

**DEFINING THE NEXT-GENERATION UMBILICAL CORD-DERIVED
CELL THERAPY FOR TREATMENT OF BRONCHOPULMONARY DYSPLASIA**

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SUMMARY

Bronchopulmonary dysplasia (BPD) is a chronic lung disease and one of the most severe complications that develop in premature infants following mechanical ventilation, exposure to supplemental oxygen, and inflammation. The hallmarks of the lung pathology are arrested lung development, including fewer and larger alveoli with less septation, thickening of alveolar septa, and impaired development of the capillary network. BPD is associated with increased mortality, respiratory morbidity, neurodevelopmental impairment, and increased healthcare costs. Significant advancements in neonatology in the last several decades, including antenatal steroids and exogenous surfactant replacement therapy, more gentle ventilation methods, and judicious oxygen use, have allowed for the survival of more preterm infants. However, the incidence of BPD still remains high and currently, there is no cure for the disease. Novel effective interventions at this stage of life are of exceptional value.

Considering their great potential in promoting tissue regeneration and modulating inflammation, mesenchymal stromal cells (MSCs) represent a promising avenue for treating several disorders, including BPD. Umbilical cord-derived MSCs (UC-MSCs) offer biological advantages over other MSC sources (easily available, high proliferative capacity, and better repair efficacy). Pioneering work in our lab showed that MSCs prevent injury to the developing lung in a rat model mimicking BPD. However, there are still considerable challenges that must be overcome before MSCs can be effectively implemented in clinical trials. As such, UC-MSC heterogeneity is poorly understood, with concerns regarding variations from donors and batches. Thus, to improve the reproducibility of basic research and clinical applications, and to identify the optimal therapeutic cell product, better molecular characterization of UC-MSCs and the development of standardized BPD models will be essential in the clinical translation of MSC

therapy for BPD. Moreover, considering that BPD is a disease of prematurity, the therapeutic potential of UC-MSCs isolated from preterm birth is of major interest.

In the study presented here, using single-cell RNA sequencing (scRNA-seq), we characterized MSCs isolated from the UC of term and preterm pregnancies at delivery (term and preterm donors), as well as non-progenitor control cell line, human neonatal dermal fibroblasts (HNDFs). Moreover, we associated UC-MSC transcriptomic profiles with their therapeutic potential in hyperoxia-induced lung injury in neonatal rats. Finally, we developed and characterized a novel two-hit (2HIT) BPD model in neonatal mice, assessed UC-MSCs' optimal route of injection, timing, and dose, and evaluated their therapeutic effects in that model.

We showed that UC-MSCs isolated from the majority of term and preterm donors, including preterm donors with pregnancy-related complications, have limited heterogeneity and possessed a transcriptome enriched in genes related to cell cycle and cell proliferation activity (termed “progenitor-like” cells). In contrast, UC-MSCs isolated from one term and two preterm donors with preeclampsia displayed a unique transcriptome comprised of many genes related to fibroblast activity, including extracellular matrix (ECM) organization (termed “fibroblast-like” cells). In addition, treatment with progenitor-like UC-MSCs, but not with fibroblast-like cells nor HNDFs, significantly improved lung structure, function, and pulmonary hypertension (PH) in hyperoxia-induced lung injury in neonatal rats. We identified marker genes for the therapeutic UC-MSCs (progenitor-like cells) and non-therapeutic cells (fibroblast-like cells and HNDFs). Among them, the high expression of major histocompatibility complex class I (MHCI) is associated with a reduced therapeutic effect.

Furthermore, we developed a novel 2HIT BPD mice model with in-depth characterization of the innate immune response and lung injury. 2HIT injury caused a transient type 1

proinflammatory cytokine response and a significant decrease in type 2 anti-inflammatory cytokine lung expression and number of anti-inflammatory M2 type alveolar macrophages. Moreover, 2HIT mice showed impaired lung compliance and growth. Repeated intravenous (*i.v.*) injections of UC-MSCs at a dose of 20×10^6 cells/kg body weight (BW) on postnatal day (PD) one and two improved survival, BW, lung compliance, and growth of 2HIT animals.

In conclusion, scRNA-seq experimentation provided evidence that UC-MSCs isolated from different donors harbor different transcriptomes with progenitor-like or fibroblast-like characteristics. Only progenitor-like cells provided a therapeutic effect in hyperoxia-induced lung injury in neonatal rats. The development of a novel murine 2HIT BPD model allowed us to characterize the innate immune response and lung pathology and confirm the optimal dose of UC-MSCs to provide therapeutic potential in that model. These results will enable better therapeutic selection of UC-MSCs and help improve treatment regimen prior to ultimate clinical translation.

ABBREVIATIONS

2HIT	2 hit
α -SMA	Alpha smooth muscle actin
A	Inspiratory capacity
AF	Amniotic fluid
AmEC	Amnion epithelial cell
ANG-1	Angiopoietin 1
ARG1	Arginase 1
AT	Adipose tissue
AT1	Alveolar epithelial type 1 cell
AT2	Alveolar epithelial type 2 cell
BASC	Bronchioalveolar stem cell
BM	Bone marrow
BPD	Bronchopulmonary dysplasia
BW	Body weight
cDNA	Complementary deoxyribonucleic acid
CM	Conditioned media
COL1 α	Collagen 1 alpha
CRTD	Center for Regenerative Therapies Dresden
Cst	Static compliance
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline

E	Embryonic day
ECM	Extracellular matrix
<i>E. coli</i>	<i>Escherichia coli</i>
ERS	European Respiratory Society
ESC	Embryonic stem cell
EV	Extracellular vesicle
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
FGF-7	Fibroblast growth factor 7
FGFR3	Fibroblast growth factor receptor 3
FGFR4	Fibroblast growth factor receptor 4
Fig	Figure
GA	Gestational age
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practices
GO	Gene ontology
H&E	Hematoxylin and eosin
HIF	Hypoxia-inducible factor
HLA-I	Human leukocyte antigen class I
HLA-II	Human leukocyte antigen class II
HLA-ABC	Human leukocyte antigen A, B and C
HNDF	Human neonatal dermal fibroblast

HSC	Hematopoietic stem cell
IDO	Indoleamine 2,3-dioxygenase
IFN γ	Interferon gamma
IL-1 α	Interleukin 1 alpha
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17	Interleukin 17
IL-33	Interleukin 33
<i>i.n.</i>	Intranasal
<i>i.p.</i>	Intraperitoneal
IP-10	Interferon gamma-inducible protein 10
iPSC	Induced pluripotent stem cell
ISCT	International Society for Cell and Gene Therapy
<i>i.t.</i>	Intratracheal

<i>i.v.</i>	Intravenous
IVIS	In Vivo Imaging Systems
KC	Keratinocyte-derived chemokine
LIF	Leukemia inhibitory factor
LIX	Lipopolysaccharide-induced C-X-C chemokine
LPP	Late preterm preeclampsia
LPS	Lipopolysaccharide
LR	Lung resident
LTBP	Latent transforming growth factor beta binding protein
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MHCI	Major histocompatibility complex class I
MHCII	Major histocompatibility complex class II
Micro-CT	Micro-computed tomography
MIG	Monokine induced by interferon-gamma
MIP-1 α	Macrophage-inflammatory protein 1 alpha
MIP-1 β	Macrophage-inflammatory protein 1 beta
MIP-2	Macrophage-inflammatory protein 2
MLI	Mean linear intercept
MMP9	Matrix metalloproteinase 9
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal/stem cell
MSC-EV	MSC-derived extracellular vesicle

NEGR	Neuronal growth regulator
NLCP	Neonatal lung cell population
NO	Nitric oxide
NOTCH2	Neurogenic locus notch homolog protein 2
NRXN	Neurexin
OHRI	Ottawa Hospital Research Institute
P	Preterm
PAAT	Pulmonary arterial acceleration time
PAET	Pulmonary arterial ejection time
PBS	1X Phosphate buffered saline
PC	Preterm chorioamnionitis
PCR	Polymerase chain reaction
PD	Postnatal day
PDGF-A	Platelet-derived growth factor A
PDGFR α	Platelet-derived growth factor receptor alpha
PDGFR β	Platelet-derived growth factor receptor beta
PH	Pulmonary hypertension
PL	Placenta
PN	Passage number
PNEC	Pulmonary neuroendocrine cell
PP	Preterm preeclampsia
Ppl	Pressure plethysmography
PRR	Pattern recognition receptor

PTX3	Pentraxin 3
PV-loop	Pressure-volume loop
qPCR	Quantitative real time-polymerase chain reaction
RA	Room air
RANTES	Regulated upon activation, normal T cell expressed and presumably secreted
RAS	Renin-angiotensin system
RDS	Respiratory distress syndrome
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
RVH	Right ventricular hypertrophy
scRNA-seq	Single-cell RNA sequencing
SD	Sprague Dawley
SDF-1	Stromal cell-derived factor 1
SGA	Small for gestational age
STD	Standard deviation
Suppl	Supplementary
T	Term
TA	Tracheal aspirate
TGF β	Transforming growth factor beta
TGF β 1	Transforming growth factor beta 1

TIMP1	Tissue inhibitor of metalloproteinases 1
TLR	Toll-like receptor
TNF α	Tumor necrosis factor alpha
UC	Umbilical cord
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VEGFR2	Vascular endothelial growth factor receptor 2
Vpl	Volume plethysmography

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CHAPTER 1: INTRODUCTION

1.1 Bronchopulmonary dysplasia

1.1.1 Epidemiology and pathophysiology

Preterm births, defined as any birth before a gestational age (GA) of 37 weeks, occur in about 8% of pregnancies in Canada (1). The range of preterm birth rates across 184 countries is between 5% to 18% (Fig. 1.1).

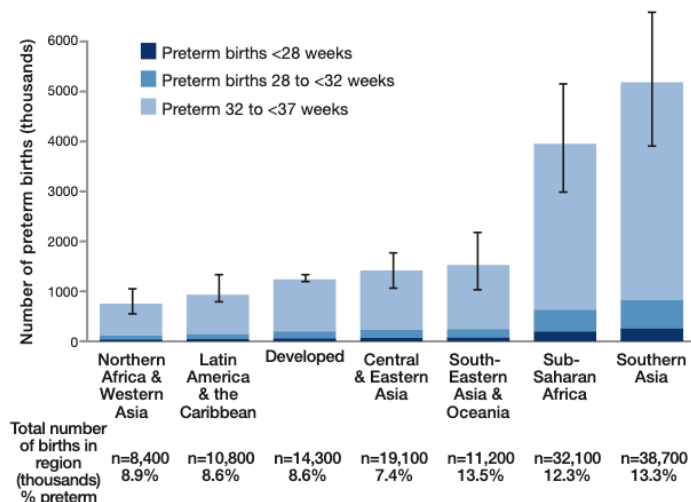


Figure 1.1 Preterm births by gestational age (GA) and region for 2010.

Premature birth rates and the relative proportions within the subcategories of extremely preterm (<28 weeks GA), very preterm (28 to <32 weeks GA), and late preterm births (32 to <37 weeks GA) vary throughout the world. Modified from (2).

This is a critical healthcare situation as preterm birth complications are the leading cause of mortality and morbidity in infants under five years of age (3). Bronchopulmonary dysplasia (BPD), a chronic lung disease, is the most severe complication of extreme prematurity (any birth before 28 weeks GA) (4). “Classic” or “old” BPD, first described by Northway and colleagues in 1967 (5), occurred in modestly premature infants with surfactant deficiency resulting in respiratory distress syndrome (RDS). Treatment interventions for RDS, which include mechanical ventilation and long-term use of high oxygen concentration, appeared to be the causes of the development of BPD (5). Advances in perinatal care to prevent and treat RDS (e.g., antenatal steroids, surfactant therapy, and less injurious ventilation techniques) have enabled the survival of more immature

infants (6). As a result, the “new” BPD mainly develops in extremely preterm infants (6). At this early developmental stage, even minimal exposure to injurious stimuli can impair the process of pulmonary microvascular and alveolar growth. Certain conditions recognized to predispose to BPD include intrauterine growth restriction (7,8), genetic predispositions (9), smoke exposure (10), preeclampsia (pregnancy-induced high blood pressure and proteinuria) (11), perinatal inflammation (12), sepsis (13), as well as pneumonia pattern (14). On the other hand, epidemiological data on the role of chorioamnionitis (acute/chronic inflammation of the membranes and fetal portion – chorion – of the placenta) as a risk factor for BPD are contradictory (15,16). This inconsistency may be attributed to the difficulty of controlling all confounding factors contributing to the disease (15,17).

In contrast with the “classic” BPD, which is characterized by collapsed alveoli, inflammation, fibrosis, and thickening of the septa, the “new” BPD is described as an arrest in normal lung growth resulting in fewer and simplified alveoli (larger air sac with fewer secondary crests), and impaired microvascular development, with less striking fibrosis (18) (Fig.1.2).

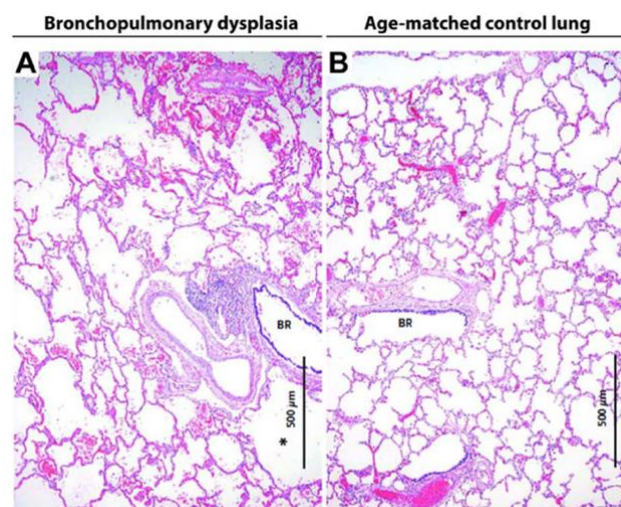


Figure 1.2 Lung histology of BPD and control donors.

