate van ontsteking in longweefsel of in sputum. Het gebrek aan effectieve behandelingen voor COPD zal een belangrijke prikkel zijn voor onderzoek op dit gebied.

De bevindingen verkregen uit de voorgaande hoofdstukken worden in een bredere context bediscussieerd in **hoofdstuk 7**. Naast een samenvatting bestaat de discussie uit vier onderdelen. Eerst komt aan bod hoe schade aan luchtwegepitheel onderzocht kan worden en wat de alternatieven zijn voor diermodellen, waarbij het nut van celkweken met primaire cellen wordt besproken. Als tweede wordt ingegaan op aspecten van de behandeling met MSCs die in toekomstige studieprotocollen verder onderzocht moeten worden (o.a. dosering, timing en toedieningsweg, het gebruik van allogene versus autologe cellen) en worden alternatieve eindpunten voor klinische studies in COPD aangedragen. In het derde deel wordt het optimaliseren van de werking van MSCs bediscussieerd, zoals pre-stimulatie met cytokines of genetische modificatie of de voorselectie van cellen met 'superieure' eigenschappen binnen de hele populatie MSCs. Als laatste worden overige ontwikkelingen in het veld van longregeneratie besproken: het activeren van de lokale stamcellen in de long, het gebruik van zogenaamde patiënt-eigen 'geïnduceerde pluripotente stamcellen', en de ontwikkeling van 'tissue engineering', wat zoveel wil zeggen als het maken van stukjes nieuw longweefsel of - als ultiem doel - een gehele long buiten het menselijk lichaam.

De toekomst van de behandeling van COPD gericht op weefselherstel ("regeneratieve geneeskunde") zou er als volgt uit kunnen zien: bij een redelijke conditie van het resterende longweefsel wordt met medicijnen geprobeerd de eigen stamcellen tot deling en weefselherstel

aan te zetten. Bij ernstigere schade is toediening van celtherapie nodig om het weefsel weer in conditie te krijgen, terwijl bij vergaande schade het weefsel vervangen dient te worden door buiten het lichaam vervaardigde stukjes longweefsel of longen.

De in dit proefschrift beschreven onderzoeken hebben een bescheiden bijdrage geleverd aan de inzichten in het herstel van epitheel en in de werking van MSCs in COPD. Verder klinisch onderzoek naar de werking van MSCs moet zich richten op optimalisatie van studieprotocollen en het vinden van beter bruikbare uitkomstparameters. Parallel aan deze ontwikkelingen zullen andere gebieden van 'regeneratieve' geneeskunde zich verder ontwikkelen om zo gezamenlijk tot een breder arsenaal aan therapeutische opties te komen om uiteindelijk COPD te kunnen genezen. De belangrijke plaats die regeneratieve geneeskunde inneemt in de Nationale Wetenschapsagenda en in het Nationaal Programma Longonderzoek (een initiatief van de Netherlands Respiratory Society (NRS) en verschillende stakeholders, inclusief patiënten) zal naar verwachting nieuwe ontwikkelingen op dit terrein verder stimuleren, die ten goede komen aan patiënten met chronisch longziekten zoals COPD.

**AFKORTINGEN EN TOELICHTING TERMINOLOGIE**

| | |
|---|---|
| BCs | basale cellen, fungeren als voorlopercellen van luchtwegepitheel |
| CD31 | cluster of differentiation 31, marker voor endotheel (bekleding bloedvaten) |
| COPD | chronic obstructive pulmonary disease (chronisch obstructieve longziekte) |
| EGF(R) | epidermal growth factor (receptor), bevindt zich op de celwand en kan na activatie meerdere signaleringsroutes in de cel aanzetten (o.a. ERK1/2 route) |
| ERK1/2 | extracellular signal-regulated kinase 1/2, een signaleringsroute die betrokken is in celdeling, spreiding en overleving |
| $FEV_1$ | forced expiratory volume in 1 second (1-seconde waarde) |
| FVC | forced vital capacity (geforceerde vitale capaciteit) |
| HGF | hepatocyte growth factor |
| IL-1β | interleukin 1β, een ontstekingsbevorderende mediator |
| IL-8 | interleukin 8, een ontstekingsbevorderende mediator |
| MSCs | mesenchymale stromale cel |
| NQO1 | NAD(P)H dehydrogenase (quinone) 1, maakt onderdeel uit van de antioxidant respons |
| RNase 7 | ribonuclease 7, een eiwit betrokken in afweer tegen microben |
| TNF-α | tumor necrosis factor-α, een ontstekingsbevorderende mediator |

**OVERZICHT PUBLICATIES**

W. Broekman, G.D. Amatngalim, Y. de Mooij-Eijk, J. Oostendorp, H. Roelofs, C. Taube, J. Stolk, P.S. Hiemstra. TNF-α and IL-1β-activated human mesenchymal stromal cells increase airway epithelial wound healing *in vitro* via activation of the epidermal growth factor receptor. *Respiratory Research*, 2016 Jan 11; 17:3.

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G.D. Amatngalim*, W. Broekman*, N.M. Daniel, E.P.M. van der Vlugt, A. van Schadewijk, C. Taube, P.S. Hiemstra (* contributed equally). Cigarette smoke modulates repair and innate immunity following injury to airway epithelial cells. *Plos One*, 2016 Nov 9; 11 (11): e0166255.

**CURRICULUM VITAE**

Winifred Broekman is geboren op 17 april 1982 te Amsterdam. Na haar middelbareschooltijd op College Hageveld te Heemstede werd zij rechtstreeks toegelaten tot de studie Geneeskunde aan de Rijksuniversiteit Groningen. In 2006 behaalde zij cum laude haar artsexamen, waarna ze als ANIOS interne geneeskunde in het Rijnland Ziekenhuis en op de Intensive Care van het Westeinde Ziekenhuis haar eerste werkervaring opdeed. In 2009 startte zij met de specialisatie tot longarts. Na afronden van de vooropleiding begon zij in april 2010 met het onderzoek naar de effecten van celtherapie met mesenchymale stromale cellen op luchtwegepitheel en in patiënten met COPD, onder begeleiding van Prof. dr. Pieter S. Hiemstra en dr. Jan Stolk in het Leids Universitair Medisch Centrum. Een deel van haar onderzoeksperiode was zij verbonden aan het NHLI Imperial College London (UK), onder supervisie van dr. Matt Hind en gesponsord door een ERS Fellowship. Het resultaat van dit werk is gebundeld in het proefschrift dat u nu in handen heeft. In januari 2014 hervatte zij haar opleiding tot longarts in het HAGA Ziekenhuis te Den Haag, waarbij haar interesse uitgaat naar immunologie en infectieziekten.

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WINIFRED BROEKMAN