Hematoxylin and eosin (H&E)-stained peripheral lung structure in a BPD patient (A) and a comparable area of lung tissue from an aged-matched donor with normal alveolarization (B). The asterisk shows one example of an abnormally large space. BR: bronchiole. Modified from (19).

Clinically, BPD is defined by the requirement of respiratory support at 36 weeks post-menstrual age (GA + chronological age). Neonatal morbidities of BPD patients include brain injury and retinopathy of prematurity (20). Beyond the neonatal period, BPD has the highest cost in initial neonatal intensive care unit stay and significantly increases the risk of long-term chronic illness and rehospitalization (21). In addition, the dysregulated pulmonary vasculature can lead to the development of pulmonary hypertension (PH) in a subgroup of BPD patients, especially prevalent in infants with severe BPD (29-58%) (22). PH may occur as an early (0 to 2 weeks of age), late (weeks to months of age), or chronic (months to years of age) form of the disease (23–26). Despite significant advancements in neonatal management, including the application of less invasive strategies, the incidence of BPD increased among survivors of extremely preterm birth between 1993 and 2012 (figure 1.3) (27). At present, around 45% of extremely preterm infants develop BPD worldwide (27,28). As the current clinical strategies fail to diminish the increasing BPD incidence, the development of innovative therapeutic approaches remains needed.

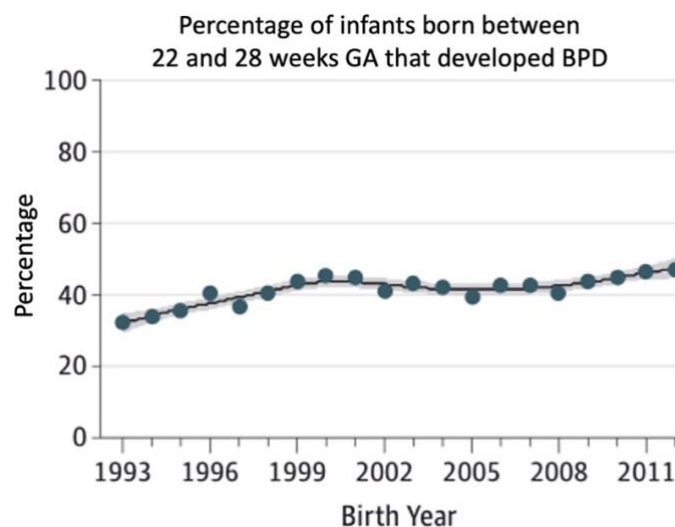


Figure 1.3 Percentage of infants born between 22 and 28 weeks GA that developed BPD. Circles show the percentage of infants born each year diagnosed with BPD, and the smoothed curve show the trend. Total number of infants (mean [range] per year): 25,000 (1,250 [746-1,534]). Study sites were distributed throughout the United States. Modified from (27).

1.1.2 Stages of lung development

Development of the respiratory system starts during the embryonic period and continues into early adulthood. The five stages of lung development are 1) embryonic, 2) pseudoglandular, 3) canalicular, 4) saccular, and 5) alveolar stages (Fig. 1.4). Extremely preterm and preterm infants are born during the late canalicular and saccular stages of lung development. Although gas exchange may take place in the distal lung at those two stages, the surface area remains small. Thus, the saccular stage is especially important for the development of the distal lung and the growth of the airspaces. Once the airspaces attain a sufficient size, the alveoli begin to form following the process of septation and capillary network maturation. This process perdures into young adulthood and facilitates gas exchange by increasing lung surface area and reducing the distance between the airspace and the microvasculature (29,30).

1.1.3 Normal alveolarization

Alveolarization is the process of generating the functional units of gas exchange: the alveoli. Due to the limited availability of human tissue, most of our knowledge about the mechanism of alveolarization has been acquired from studies in rodent models, but important differences exist between rodents and human lung development. Rodents delivered at term are at the saccular stage of lung development (25) (Fig. 1.4), which is the stage at which most preterm infants are born (30). Therefore, rodents born at term are often used as an *in vivo* model to mimic the lungs of preterm infants. However, it is essential to note that rodent lungs at the saccular stage are fully functional, whereas human lungs at the saccular stage are not.

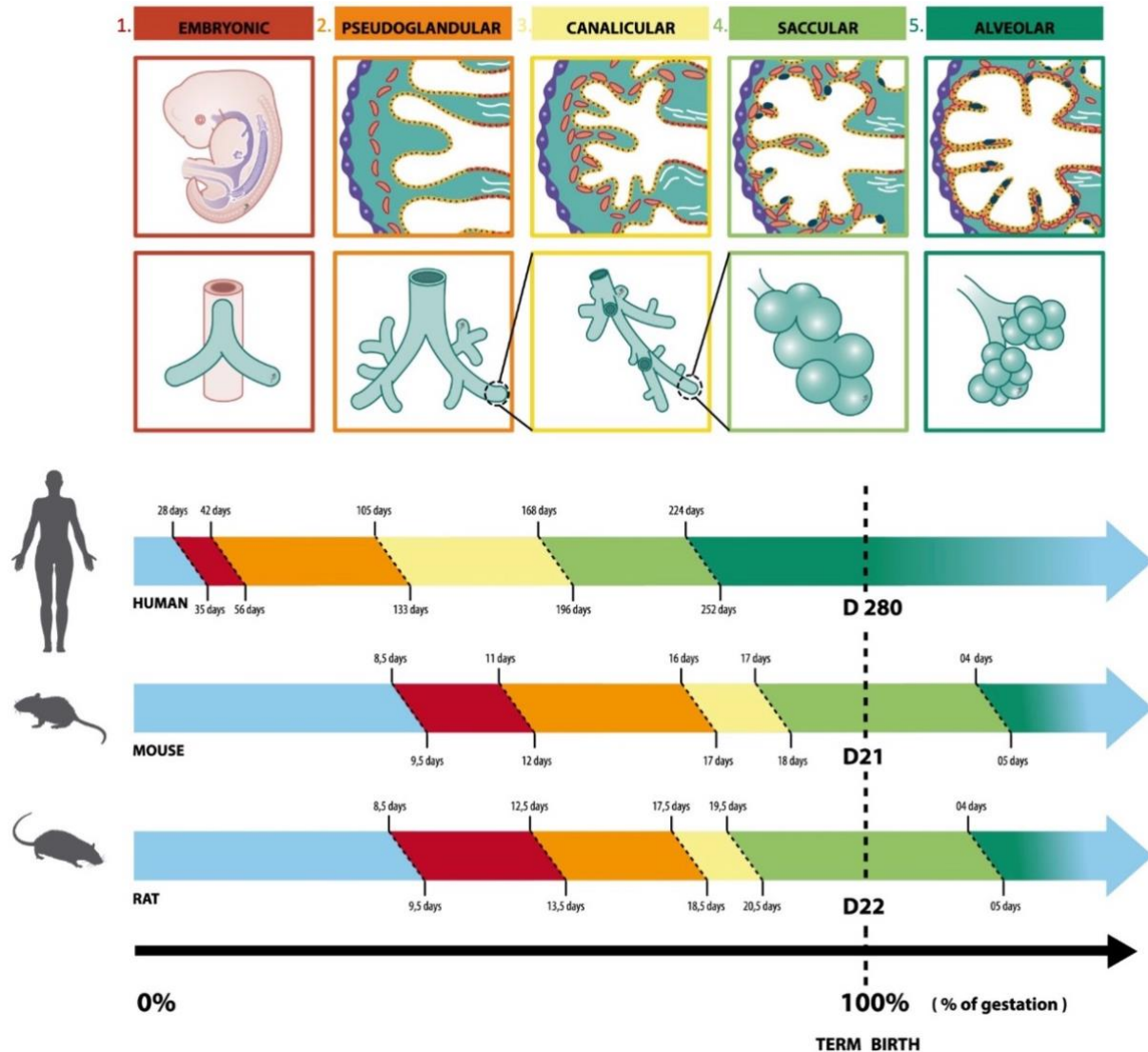


Figure 1.4 Mammalian stages of lung development.

Lung development occurs in five stages: **1)** The embryonic stage involves the development of the larynx and the trachea, which are created from the foregut. Two small buds are formed, which eventually become the lungs. **2)** The pseudoglandular stage is when the remaining conducting airways develop; the endodermal tubules develop into terminal bronchioles and are surrounded by a modest capillary network. **3)** During the canalicular stage, the terminal bronchioles develop into alveolar ducts. Development of vascular beds and capillaries takes place. **4)** During the saccular stage, the alveolar saccules are formed, and surfactant is detectable in amniotic fluid (AF). **5)** During the alveolar stage, more surfactant is produced, and the terminal sacs develop into alveoli due to secondary septation. By the age of eight years, the lung will comprise 300 to 400 million alveoli. Modified from (31).

The formation of mature alveoli is regulated via a complex network of signaling pathways involving many pulmonary cell types (Fig. 1.5).

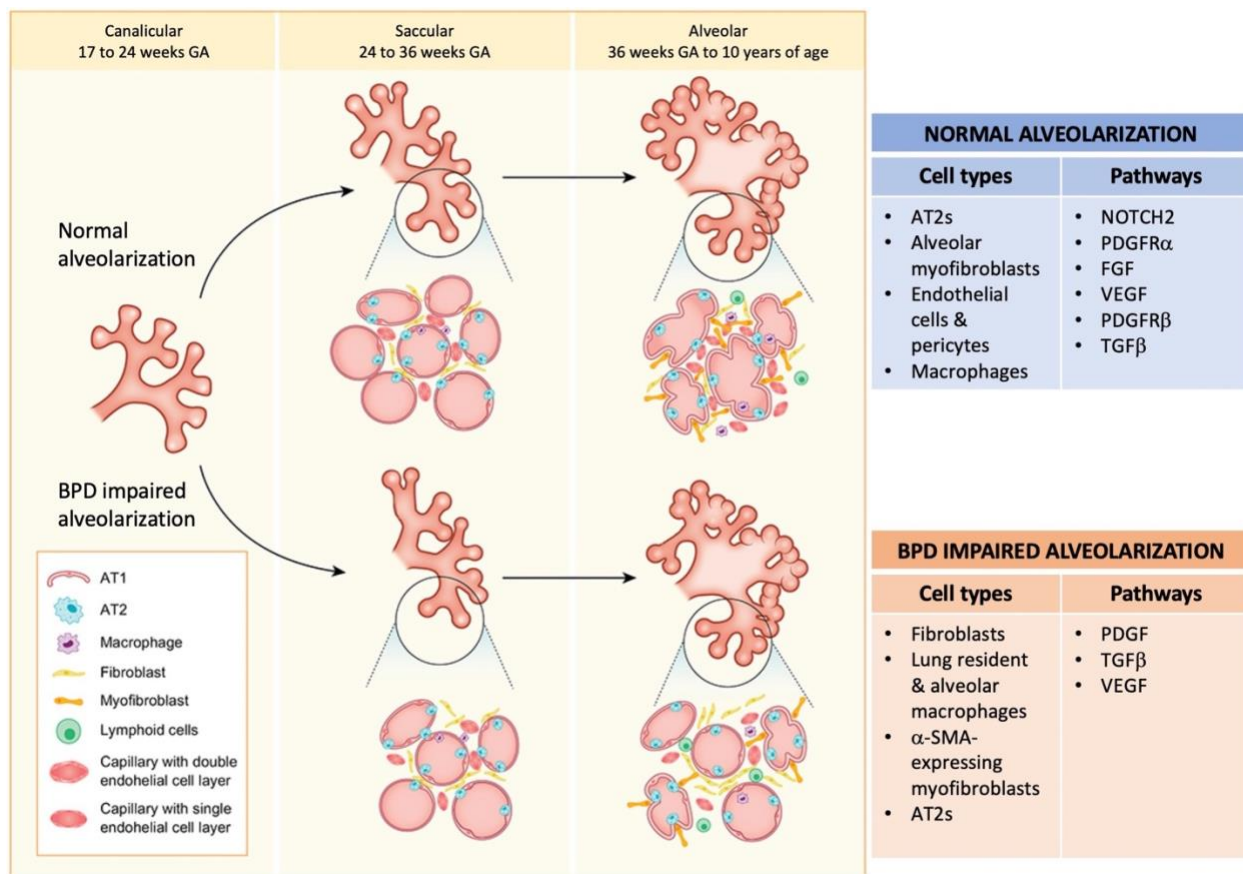


Figure 1.5 Schematic illustration of normal and impaired lung development. Suggested pathways regulating lung development and key cell types involved in normal and BPD alveolarization. Modified from (32).

Alveolar lung tissue is formed by cells of four lineages: mesenchymal, endothelial, epithelial, and immune cells. While the majority of these cells are present and functional at birth, they continue to differentiate and proliferate postnatally to generate additional novel alveoli.

From the mesenchyme, fibroblasts are essential for the modification of the extracellular matrix (ECM) and the formation of secondary septa. Platelet-derived growth factor receptor alpha (PDGFR α) signaling plays a critical role in this process, as evidenced by the abnormally enlarged alveolar air spaces in platelet-derived growth factor-A (PDGF-A) deficient mice (33–37).

Secretion of PDGF-A, which is required for elastin production by alveolar myofibroblasts (38–40), is induced by neurogenic locus notch homolog protein 2 (NOTCH2) signaling from alveolar epithelial type 2 cells (AT2s) (34). Elastin is deposited at the tip of the septa and provides a scaffold for secondary septa formation. Fibroblast growth factor (FGF) signaling through fibroblast growth factor receptors 3 and 4 (FGFR3 and FGFR4) is also involved in this process, as it controls the orderly formation of the elastin ECM in the lung mesenchyme (41). Another critical molecule for elastogenesis of the developing lung is transforming growth factor-beta (TGF β). TGF β , in its inactive form, binds to latent TGF β binding proteins (LTBPs) in the ECM. In turn, LTBPs bind to other important ECM proteins, such as fibronectin and fibrillins, enabling elastogenesis (42).

The alveolarization process occurs simultaneously with microvascular maturation, where the immature septa, which contain a double endothelial cell layer, are replaced by the expanding secondary septa with a single layer of capillary endothelial cells. Vascular endothelial growth factor (VEGF) is crucial for normal vascular development during alveolarization (43). In order to form functional vasculature, endothelial cells of developing vessels require stabilization through interaction with pericytes. This interaction is partially mediated by the platelet-derived growth factor receptor beta (PDGFR β) signaling pathway. Subsequently, the pericytes differentiate into smooth muscle cells (44,45).

From the epithelium, type 1 alveolar epithelial cells (AT1s) promote the growth of the alveoli by expanding through a flattening and folding process. Proper epithelium expansion and AT1s spacing occur following TGF β -dependent mechanisms (46,47). Elongated AT1s eventually cover most of the alveolar surface area (95%) and allow gas diffusion. AT2s produce surfactant, which prevents alveolar collapse, and provides immune protection against inhaled pathogens and toxins. Moreover, AT2s have the ability to differentiate into AT1s.

In addition, as pathogenic microbes and air particles enter the lungs through the airways, increased numbers and types of immune cells emerge to help protect the lung from infection (48–50). The influx of macrophages reaches the lungs in three developmental waves (51). In the first wave during embryonic day (E) 10.5 in mice, a population of F4/80⁺ macrophages in the interstitial space arises from the yolk sac. In the second wave, at E12.5, Mac2⁺ macrophages originate from the fetal liver (52). Around birth time, these macrophages penetrate the alveolar spaces and become self-renewing alveolar macrophages. Finally, circulating monocytes are recruited into the interstitial spaces during the third wave, corresponding to the first three weeks of postnatal life, to generate F4/80⁺ definitive interstitial macrophages. However, the complete understanding of macrophages' contribution to normal lung development remains to be elucidated (51,53).

1.1.4 Impaired alveolarization

In BPD patients, the process of secondary crests and microvascular network formation leading to normal alveolarization is impaired. Exposure to pre- and postnatal inflammation in both patients and animal models appears to play an important role in the development of many of these pathophysiological changes (54–57). Elevated levels of proinflammatory cytokines were detected in both tracheal aspirates (TAs) (58,59) and serum (60–62) of BPD patients. Similarly, an increase in proinflammatory cytokines was observed in neonatal mice with hyperoxia-induced lung injury (63–65). Studies in experimental BPD using transgenic mice have highlighted the role of proinflammatory cytokines such as interleukin 1 beta (IL-1 β) (66,67), interferon-gamma (IFN γ) (68), and TGF β (69), as well as the nucleotide-binding domain, leucine-rich repeat family, pyrin domain-containing protein 3 inflammasome (56). Furthermore, the lung-resident macrophages seem to contribute to the disease process as the depletion of macrophage/monocyte lineages

resulted in protection from hyperoxia-induced lung injury (70). Besides the inflammatory pathways, the PDGFR α and VEGF signaling pathways, known to be critical for normal alveolarization, are also targets of neonatal lung injury and may have cross-regulatory effects on one another along with TGF β (71,72).

1.1.4.1 PDGF signaling in impaired alveolarization

Lungs of hyperoxia-exposed neonatal mice, as well as mesenchymal stem/stromal cells (MSCs) isolated from infants that developed BPD, showed decreased expression of PDGFR α and PDGFR β (73). In ventilated newborn mice and lambs, dysregulated PDGFR α signaling and PDGF-A expression play an important role in the development of BPD-like injury (74,75). Furthermore, in a three-dimensional study in the lungs of hyperoxia-exposed mice, the mislocalization of alpha-smooth muscle actin (α -SMA)-expressing myofibroblasts, which are required to deposit elastin during secondary septation (a PDGFR α -dependent mechanism), caused the disruption of the pulmonary elastin network (76), resulting in BPD-like lung pathology (77). Thus, defective PDGF signaling is a primary feature of BPD.

1.1.4.2 TGF β signaling in impaired alveolarization

Increased TGF β expression by innate immune cells (e.g., monocytes and macrophages) in the lung is associated with BPD in several species, including humans, lambs, and baboons (78–80). Consequently, this causes upregulation of lysyl oxidases and transglutaminases, which is essential for ECM formation, and dysregulated in BPD (81,82). Moreover, TGF β can regulate the expression of *PDGFR α* , as evidenced by the downregulation of *PDGFR α* expression following TGF β 1 treatment in neonatal mesenchymal cells derived from the lungs of BPD patients (73).

Inhibition of TGF β signaling can protect against hyperoxia-lung injury in neonatal rodent models of BPD, partly by a VEGF-dependent mechanism (83–85). In another report, activin A, a member of the TGF β superfamily, contributed to hyperoxia-induced lung injury in newborn mice (86). In contrast, TGF β 1 has also been shown to be necessary for the survival and repair of AT2s, as stimulation of hyperoxia-induced AT2s with TGF β 1 improved cell survival, migration, and secretion of VEGF and matrix protein such as fibronectin (87). Taken together, these results highlight the multifaceted role of TGF β signaling in alveolarization. However, the exact involvement of TGF β signaling in the progression of BPD remains to be better clarified.

1.1.4.3 VEGF signaling in impaired alveolarization

Endothelial cell signaling via VEGF receptors (VEGFRs) is crucial for the formation of the alveolar structure (43). Vascular endothelial growth factor receptor 2 (*Vegfr2*) expression was reduced in lung endothelial cells of hyperoxia-exposed neonatal rats (88). Furthermore, mechanical ventilation decreased *Vegfr2* expression (74,75) and VEGF signaling in the lung (65,66,80). Both the lack (89,90) and excess (91) of VEGF signaling resulted in impaired alveolarization and pulmonary angiogenesis, suggesting the importance of temporal regulation of this pathway. Regarding the upstream regulators of VEGF, hypoxia-inducible factor (HIF) family plays an important role as VEGF expression has been identified as a target of HIF (92). Finally, PDGF signaling seems to co-regulate VEGF signaling, as PDGF-A treatment restored VEGF signaling in neonatal mice (71).

1.1.5 Type 1 and type 2 immune response in the normal and hyperoxic developing lung

Recent research suggests that normal mouse lung development is partly driven by a type 2 immune response (49,93). The epithelial stretch triggered by the first breath is followed by interleukin 33 (IL-33) secretion from AT2s. In the presence of IL-33, lung resident basophils and recruited innate type 2 lymphoid cells produce type 2 cytokines, in particular interleukin 13 (IL-13). This results in the maturation of alveolar macrophages to mature M2 (anti-inflammatory/alternatively activated) phenotype characterized by the expression of C-C motif chemokine ligand 17, arginase 1 (*Arg1*), found in inflammatory zone 1, and chitinase-like protein 3 (48,53). These M2 macrophages are programmed to clear their local environment from debris and produce L-ornithine and polyamines to advance regeneration and wound healing (94). Conversely, under hyperoxic conditions, type 1 cytokines will induce the proinflammatory M1 phenotype in the normally M2 polarized macrophages (95,96). M1 macrophages produce lactate, reactive oxygen species (ROS), and nitric oxide (NO), which have cytotoxic effects (94). The exact link between the altered cytokine environment and impaired lung development in hyperoxia-induced BPD remains to be established.

1.1.6 Animal models of BPD

To mimic the pathological perturbations observed in the lung structure of infants with BPD, different injurious stimuli such as hyperoxia exposure, mechanical ventilation, and antenatal or postnatal administration of substances such as lipopolysaccharide (LPS) are used (97–100). The most common experimental models of BPD involve the exposure of term rodents to hyperoxia of varying concentrations (40% up to 100%) and lengths (3 days up to 4 weeks) (101). Bonikos *et al.* were the first to report that exposure of mice to hyperoxia results in chronic lung injury

characterized by alveolar simplification and fibrosis reminiscent of BPD (102). Subsequently, many groups have conducted more in-depth studies of physiological and structural effects of hyperoxia on newborn rodents (101,103). Furthermore, to broaden our understanding of BPD pathobiology, the development of novel models recapitulating pathological characteristics of currently prevalent forms of BPD has been suggested (104,105). In this respect, the inflammatory injury in hyperoxia models is moderate (106,107), and the use of a second hit to induce systemic inflammation has been suggested as a strategy to better mimic clinical BPD (105,107,108).

1.1.7 Limited therapeutic options for the management of BPD

Treatments for BPD remain limited; currently, there is no cure for the disease. Among the therapeutics available that are aimed at improving outcomes of preterm infants, great enthusiasm was generated when exogenous surfactant triggered immediate benefits on gas exchange. However, it only had a modest impact on the outcome of BPD (109). Similarly, the administration of prenatal and postnatal corticosteroids seemed initially promising, given their impressive anti-inflammatory action in animal studies. Indeed, administering corticosteroids has positive outcomes in preventing and treating BPD in preterm infants. However, their use needs to be limited due to severe adverse effects, such as poor neurodevelopmental outcomes and increased risk for cerebral palsy (110,111). Other potential preventive and treatment strategies have been proposed to limit the extent of the injury. These include the administration of vitamin A, caffeine, azithromycin, diuretics, and bronchodilators. Most of these interventions have shown no or minimal reduction in the incidence of BPD and provide minimal help in decreasing the burden of morbidities in preterm infants (112–116). Thus, novel intervention strategies would be of exceptional value.

1.2 Mesenchymal stromal/stem cells

Despite the significant progress in our understanding of the pathological mechanisms of BPD, therapeutics to treat the neonatal lung disease remain limited. Most likely, the limited therapeutic efficacy of established drugs may be attributed to the variety of mechanisms driving BPD development, which may be difficult to target simultaneously. Primarily because of their vast range of potential mechanisms of action, MSCs represent one avenue for the treatment of BPD.

1.2.1 Stem cells

Stem cells are undifferentiated cells that can replicate themselves indefinitely and differentiate into many types of tissues. They can be classified into three main groups on the basis of sources; 1) embryonic stem cells (ESCs), 2) induced pluripotent stem cells (iPSCs), and 3) somatic (or adult) stem cells. ESCs are derived from early-stage embryos. ESCs from the zygote and morula are totipotent cells (they can give rise to all embryonic and extraembryonic tissues), whereas ESCs from the blastula are pluripotent cells (they are capable of forming any type of cells in the developing embryo). iPSCs are adult cells that have been reprogrammed by activating specific genes, the Yamanaka factors, Oct4, Sox2, Klf4, and c-Myc, to become pluripotent (117). Somatic stem cells are either multipotent (able to differentiate into several cell types, e.g., MSCs and hematopoietic stem cells [HSCs]), or unipotent (able to differentiate into only one cell type, e.g., AT2s differentiating into AT1s), and are found in the adult body. Out of these three categories, somatic stem cells, and more particularly MSCs, have attracted the most attention as a prospective source for cell therapy. This is due to their easy access and easy growth *in vitro*, the smaller number of ethical issues compared to ESCs, and reduced tumorigenicity potential compared to both ESCs and iPSCs (118–120).

1.2.2 MSC identification and characterization

MSC, an adherent fibroblast-like population, was first discovered within the non-hematopoietic fraction of the bone marrow (BM) by Friedenstein and coworkers in a series of seminal studies in the 1960s and 1970s (121–123) and reviewed by Friedenstein in 1990 (124). These cells provide the supporting microenvironmental niche for HSCs. In addition, MSCs were shown to have proliferative and self-renewal abilities and were able to differentiate into osteoblasts when re-transplanted *in vivo* (121). Subsequently, the cells were detected in multiple other tissues, such as the adipose tissue (AT) and perinatal tissues, including the placenta (PL), the amniotic membrane and amniotic fluid (AF), as well as the umbilical cord tissue (UC) and umbilical cord blood (UCB) (125). Since coined by Caplan in 1991, the term “mesenchymal stem cells” has been interchangeably used with “mesenchymal stromal cells,” with both terms sharing the same acronym, “MSCs” (126). In recognition that MSCs can be isolated from multiple tissues using a multitude of procedures, the International Society for Cell and Gene Therapy (ISCT) developed a set of minimal criteria to identify MSCs based on phenotypic characteristics rather than tissue of origin. The cells must: 1) adhere to plastic, 2) express specific cell surface markers identified by flow cytometry (CD73⁺, CD90⁺, CD105⁺, CD34⁻, CD45⁻, CD14/CD11b⁻, CD79 α /CD19⁻, HLA-DR⁻), and 3) differentiate into osteoblasts, adipocytes, and chondrocytes (127). These criteria helped increase the reproducibility and comparability of researchers’ cell-based experiments, ultimately leading to 4,000 to 5,000 new publications related to MSCs on *PubMed* each year.

Aside from their vital role in supporting the maintenance and proliferation of HSCs, MSCs play an important part in tissue regeneration and demonstrate a strong immunomodulatory effect. Clinical interest in regard to MSCs has dramatically increased over the last few years, as MSCs are considered an ideal candidate cell type for regenerative medicine, as well as for the treatment

of immune and inflammatory diseases. MSC-based therapy has already been approved in many countries for the treatment of autoimmune diseases like graft-versus-host disease (Canada, Japan, New Zealand, and South Korea) (128,129). Despite the refinement of the definition of MSCs by the aforementioned ISCT minimal criteria, MSCs are heterogeneous and may comprise different cell subpopulations with a diverse range of therapeutic potencies and safety profiles. It should be noted, however, that the purpose of the initial minimal criteria was to eliminate/select against the possible contaminant cells (CD34⁻: eliminates hematopoietic and endothelial cells, CD45⁻: eliminates leukocytes, CD14/CD11b⁻: eliminates monocytes and macrophages, CD79 α /CD19⁻: eliminates B cells), and not to detect therapeutic cells per se. As a result, the current broad definition of MSCs has led to confusion not only in the scientific community but also in the general public. This, in turn, has led to an alarming situation in which certain companies have succeeded in marketing unproven stem cell interventions to misinformed consumers (130). Therefore, there is an urgent need to better define MSC subpopulations and elucidate what fraction of the newly defined subpopulations can demonstrate effectiveness during cellular therapy. Thus, in-depth analysis of MSCs' quality attributes and novel quality control to better define the therapeutic values of MSCs have to be further evaluated.

1.2.3 Sources of MSCs – UC-MSCs

MSCs can be isolated from many tissues and organs (e.g., BM, AT, UC, PL, AF...), with BM-MSCs being the most extensively studied type of MSCs. This is primarily attributed to BM transplants' success in treating different cancers, such as leukemia and myeloma (131–133). UC-MSCs, on the other hand, remain less frequently used but possess multiple advantages over the more widely used MSC types. UC-MSCs are readily available (the collection is painless and

non-invasive for the donor), have significant proliferative and differential potential, high immunomodulatory activity, and possibly superior repair power (134–139). UC-MSCs can be isolated from donors at different GAs at birth, including both term and preterm babies. Whether or not intrinsic characteristics of UC-MSCs are altered as a consequence of antenatal complications remains to be elucidated. Importantly, given that BPD is a disease of prematurity, characterization of UC-MSCs isolated from human preterm birth is of major interest for the eventual development of autologous cell based-therapy for BPD.

1.2.4 UC-MSCs methods of isolation

UC-MSCs can be isolated from the entire UC or subparts (e.g., Wharton's Jelly, the soft connective tissue within the UC) by two main methods: the explant outgrowth method and the enzymatic digestion method. In the explant outgrowth method, the UC is sliced into fragments of a few centimeters and cultured in a plastic Petri dish for seven days (until cells migrate out of the tissue) (140–142). The enzymatic digestion method can be done using different protocols, which vary in the concentrations and types of enzymes, as well as in the digestion times (143–145). In general, an enzyme mix solution is added to the UC fragments, which are then mechanically stirred using automatized agitators. The solution is then filtered, and MSCs are extracted through density centrifugation (140). The cells are then cultured in a plastic Petri dish. With both methods, once the cells reach around 80-90% confluency, they are detached using a trypsin solution and passaged to be expanded for several passage numbers (PNs) until the desired PN/cell quantity. The enzymatic digestion method is faster in obtaining UC-MSCs (shorter culture cycle) compared to the explant outgrowth method. Depending on the protocol used, enzymatic digestion might result in higher culture efficiency than explant outgrowth methods (143). In contrast to the enzymatic

digestion method, the explant method is a cost-effective cultivation technique with lower damage to the vitality of the cells (143).

1.2.5 MSC mechanisms of action

The utility of MSCs in regenerative medicine was initially attributed to their capacity to differentiate into specific mature cell types. However, recent evidence suggests that MSCs exert their regenerative effects primarily through paracrine modes of action, where they secrete different molecular factors, as well as extracellular vesicles (EVs), which can generate immunomodulatory, anti-inflammatory, anti-apoptotic, antioxidant, anti-fibrotic and pro-angiogenic influences. In addition, MSCs have been shown to possess homing properties, as systemic infusion of cells resulted in efficient migration and homing to the injury site (146).

1.2.5.1 Immunomodulation and anti-inflammatory effects

The intensive investigation of the immunomodulatory properties of MSCs was prompted by the discovery that MSCs can suppress allogeneic T cell response and inhibit the formation of cytotoxic T cells and natural killer cells (147). Additionally, MSCs have been shown to reduce inflammation by modulating macrophages and neutrophils. Further studies demonstrated that the mechanisms by which MSCs modulate the immune system vary among individuals (148). Also, depending on the signal type and strength, MSCs secrete specific cytokines that can either promote or suppress the immune responses with the ultimate goal of maintaining the immune balance (148). Prostaglandin E2 has been suggested as one of the most important molecules implicated in the immunosuppressive activity of MSCs (149,150). Other essential factors that play a role in the immunomodulatory function of MSCs are indoleamine 2,3-dioxygenase (IDO), NO, human

leukocyte antigen G, insulin-like growth factor-binding proteins, and tolerogenic antigen-presenting cells (151). In addition, MSCs can inhibit the production of inflammatory cytokines and chemokines such as IL-1 β , IL-6, tumor necrosis factor-alpha (TNF α), and IFN γ and increase the production of anti-inflammatory molecules such as interleukin 4 (IL-4) and interleukin 10 (IL-10).

1.2.5.2 Pro-regenerative effects

In addition to their anti-inflammatory effect, MSCs have been shown to possess pro-regenerative properties through cell-to-cell interactions and secretion of growth factors (152). VEGF, FGF, hepatocyte growth factor, placental growth factor, and TGF β , as well as cytokines and chemokines such as monocyte chemoattractant protein 1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and other proteins including stromal cell-derived factor-1 (SDF-1), B-cell lymphoma 2 and angiopoietin 1 (ANG-1) play a significant role in MSC-induced tissue repair and regeneration (152).

1.2.5.3 MSC-derived extracellular vesicles

MSCs production of EVs, including exosomes (40-100 nm in diameter) and microvesicles (50-1000 nm in diameter), plays an important role in their repair/regenerative activity. EVs are membrane-derived nanoparticles containing cytoplasmic and membrane constituents, including mitochondria (153), proteins, mRNAs, and microRNAs (154). Several studies using MSC-derived EVs (MSC-EVs), as well as metabolites-containing MSC conditioned media (CM), have shown that they can prevent hyperoxia-induced arrest in alveolarization and repair the lung by stimulation of bronchoalveolar stem cells, which will be discussed in more detail below (96,155–159).

1.2.6 MSCs as a treatment for BPD

MSCs are a promising treatment for BPD for several reasons. MSCs have multiple beneficial immunomodulatory effects and may “reprogram” the immune system to reduce host tissue damage, while preserving the immune system in a state in which it is able to effectively respond to potential severe infection (e.g., sepsis). Moreover, MSCs have the capacity to inhibit inflammation, mediated in part by secretion of anti-inflammatory cytokines and modulation of macrophage polarization, and are able to restore endothelial cell growth. As such, administration of MSCs could not only enhance lung development and repair but might also halt the progression of secondary insults targeting other organs, including the brain and eyesight. Furthermore, MSCs are immune-privileged as they express low levels of human leukocyte antigen (HLA)/major histocompatibility complex (MHC) class I and lack the expression of HLA/MHC class II on their surface. Thus, MSCs do not induce a classical cytotoxic T cell (rejection) response and could be used as allogeneic cell treatment without the necessity for immunosuppression. However, several authors have expressed doubts about this concept and suggested that MSCs may not be completely immune-privileged, as some researchers have shown that rejection occurs after transfusion of MSCs in MHC-mismatched animals (160–162). Finally, MSCs are the most widely studied cell type for regenerative stem cell-based therapy, with several studies demonstrating safety and feasibility of MSCs administration in clinical trials. Importantly, administration of MSCs is unlikely to induce tumor formation (163), as the cells are cleared from the tissue a few days after delivery (155).

1.2.6.1 Dysregulation of lung resident MSCs in BPD

One key lung cell population affected in BPD patients is the lung resident (LR)-MSC. Dysfunction of LR-MSCs is observed in BPD and is associated with the inflammatory response (164). This was confirmed in BPD models and studies using TAs and lungs from preterm infants (73,165,166). In addition, MSCs obtained from TAs of preterm infants were characterized by a proinflammatory phenotype with increased levels of cytokines and chemokines, including chemokine (C-X-C motif) ligand 1, interleukin 6 (IL-6), and interleukin 8 (IL-8) (167). Preterm infants who later developed severe BPD displayed the most pronounced changes in protein expression. Thus, reprogramming LR-MSCs has been hypothesized as a therapeutic option for BPD, and exogenous MSCs may help this process (168).

1.2.6.2 Preclinical evidence for MSC-based therapy in experimental BPD

Early pre-clinical studies in rodent models of hyperoxia-induced BPD provided a proof of concept for MSC therapeutic potential for neonatal lung injury, as administration of BM-MSCs, BM-MSCs-CM and UCB-MSCs prevented the arrest of alveolar growth and subsequent lung injury (157,169,170). Later on, the lung-protective effect of MSCs and MSCs-CM were also confirmed using UC-MSCs (171,172), AT-MSCs (173), PL-MSCs (174), AF-MSCs (175), and BM-MSCs-derived exosomes (158). Various routes of administration of MSC products have been demonstrated so far, including intravenous (*i.v.*) (156,170,176–182), intratracheal (*i.t.*) (155,157,169,173–175,183–190), intraperitoneal (*i.p.*) (169,172,191–195) and intranasal (*i.n.*) route (171). Finally, in addition to improving alveolarization, MSC treatment was shown to reverse BPD-associated PH (155–158,183), attenuate lung inflammation (173,174,177,178,181,183,187), as well as pulmonary fibrosis (191,195) and lung cell apoptosis (182).

1.2.6.3 MSC mechanisms of action in experimental BPD

Preclinical studies to assess the therapeutic effect of MSCs in experimental BPD made it clear that MSC therapeutic efficacy is attributed to a concerted effort targeting multiple biological processes, including immunomodulation, inflammation, angiogenesis, fibrosis, and cell survival (Fig. 1.6). In addition, these preclinical experiments provided strong evidence for the paracrine action as an important player in these processes, which will be discussed below.

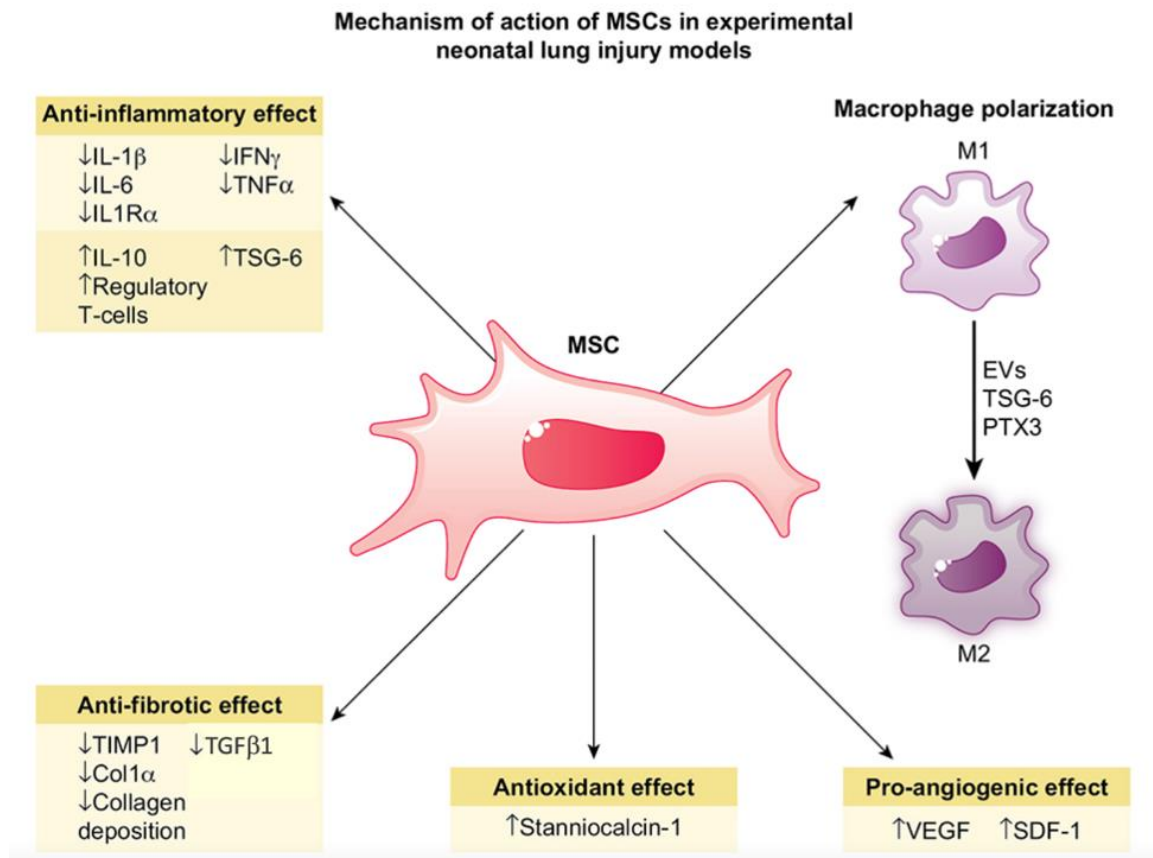


Figure 1.6 Mechanism of action of MSCs in experimental BPD.

After transplantation to the injured lung, MSCs exert their lung repair and regenerative functions via multiple mechanisms of action, as shown in different preclinical neonatal lung disease models in rodents. MSCs have anti-inflammatory effects: they attenuate the release of proinflammatory cytokines and increase the release of anti-inflammatory cytokines. Moreover, they induce M1-to-M2 macrophage polarization. MSCs also exert an anti-fibrotic, antioxidant, and pro-angiogenic effect. IL1R α = IL-1 α . Modified from (32).

1.2.6.3.1 MSC anti-inflammatory effect

BPD is characterized, in part, by an unbalanced immune response. As a result, multiple inflammatory cytokines and chemokines are upregulated in the lung tissue and the bronchoalveolar lavage fluid of hyperoxia-exposed neonatal rodents, as well as in the TAs of preterm infants (58,59,63–65). MSCs were shown to attenuate lung inflammation in experimental BPD by 1) reducing the hyperoxia-induced increase in cytokines, including $\text{TNF}\alpha$, $\text{TGF}\beta 1$, $\text{IL-1}\alpha$, $\text{IL-1}\beta$, IL-6 , IL-8 , 2) downregulating the receptor for advanced glycation end-products/nuclear factor- κB (173,174,177,178,181,183,187), and 3) by increasing anti-inflammatory cytokines such as IL-10 (177).

1.2.6.3.2 MSC paracrine effect on macrophage response

Macrophages are present in all tissues and play a critical role in tissue homeostasis. Macrophage dysfunction is of great importance in the pathogenesis of many diseases, including BPD, and thus, is an attractive target for drug development (70,94). Macrophages respond to microenvironmental signals by shifting their phenotype according to the inflammatory phase and acquiring different functional stages. It has been postulated that in the early stages of injury, macrophages are polarized toward a proinflammatory M1 phenotype (classically activated) with an enhanced phagocytic activity that targets and destroys intracellular pathogens. Conversely, macrophages in the later stages of injury are polarized toward an anti-inflammatory/pro-repair/resolution M2 phenotype (alternatively activated), prompting the removal of dead/injured host cells and tissue repair (94). Depending on the environment, macrophages express specific M1 (major histocompatibility complex class II [MHCII], IL-6 , and inducible nitric oxide synthase) or M2 (IL-4 , IL-10 , IL-13 , and ARG1) markers (94). Under hyperoxic conditions mimicking the

pathology of BPD in rodents, both alveolar and interstitial resident macrophages showed a phenotypic shift from anti-inflammatory M2 to a proinflammatory M1 state (196,197).

The ability of MSCs to modulate the phenotype and function of macrophages has been extensively studied. MSCs were shown to modify macrophage phenotype and increase the number of M2 macrophages in the injured mice lungs, which in turn reduced collagen deposition and improved lung structure (95). Moreover, secreted MSC-EVs improved lung structure and function in mice with BPD by promoting an M1 to M2 polarization (96), with TNF α stimulated gene 6 and pentraxin 3 (PTX3, also known as TNF-inducible gene 14) proposed as key mediators (159,198). Thus, the alteration of macrophage subtypes plays an important role in injury resolution, and the ability of MSCs to modulate macrophage polarization states seems to have important implications for their therapeutic function in experimental BPD.

It should be noted that most recent evidence suggests that in response to environmental variations, macrophages can rapidly change their phenotype to a wider spectrum of subtypes, with M1 and M2 as two extremes (199). Dozens of subtypes have been identified, depending on macrophage origin, tissue of residence, and whether previous exposure to the same insult has occurred or not (200). Therefore, many factors may influence the effect of MSCs on macrophage polarization, and the shift from M1 to M2 induced by MSCs may be an oversimplification of their mechanism of action. Nevertheless, modulation of the immune response by MSCs via macrophage polarization appears to play an important role in their therapeutic effect.

1.2.6.3.3 MSC paracrine effect on angiogenesis

Proliferation of microvascular endothelial cells characterized by rapid angiogenesis and alveolarization is imperative for normal lung development. VEGF and ANG-1 play essential roles

in postnatal lung angiogenesis, and MSCs may partially alleviate lung damage by producing these angiogenic factors. Accordingly, it was observed that in rodent lung tissue, treatment with MSCs significantly restored the levels of VEGF and *Ang-1* that had been decreased by exposure to hyperoxia (173,178,183). Moreover, in a similar hyperoxia rodent model, the protective effect of *in vivo* treatment was lost when VEGF in UCB-MSCs was knocked down (185). Similarly, the therapeutic effects of BM-MSCs on alveolarization and PH in a hyperoxic rodent BPD model were significantly reduced when a different pro-angiogenic/anti-inflammatory cytokine, SDF-1, was knocked down (201). Thus, MSCs alleviate lung injury in rodent hyperoxia-induced BPD, in part, by the modulation of angiogenic factors VEGF, ANG-1, and SDF-1.

1.2.6.3.4 MSC paracrine effect on fibrosis

Widespread interstitial fibrosis is recognized as a feature of BPD. Bleomycin exposure is a commonly used model of lung fibrosis in rodents (202). In the bleomycin mouse model, MSC treatment has been shown to be an effective strategy for preventing lung injury and fibrotic remodeling (203). Oxygen toxicity also triggers the progression of lung fibrosis. In several studies, MSCs attenuated hyperoxia-induced parenchymal fibrosis and decreased collagen density (191,195,204). Interestingly, MSCs pretreated with fibroblast growth factor 7 (FGF-7, also known as keratinocyte growth factor) exhibited enhanced preventive effects against lung fibrosis (195). Numerous studies have reported the fibrotic and inflammatory effects of TGF β 1 expression in lung fibrosis (205,206), and over-expression of TGF β 1 in the lungs of newborn rats resulted in BPD-like histopathology (207). In hyperoxia-exposed mice, it was shown that MSCs reduce pulmonary expression of TGF β 1, as well as collagen 1 alpha (*Col1 α*) and ECM-remodeling enzyme tissue inhibitor of metalloproteinases 1 (*Timp1*) (177,191). However, the exact anti-

fibrotic role that MSCs play via modulation of those molecules remains to be elucidated.

1.2.6.3.5 MSC paracrine effect on oxidative stress

Preterm infants, especially BPD patients, are particularly susceptible to oxidative stress due to increased production of free oxygen radicals secondary to high oxygen levels and inflammation exposure. These patients also have reduced antioxidant defenses towards ROS, further adding to their susceptibility to damage induced by oxidative stress (208). Co-culture with MSCs or their CM for 24 hours was shown to protect alveolar epithelial cells and pulmonary microvascular endothelial cells from oxidative stress *in vitro* (209,210), an effect potentially mediated by MSC-secreted antioxidant stanniocalcin 1 (194,211).

1.2.6.3.6 MSC paracrine effect on apoptosis

Epithelial cell apoptosis is a critical factor in the pathological arrest of alveolar development, and methods to decrease cell apoptosis might prove beneficial for lung growth. Interestingly, MSCs and MSCs-CM were shown to reduce apoptosis of alveolar epithelial cells following an H₂O₂-induced cell oxidative injury (209). Similarly, BM-MSCs-CM prevented hyperoxia induced-apoptosis of AT2s (157). The renin-angiotensin system (RAS) is believed to function as an apoptosis regulator in alveolar epithelial cells (212), and hyperoxia was shown to activate RAS in the lungs of newborn rats (213). Administration of PL-MSCs to hyperoxia-exposed rats improved alveolarization and decreased the expression of angiotensin II, angiotensin II type 1 receptor, and angiotensin-converting enzyme, resulting in reduced RAS activity (174). Finally, MSC-mediated increase in aquaporin 5 and surfactant protein C expression in hyperoxia-exposed neonatal rats was shown to be likely due to the suppression of alveolar cell

apoptosis (182). These results indicate that MSCs might prevent lung epithelial cell apoptosis via inhibition of RAS, ultimately improving lung growth in experimental BPD.

1.2.6.3.7 MSC paracrine effect on endogenous lung stem cells

Recent evidence highlights the dysfunction of LR-stem cells in experimental BPD (168). Bronchioalveolar stem cells (BASCs) are epithelial stem cells residing in the bronchioalveolar duct junction of terminal bronchioles with the ability to self-renew and differentiate *in vitro* and proliferate in response to bronchiolar and alveolar lung damage *in vivo* (214). Moreover, BASCs contribute to alveolar repair following bleomycin-induced injury in mice (215). In a mouse hyperoxia model of BPD, systemic treatment with BM-MSCs or their CM increased the number of BASCs (159), indicating that exogenous MSCs may exert their therapeutic potential by stimulating LR-stem cells (176).

1.2.6.4 Clinical trials using MSCs and other cell therapy for treatment of BPD

The promising results obtained from pre-clinical studies in animal models of BPD raised high hopes for the therapeutic potential of MSCs to effectively alleviate the consequences of lung injury in preterm infants. Several clinical trials have commenced testing the safety, feasibility, and efficacy of MSCs for treating premature infants at high risk of developing BPD. So far, three research groups have published results demonstrating the safety of MSCs in preterm infants. In the first study, allogeneic human UCB-MSCs were administered (*i.t.*) to preterm infants at high risk of developing BPD at a dosage of 1×10^7 or 2×10^7 cells/kg of body weight (BW) (216). No acute toxicities or side effects were reported. In addition, TA analysis revealed a reduction in proinflammatory mediators IL-6, IL-8, TNF α , and matrix metalloproteinase 9 (MMP9) seven days

following MSCs' administration (216). In a follow-up study at two years of age, no adverse respiratory, growth, or neurodevelopmental effects were observed (217). In the subsequent phase two double-blind, randomized, placebo-controlled trial, MSCs (1×10^7 cells/kg BW) were administered (*i.t.*) to a total of 33 preterm infants (23-28 weeks GA) with mechanical ventilator support and respiratory deterioration (218). Although the levels of inflammatory mediators (IL-1 β , IL-6, IL-8, TNF α , and MMP9) in the TA were significantly decreased, the primary outcome of death or severe/moderate BPD was not significantly improved with MSC treatment. However, the secondary outcome (severity of BPD) was significantly improved in the lowest GA group (23 to 24 weeks GA). It is important to note that the authors themselves considered the study to be underpowered. Results from the second phase one clinical trial showed that treatment of 12 preterm infants with *i.t.* allogeneic human UCB-MSCs at a dosage of 1×10^7 or 2×10^7 cells/kg BW was safe and feasible (219). In the most recent phase one trial, the administration of two *i.v.* doses of allogeneic UC-MSC of 1×10^7 cells/kg BW (per dose) were also found to be safe as there were no adverse effects in four patients with established BPD (220).

Another cell type investigated for safety in the treatment of BPD is the amnion epithelial cell (AmEC). AmECs are isolated from the amniotic membrane and share similar pleiotropic effects with MSCs, including immunomodulatory properties (221). AmECs were shown to have protective effects in rodent and ovine models of neonatal lung injury (222,223). In a phase one clinical trial, severely affected infants with established BPD were subjected to *i.v.* infusion of 1×10^6 AmECs/kg BW (224). The treatment was well tolerated, and no adverse effects were observed.

Four more trials aiming to test the therapeutic potential of MSCs on lung injury in preterm infants are listed on clinicaltrials.gov. One study completed the recruitment of patients (identifier:

NCT02443961), while the remaining three are either actively recruiting (identifier: NCT03774537 and NCT03378063) or will soon begin recruiting patients (identifier: NCT04255147).

1.2.6.5 Drawbacks and necessary improvements for MSC therapy for BPD

Many challenges remain to be overcome before MSCs can be successfully translated from the bench to the bedside. One major drawback that complicates the selection of MSCs is their high heterogeneity. MSC phenotype and level of stemness referred to as the molecular processes underlying the stem cell properties of self-renewal and differentiation ability, differ depending on the tissue source and the donor they originate from, leading to a wide range of therapeutic strengths (46). For example, as previously mentioned, UC-MSCs have higher proliferative potential and stronger paracrine potency compared to BM- and AT-MSCs (225). Furthermore, MSCs expressing high procoagulant TF/CD142 can trigger instant blood clotting after *i.v.* infusion, ultimately increasing the risk of thromboembolic events (226). Thus, potency assays must be established to select the most effective cell population. The large-scale manufacture of the cells required for therapy purposes poses another challenge. In the ideal scenario, MSCs would be prepared as an off-the-shelf product. However, the extensive proliferation and cryopreservation processes required to manufacture the off-the-shelf product might negatively impact the cells' therapeutic potential. Large-scale proliferation of MSCs might result in less effective cells due to the high percentage of senescent cells, while the cryopreservation of MSCs might impair their immunomodulatory properties (227). In addition, allotransplantation risks (e.g., potential immune rejection) also need to be considered, as the available clinical data remain inconclusive regarding whether the use of autologous or allogeneic MSCs is preferable. Although many pre-clinical trials using MSCs in animal models of BPD are successful, no optimal treatment regimen (e.g., route of

administration, dose concentration, dosing frequency, and timing) for neonatal BPD has yet been validated. Finally, standardized methods to isolate and expand MSCs are lacking. To address this problem, the possibility of using a closed, fully automated system with integrated software control for manufacturing MSCs is of great interest (228). This approach would standardize the production process, minimize the risk of contamination, decrease the likelihood of human error, and generate a consistently reproducible cell product.

1.3 Single-cell RNA sequencing, a valuable tool to better understand the heterogeneity of MSCs

Novel technologies, such as single-cell RNA sequencing (scRNA-seq), have emerged as powerful tools to explore the cellular heterogeneity of complex systems and cell populations. Indeed, genome-wide transcriptome data from single cells can be obtained by scRNA-seq using high throughput sequencing. The main advantage compared to traditional RNA-seq is the ability to measure the gene expression at the single-cell level rather than averaging the expression across multiple cells within the sample. Thus, single-cell analysis identifies subpopulations with a similar gene expression profile within a larger heterogeneous population. As such, it is particularly suitable for studying biological questions in which cell-specific changes in transcriptome are important, such as identifying MSCs providing a therapeutic effect.

1.4 Rationale and aims

BPD is the most severe and common complication of extreme prematurity, and no effective treatment exists for the disease. MSCs have been identified as potent repair cells with pleiotropic effects. Pre-clinical studies in animal models provided proof of concept for their therapeutic potential in neonatal lung diseases, including BPD.

However, MSCs are a heterogeneous population, and variations in phenotypes and functions have limited consensus, thus hampering the efficiency and reproducibility in basic research and clinical application. Bulk RNA-seq is limited in its ability to reveal the differences between individual cells within heterogeneous populations with high accuracy. Furthermore, flow cytometry using only a few cell population markers cannot fully explore subcellular heterogeneity within MSCs. Many advantages exist to using UC-MSCs rather than other sources of MSCs. Considering that BPD is a disease of prematurity, UC-MSCs could provide the unique opportunity to have an autologous treatment without the risk of immunological rejection. Studies characterizing UC-MSCs isolated from preterm delivery with and without other complications often associated with preterm birth, such as chorioamnionitis and preeclampsia, as potential treatments for BPD remain absent.

Moreover, preclinical testing of novel therapies in animal models is crucial in order to reliably reproduce the disease phenotype observed in preterm infants that develop BPD. Since BPD is a multifactorial disease, different models are needed to reflect the diverse injury profiles. Exposure of newborn rodents to hyperoxia is extensively used as it results in alveolar simplification similar to that seen in BPD. However, hyperoxia only moderately recapitulates the inflammatory aspect of BPD. The use of a second hit to induce systemic inflammation has been

suggested to better mimic BPD with perinatal inflammation. However, a thorough characterization of such models demonstrating the evolution of the immune response is lacking.

In this context, the main aims of this project were:

- 1) To explore cellular heterogeneity within the UC-MSC populations derived from term donors and from preterm donors, with or without other pregnancy-related complications using scRNA-seq.
- 2) To assess the therapeutic potential of selected UC-MSC candidates in hyperoxia-induced lung injury in neonatal rats.
- 3) To develop and characterize the innate immune response and injury in a novel 2-hit (2HIT) mouse BPD model with postnatal LPS and hyperoxia exposure.
- 4) To assess the therapeutic potential of selected UC-MSC candidates in the novel 2HIT mouse BPD model and determine the optimal route, timing, and dose of injection.

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CHAPTER 2: Single-cell RNA sequencing reveals reparative features of human umbilical cord mesenchymal stromal cells

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2.1 Abstract

Rationale: The chronic lung disease bronchopulmonary dysplasia (BPD) is the most severe complication of extreme prematurity. BPD results in impaired lung alveolar and vascular development and long-term respiratory morbidity, for which there is no treatment. Umbilical cord-derived mesenchymal stromal cells (UC-MSCs) improve lung structure and function in experimental BPD. Results of clinical trials with MSCs do not yet match the promising preclinical studies. Transcriptional MSC heterogeneity may constrain their clinical application. **Objectives:** To determine and correlate single-cell MSC transcriptomes with their therapeutic potential in experimental BPD. **Methods:** UC-MSCs from five term donors and human neonatal dermal fibroblasts (HNDFs) transcriptomes were investigated by single-cell RNA sequencing analysis (scRNA-seq). The lung-protective effect of MSCs with a distinct transcriptome and control HNDFs was tested *in vivo* in hyperoxia-induced neonatal lung injury in rats. **Measurements and main results:** UC-MSCs showed limited transcriptomic heterogeneity, but were different from HNDFs. Gene ontology enrichment analysis revealed distinct - progenitor-like and fibroblast-like - UC-MSC subpopulations. Only the treatment with progenitor-like UC-MSCs improved lung function and structure and attenuated pulmonary hypertension in hyperoxia-exposed rat pups. Moreover, scRNA-seq identified major histocompatibility complex class I as a molecular marker of non-therapeutic cells, associated with decreased lung retention. **Conclusions:** UC-MSCs with progenitor-like transcriptome, but not with fibroblast-like transcriptome, provide lung protection in experimental BPD. Additionally, high expression of major histocompatibility complex class I is associated with reduced therapeutic benefit. scRNA-seq may be useful to identify subsets of MSCs with superior repair capacity for clinical application.

2.2 Introduction

Bronchopulmonary dysplasia (BPD) is the most common and severe complication of prematurity (1). BPD develops in extreme preterm newborns exposed to mechanical ventilation, oxygen supplementation, and inflammation. Improvements in neonatal intensive care, such as antenatal steroids, exogenous surfactant therapy, and more judicious use of positive pressure ventilation and oxygen, have led to an increase in survival of more immature infants (2). BPD pathology is characterized by arrested lung development, including fewer and larger alveoli, decreased septation, thickening of alveolar septa, and impaired capillary network development (3). These changes may lead to life-long respiratory morbidity, including asthma, reduced exercise capacity, early onset emphysema, and pulmonary hypertension (PH) (4–6). Currently, there is no cure for BPD.

Mesenchymal stromal cells (MSCs) have immunomodulatory and tissue regenerative properties, and are being investigated as a therapeutic candidate for inflammatory respiratory diseases. Pre-clinical studies provided proof of concept for the therapeutic potential of MSCs in experimental BPD (7,8). Early-phase clinical trials have shown safety of MSCs in BPD and other lung inflammatory diseases, such as acute respiratory distress syndrome (9–14). Nevertheless, a number of translational issues remain to be addressed, including their cell-to-cell variability among donors and within cell populations, before their therapeutic potential can be fully exploited (15,16).

The International Society for Cell and Gene Therapy (ISCT) has proposed minimal criteria to define MSCs, which are widely accepted by the research community (17), but failed to functionally distinguish cells. In fact, MSCs in culture show intrinsic heterogeneous phenotypes and functions even when they are isolated from the same tissue of origin (17–19). The lack of knowledge about MSC subpopulations and the absence of more specific markers to identify

therapeutic MSCs hinder the development of more effective cell-based treatments.

Multiple advantages of using umbilical cord (UC) over other MSC sources in clinical applications exist: UC-MSCs are easily available, have excellent expansion potential, and may have superior repair capabilities (20–22). However, deeper characterization of UC-MSCs is required to unveil their true therapeutic capacities. Single-cell RNA sequencing (scRNA-seq) has become a powerful method to analyze gene expression and cellular heterogeneity in an unbiased way (23).

In this study, we used scRNA-seq to investigate the transcriptome of UC-MSCs from five donors born at term. We assessed the therapeutic efficacy of UC-MSCs from two donors with a distinct transcriptome in a hyperoxic rat BPD model. We hypothesized that UC-MSCs' therapeutic potential could be identified based on their transcriptome. We found that administration of UC-MSCs from one donor that displayed a transcriptome with enrichment in genes involved in DNA transcription and translation (termed “progenitor-like”), but not UC-MSCs from one donor with a “fibroblast-like” transcriptome, reduced lung damage and PH. Additionally, administration of a subpopulation of UC-MSCs that express high levels of major histocompatibility complex class I (MHC I) (HLA-A, HLA-B, and HLA-C) showed reduced therapeutic potential. Our results demonstrate for the first time that a distinct transcriptome can predict UC-MSCs' beneficial effects in experimental BPD.

2.3 Materials and methods

Please refer to section 2.7.1 “Supplementary materials and methods” for detailed methods in addition to the following information.

Ethics approval

Human Research Ethics Board (Ottawa, Canada) and “Ethikkommission der Technischen Universität Dresden” (Dresden, Germany) approved human samples collection. Animal Care Committee of the University of Ottawa (OHRI-1696) approved animal experiments.

UC-MSCs isolation

UC-MSCs were isolated from five term donors (Table 2.1) and expanded as previously described (24).

scRNA-seq library preparation and sequencing

Passage number (PN) three UC-MSCs and human neonatal dermal fibroblasts (HNDfFs) (cat#CC-2509, Lonza, Hayward, CA, USA) were processed for scRNA-seq on the 10x Genomics Chromium Single Cell 3' platform (StemCore Laboratories, OHRI). Gene expression libraries were prepared and sequenced using NextSeq500 (Illumina) high-output 75-cycle runs.

scRNA-seq data processing and pipeline

Following sequencing, fastq files were generated using the Cell Ranger mkfastq tool (10x Genomics). Gene expression libraries were processed using the Cell Ranger count function, aligning to the human transcriptome (GRCh38) with default parameters other than explicitly setting `--expect-cells 15000*`. MULTI-seq barcode fastq files were processed using the deMULTiplex tool developed for MULTI-seq (<https://github.com/hris-mcginnis-ucsf/MULTI-seq>), providing a sample annotation for each cell barcode. **scRNA-seq experiment was performed once in this thesis, including cells from preterm donors that were analyzed only in **Chapter 3**.*

Thus, “expect-cells 15000” is used, but less than 15,000 cells are expected in the term UC-MSCs and HNDFs study (**Chapter 2**), which explains the total of 2,834 cells analyzed in this Chapter.

Gene expression libraries were imported into R and processed with Seurat v.4.0.1 (25). Cells containing high proportions of mitochondrial reads (>20% of the transcripts) were removed before normalization using SCTransform (26). This was also used to regress out cell cycle effects and stress, to ensure that downstream clustering would not be influenced by cell cycle activity. The data were processed with principal component analysis (PCA) and clustered using the Louvain algorithm (resolution=0.25) on a nearest neighbor graph derived from first 30 principal components. UMAP embeddings were generated using the RunUMAP() function on the first 30 principal components. Differential gene expression analysis was performed using the Wilcoxon test implemented in the FindMarkers() function in Seurat.

Animal model

Hyperoxia-induced neonatal lung injury model was achieved in Sprague Dawley (SD) rats (Charles Rivers, Saint-Constant, QC, Canada) by maintaining a hyperoxic environment (85% O₂) from postnatal day (PD) zero to PD14 (8,27). At PND21, the animals were euthanized by intraperitoneal (*i.p.*) injection of pentobarbital sodium (Euthanyl, 0.2 mL, 65 mg/mL).

Administration of UC-MSCs

At PD4, hyperoxic pups received intratracheal (*i.t.*) vehicle (1× phosphate buffered saline [PBS], 25 µL), PN3 HNDFs or PN3 UC-MSCs (in 25 µL PBS) at a dose of 10×10⁶ cells/kg body weight (BW). The experimental groups were as follows: 1) normoxia control (Ctrl), 2) hyperoxia vehicle control (PBS), 3) hyperoxia non-progenitor cells (HNDFs), and 4) hyperoxia UC-MSCs.

Lung structure analysis

Lungs were prepared as described before (28) for morphometry analysis by mean linear intercept (MLI) assessment (29).

Statistics

Values are represented as the mean $\pm$ standard deviation (STD). Outliers were identified using Grubb's test and normal distribution using Shapiro-Wilk normality test. *P* values ($P \leq 0.05$ was considered significant) were calculated with *t* test (unpaired, two-tailed), one-way ANOVA with Tukey's multiple comparison post hoc test, or log-rank (Mantel-Cox) test. All group assignments were blinded before analysis.

2.4 Results

2.4.1 scRNA-seq reveals distinct UC-MSC subpopulations

We performed scRNA-seq analysis of cultured PN3 UC-MSCs isolated from five term donors (T1 to T5) and HNDFs as control cells (Table 2.1).

Table 2.1 – Characteristics of UC-MSC donors

Donor	Gestational age (weeks + days)	Birth weight (g)	Sex	Maternal age (years)	Delivery
T1	39 + 5	3470	Male	24	Cesarean section
T2	39 + 3	4490	Male	34	Cesarean section
T3	39 + 1	2635	Female	39	Cesarean section
T4	39 + 4	3455	Female	38	Cesarean section
T5	37 + 2	2385	Female	23	Cesarean section

After quality control, a total of 2,834 cells were analyzed (Suppl. Table 2.1). Based on mRNA expression profiles, we identified four cell populations (clusters 0 to 3) (Fig. 2.1A). Clusters 0 and 2 comprised cells from multiple UC-MSC donors, whereas cluster 1 was associated with donor T2 and cluster 3 with HNDFs only (Fig. 2.1B, C). By analyzing the expression of epithelial (*EPCAM*), endothelial (*PECAMI*), immune (*PTPRC*), mesenchymal (*COL1A1*), red blood cell (*HBA-A1*), and pericyte (*RGS5*) markers, we determined that only the mesenchymal marker *COL1A1* was expressed (Fig. 2.1D). Then, we analyzed the expression of MSC markers (17). The cells expressed *CD105*, *CD73*, and *CD90*, and almost or completely lacked the expression of *CD45*, *CD34*, *CD14*, *CD11B*, *CD79A*, *CD19*, and *HLA-DR* (Fig. 2.1E). Moreover, UC-MSCs from all donors and HNDFs differentiated into adipocytes, chondrocytes, and osteoblasts (Suppl. Fig. 2.1A-C).

Based on differentially expressed genes (DEGs), we identified transcriptomic profiles specific to each cluster (Suppl. Table 2.2, Fig. 2.1F). We then identified enriched gene ontology (GO) terms associated with each cluster. Among the most enriched GO terms in cluster 0 – multiple donors – were those associated with cell division, cell proliferation, cell cycle, gene, and protein expressions, as well as cell signaling functions, while cluster 2 – multiple donors – was characterized by terms related to protein folding among others (Fig. 2.1G, Suppl. Table 2.3). Interestingly, cluster 1 – donor T2 – shared a number of terms with cluster 3 – HNDFs – including extracellular matrix (ECM) organization, collagen catabolic process, collagen fibril organization, and other fibroblast-specific terms (Fig. 2.1G, Suppl. Table 2.3).

Together, this analysis revealed that the transcriptome of different UC-MSC donors is distinct from HNDFs, and exhibits little heterogeneity. UC-MSCs from donors T1, T3, T4, and T5 displayed a progenitor-like transcriptome, whereas UC-MSCs from donor T2 displayed a fibroblast-like transcriptome resembling the profile of HDNFs.

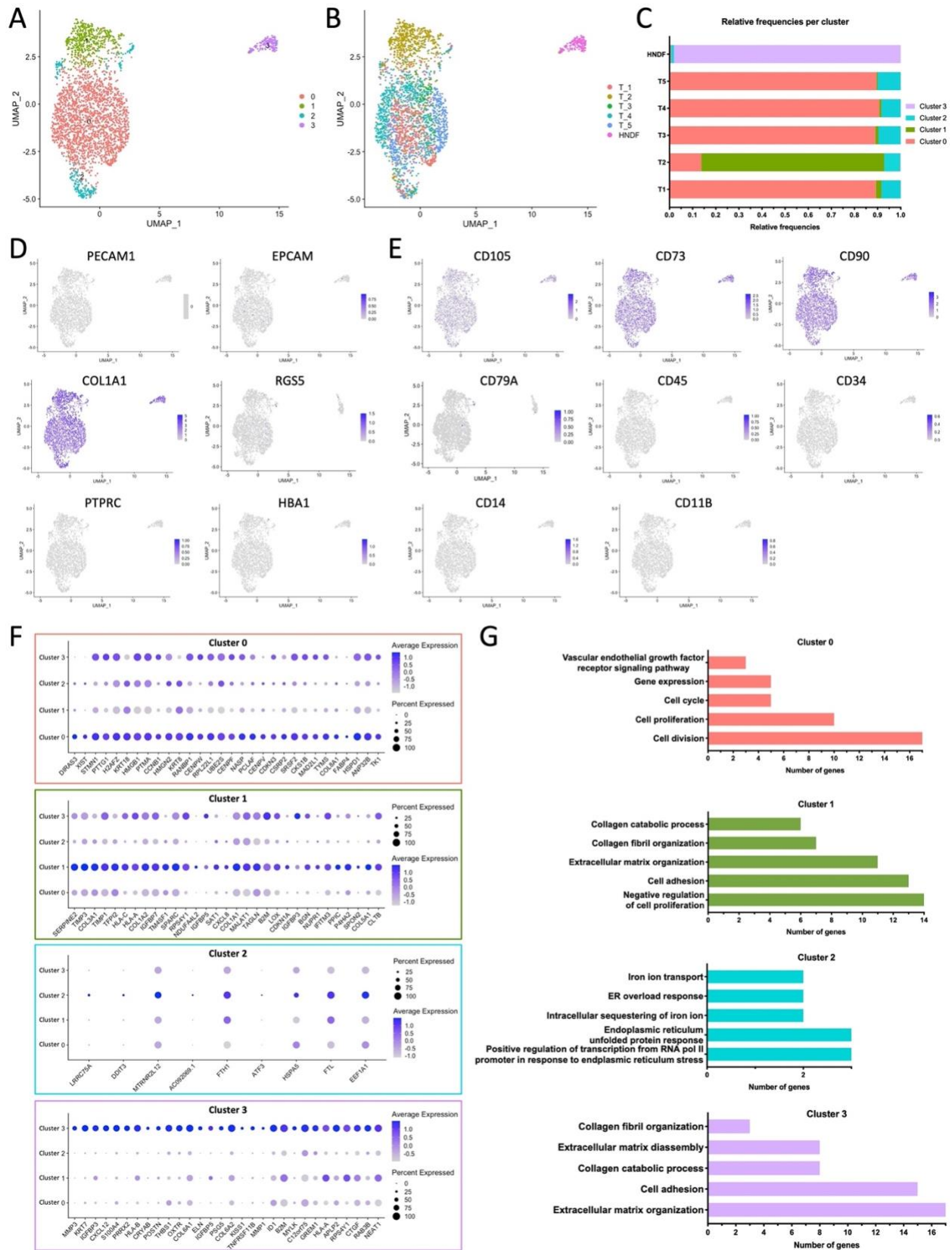


Figure 2.1 scRNA-seq analysis of UC-MSCs isolated from five term donors and HNDFs.

A) UMAP showing the four clusters that were identified based on mRNA expression profiles. Cell clusters are colored as indicated by the legend on the right of the UMAP. **B)** UMAP showing the distribution of cells from each donor and HNDFs within the clusters. Cells from each donor and HNDFs are colored as indicated by the legend on the right of the UMAP. **C)** Relative contribution of the four clusters to each UC-MSC donor and HNDFs. Clusters are colored as indicated by the legend on the right of the graph. **D)** UMAP showing expression levels of canonical markers of epithelial (*EPCAM*), endothelial (*PECAMI*), immune (*PTPRC*), mesenchymal (*COL1A1*), red blood cell (*HBA-A1*), and pericyte (*RGS5*). **E)** UMAP showing expression levels of ISCT MSC markers *CD105*, *CD73*, *CD90*, *CD79A*, *CD45*, *CD34*, *CD14*, and *CD11B* (no graphs available for *CD19* and *HLA-DR*, as the cells completely lacked the expression). In panels **D** and **E**, the intensity of expression is indicated by the purple color. **F)** Dot plots depicting expression of the top 30 marker genes for clusters 0, 1, and 3 or the full list of marker genes for cluster 2. The intensity of expression is indicated by the purple color and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. **G)** Selected enriched GO terms for each cluster. All terms are significantly enriched (p value < 0.05). Expression levels in UMAP plots and Dot plots are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to log-transformed UMIs per 10K.

2.4.2 Only UC-MSCs with a progenitor-like transcriptome - T4MSCs - improve lung structure and function

Next, we explored the therapeutic potential of UC-MSC subpopulations identified by scRNA-seq in a neonatal rodent hyperoxic model of BPD (Fig. 2.2A). UC-MSCs with a progenitor-like transcriptome (T4MSCs) and a fibroblast-like transcriptome (T2MSCs) were selected for this comparative study. Cells or PBS only were administered at PD4. Two hyperoxia controls were used: PBS as the vehicle control and HNDFs as control cells. Survival at PD21 was significantly decreased in the HNDFs treated group (Suppl. Fig. 2.2A). Significantly lower BW was observed at PD4 between the normoxic control group and T2MSCs or T4MSCs hyperoxic groups, which was not observed anymore at PD14 and PD21 (Suppl. Fig. 2.2B). Hyperoxia exposure resulted in simplification of alveolar structures in vehicle-injected pups compared to normoxic control pups, as indicated by thickening of the alveolar wall and enlarged alveoli with

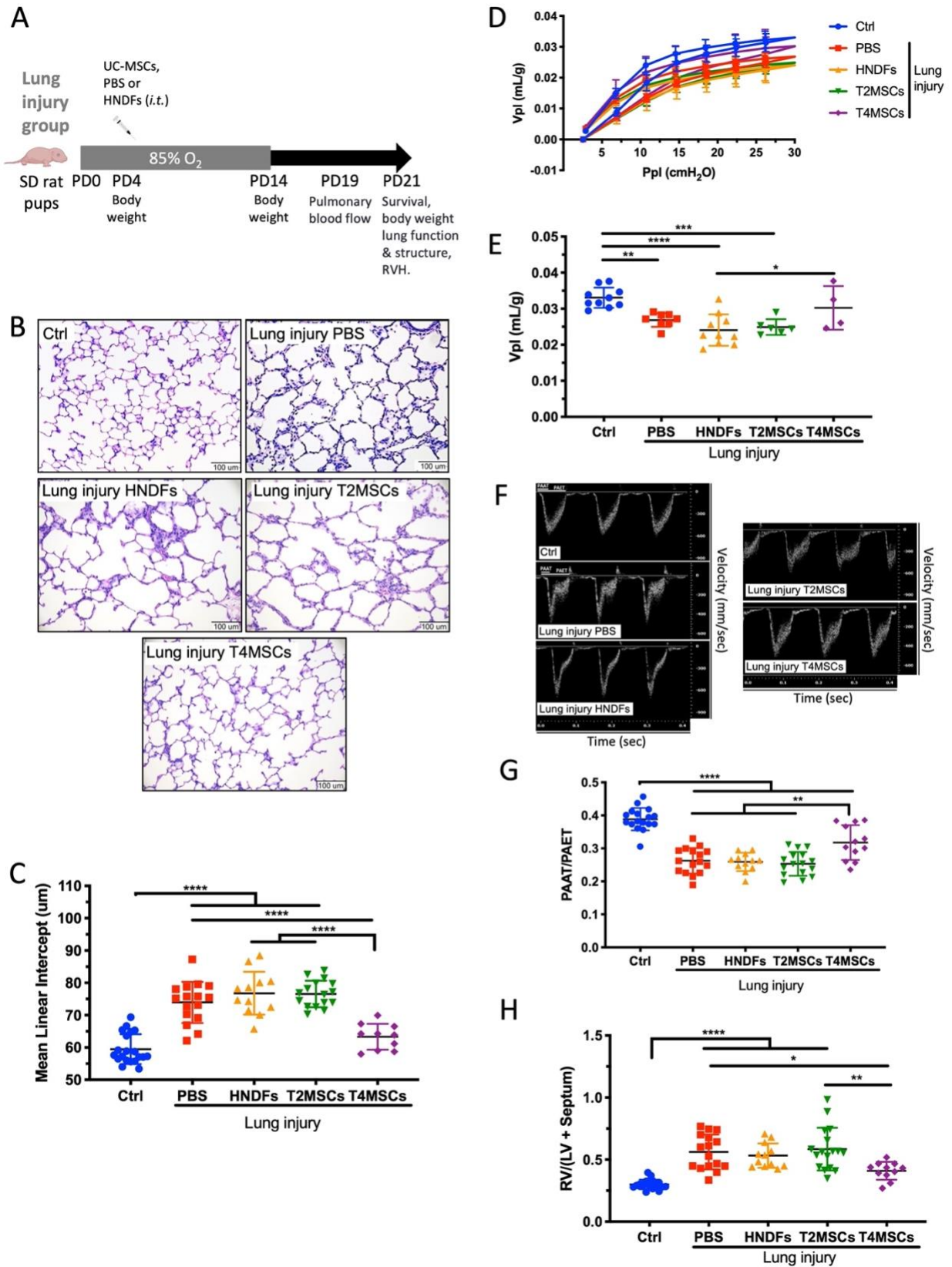


Figure 2.2 Treatment with UC-MSCs with a progenitor-like transcriptome (T4MSCs), but not with UC-MSCs with a fibroblast-like transcriptome (T2MSCs), reversed altered lung morphology and function, as well as PH and RVH associated with hyperoxia-induced lung injury in a BPD rat model.

A) Schematic representation of hyperoxia-induced lung injury model in neonatal rodents, as well as injection regimen and endpoints. Newborn SD rat pups were exposed to 85% O₂ until PD14 and kept under normoxic conditions until harvest. At PD4, PBS, HNDFs, or UC-MSCs were administered (*i.t.*). PAAT/PAET was assessed at PD19, while left lung histology, lung function, and Fulton's index (RVH) were assessed at PD21. **B)** Representative images of lung histology stained with H&E from the normoxic group (Ctrl) and hyperoxic groups injected with either vehicle (PBS), non-progenitor cell (HNDFs), or UC-MSCs from donor T2 (T2MSCs) or T4 (T4MSCs). **C)** Graph depicting the MLI (Ctrl: n=20, PBS: n=16, HNDFs: n=12, T2MSCs: n=16, T4MSCs: n=10). **D)** BW-adjusted PV-loop. PV-loops are shown as the mean volume of all animals within one group at each pressure point (Vpl: volume plethysmography, Ppl: pressure plethysmography) (Ctrl: n=10, PBS: n=8, HNDFs: n=10, T2MSCs: n=6, T4MSCs: n=4). **E)** BW-adjusted lung volume at peak pressure 30 cmH₂O extracted from the PV-loop in **D** (Ctrl: n=10, PBS: n=8, HNDFs: n=10, T2MSCs: n=6, T4MSCs: n=4). **F)** Representative images of echocardiography for each experimental group. **G)** Graph depicting PAAT/PAET ratio. PAAT and PAET were measured from echocardiography images, such as the ones in **F** (Ctrl: n=17, PBS: n=16, HNDFs: n=12, T2MSCs: n=16, T4MSCs: n=12). **H)** Fulton's index, reflecting RVH (Ctrl: n=20, PBS: n=16, HNDFs: n=12, T2MSCs: n=17, T4MSCs: n=11). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001.

significantly increased MLI (Fig. 2.2B, C). Treatment of hyperoxia-exposed pups with T4MSCs, but not with T2MSCs nor HNDFs, improved lung structure (Fig. 2.2B) and significantly decreased the MLI (Fig. 2.2C).

Lung function measurements of hyperoxia-exposed vehicle- and HNDFs-treated pups showed a downward shift in the pressure-volume (PV)-loop and a significant reduction in lung volume at a pressure of 30 cmH₂O compared to normoxic control pups (Fig. 2.2D, E). Treatment with T4MSCs, but not T2MSCs, partially restored PV-loop and lung volume in hyperoxia-exposed pups (Fig. 2.2D). Moreover, the lung volume of pups injected with T4MSCs was not significantly different compared to normoxic control pups (Fig. 2.2E).

2.4.3 Only UC-MSCs with a progenitor-like transcriptome - T4MSCs - reduce PH

Pulmonary arterial acceleration time (PAAT) and pulmonary arterial ejection time (PAET) values were obtained at PD19 (Fig. 2.2F). Echo Doppler showed a significant decrease in the PAAT/PAET ratio in hyperoxia-exposed vehicle- and HNDFs-treated pups (Fig. 2.2G). Treatment with T4MSCs, but not T2MSCs, significantly increased the PAAT/PAET ratio compared to hyperoxia-exposed control pups (Fig. 2.2G). Similarly, at PD21, we observed right ventricular hypertrophy (RVH) in hyperoxia-exposed vehicle- and HNDFs-treated pups (Fig. 2.2H). Treatment with T4MSCs, but not T2MSCs, reduced RVH compared to hyperoxia-exposed vehicle-treated pups (Fig. 2.2H).

Thus, T4MSCs (progenitor-like), but not T2MSCs (fibroblast-like) nor HNDFs provide a therapeutic effect in a hyperoxia neonatal lung injury model.

2.4.4 DEGs identified in progenitor-like UC-MSCs and non-therapeutic cells (fibroblast-like cells and HNDFs)

To further explore the differences between progenitor-like UC-MSCs and non-therapeutic cells at the transcriptomic level, we identified specific markers of UC-MSCs with a progenitor-like transcriptome, which includes UC-MSCs from the therapeutic donor T4, and specific markers of non-therapeutic UC-MSCs - T2MSCs - together with HNDFs. We generated a list of DEGs between the four UC-MSC donors with progenitor-like transcriptomes (T1, T3, T4, and T5) and the remaining non-therapeutic cell populations (T2 and HNDFs) (Suppl. Table 2.4). Specific markers of UC-MSCs with a progenitor-like transcriptome and of the non-therapeutic cells are shown in figure 2.3A and B. GO analysis further confirms that those genes enriched in progenitor-like UC-MSCs were involved in biological processes like cell division, cell cycle, cell

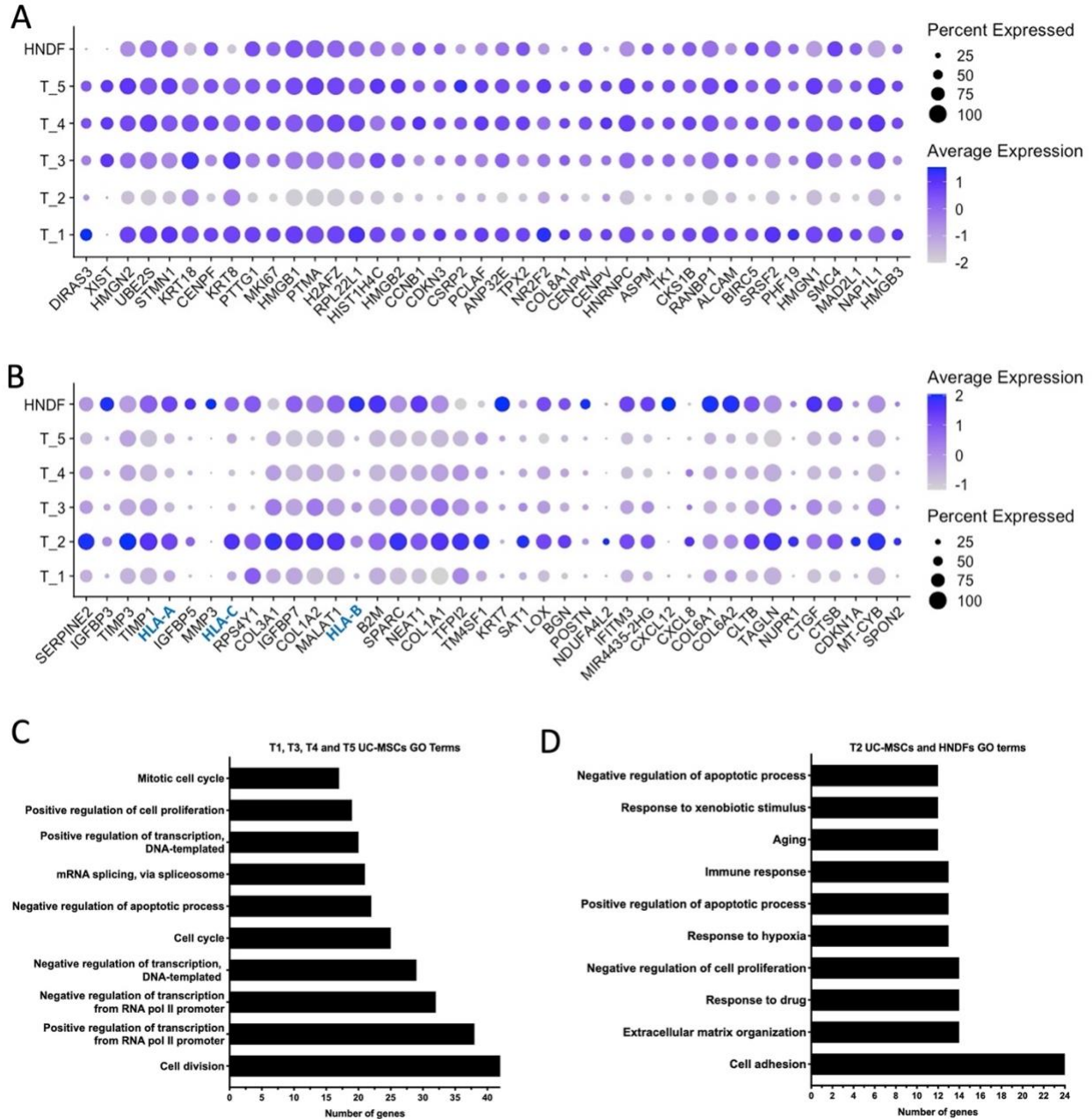


Figure 2.3 Marker genes of progenitor-like UC-MSCs and non-therapeutic cells.

A) Dot plot depicting expression of the top 40 marker genes of UC-MSCs with a progenitor-like transcriptome, which include UC-MSCs from the therapeutic donor T4. **B)** Dot plot depicting expression of the top 40 marker genes of non-therapeutic populations (UC-MSCs from donor T2 and HNDFs). Cell-surface molecules *HLA-A*, *HLA-B*, and *HLA-C* are identified in blue. In panels **A** and **B**, the intensity of expression is indicated by the purple color and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. **C)** Top 10 enriched GO terms for UC-MSCs with a progenitor-like transcriptome, which include UC-MSCs from the therapeutic donor T4. **D)** Top 10 enriched GO terms for non-therapeutic cells. All terms are significantly enriched (p value < 0.05). Expression levels in Dot plots are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to $\log$ -transformed UMIs per 10K.

proliferation, DNA transcription and chromatin organization (Fig. 2.3C), while those enriched in the non-therapeutic cell population (T2MSCs and HNDFs) were involved in cell adhesion, ECM organization, and collagen processes (Fig. 2.3D).

Progenitor-like UC-MSCs, including therapeutic donor T4 UC-MSCs, can be identified by a specific gene expression pattern involved in cell proliferation.

2.4.5 More therapeutic UC-MSCs are retained in the lungs of hyperoxic rat pups two to three days following administration compared to non-therapeutic UC-MSCs

Among the markers identified above for the non-therapeutic cells, three genes, *HLA-A*, *-B*, and *-C* (*HLA-ABC*), encode MHC I molecules (Fig. 2.3B). *HLA-ABC* play a dominant role in transplant rejection. To explore the possibility that non-therapeutic UC-MSCs (T2MSCs) might be rejected faster from the host than therapeutic UC-MSCs (T4MSCs), we assessed UC-MSCs' time of retention in the lungs of hyperoxia-exposed pups using two parameters: In Vivo Imaging Systems (IVIS) and PCR quantification of human DNA.

Bioluminescence signal from non-therapeutic T2MSCs and therapeutic T4MSCs transduced with luciferase lentivirus, as measured by total flux from the ventral area of rat pups, was observed at PD4 (3 to 4 hours after injections) (Fig. 2.4A, B). A time-dependent decrease in signal intensity was observed in both groups (Fig. 2.4A, C). However, the signal observed in T4MSCs-injected animals was significantly higher at PD6 and PD7 (Fig. 2.4C). No signal could be observed in any of the animals at PD11. Quantification of human DNA in the right lungs of hyperoxia-exposed pups also revealed a time-dependent decrease in number of cells in both groups (Fig. 2.4D). Moreover, the number of therapeutic T4MSCs in the lungs of hyperoxic pups at PD6 and PD7 was significantly higher compared to the number of non-therapeutic T2MSCs (Fig. 2.4D).

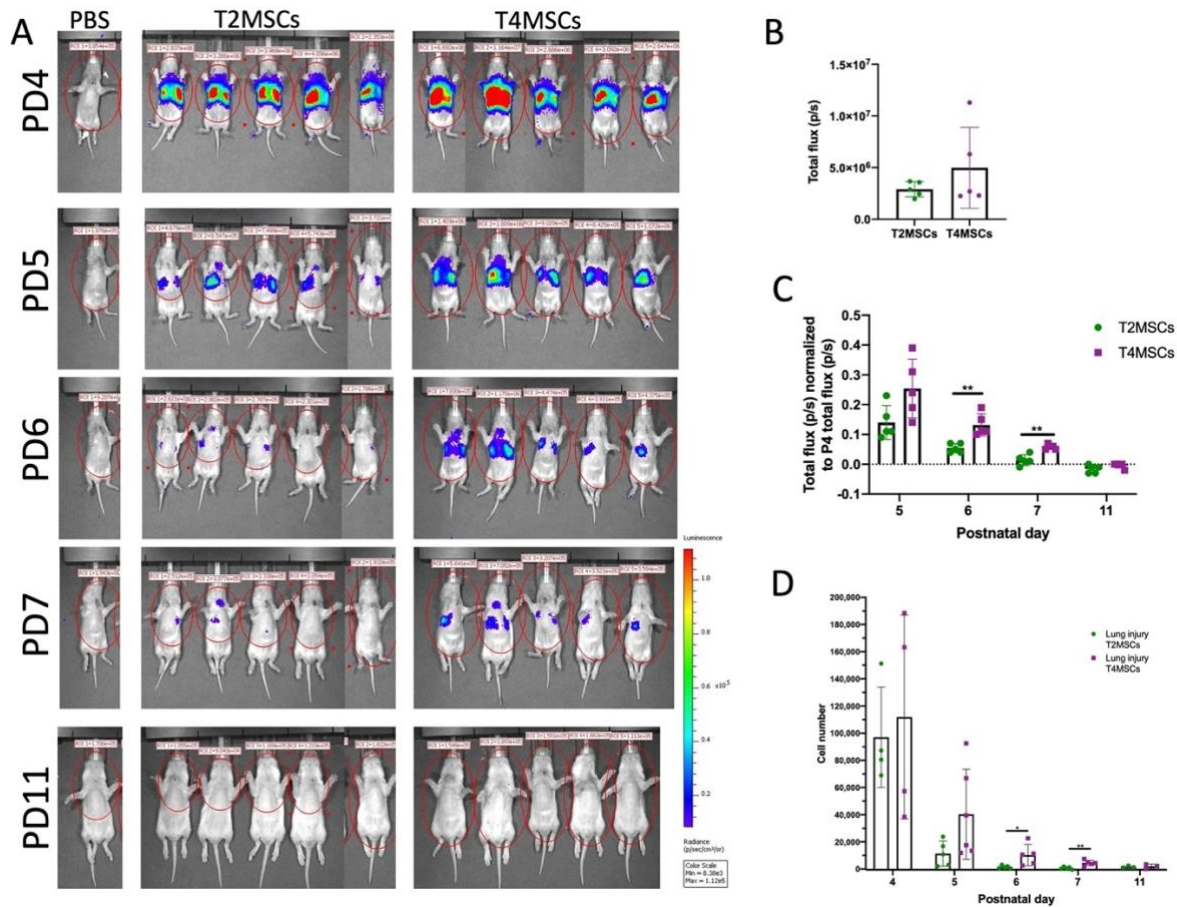


Figure 2.4 Time-dependent decrease of UC-MSCs in the lungs of hyperoxia-exposed pups.

A) Bioluminescence detection using IVIS in hyperoxia-exposed rat pups administered *i.t.* either with PBS or UC-MSCs from donor T2 (T2MSCs) or T4 (T4MSCs) transduced with Firefly Luciferase Lentivirus on PD4. Images were taken at PD4 (3 to 4 hours after *i.t.* injections), PD5, PD6, PD7, and PD11. All IVIS images were normalized for signal count. **B)** Total flux quantification of the IVIS images from the ventral region in hyperoxia-exposed rat pups injected with T2MSCs T4MSCs normalized to total flux of hyperoxia-exposed rat pups injected with PBS at PD4. Data are presented as the total flux (p/s) (T2MSCs: n=5, T4MSCs: n=5). **C)** Total flux quantification of the IVIS images from the ventral region in hyperoxia-exposed rat pups injected with T2MSCs or T4MSCs normalized to both total flux of hyperoxia-exposed rat pups injected with PBS, as well as to the total flux of the at PD4 of corresponding hyperoxia-exposed rat pup. Data are presented as the total flux (p/s) normalized to PD4 total flux (p/s) (T2MSCs and T4MSCs: n=5 at all time points). **D)** Detection of number of human cells in the lung tissue of hyperoxia-exposed rat pups at PD4 (3 to 4 hours after UC-MSCs *i.t.* injections), PD5, PD6, PD7, and PD11 after the injection of T2MSCs or T4MSCs (*i.t.* at PD4). The number of UC-MSCs was calculated from the amount of human genomic DNA extracted from the lung. Results were normalized to hyperoxia-exposed rat pups injected with PBS instead of UC-MSCs. qPCR was performed in triplicate samples in one run (PD4: T2MSCs and T4MSCs: n=4, PD5: T2MSCs: n=5, T4MSCs: n=6, PD6 and PD7: T2MSCs and T4MSCs: n=5, PD11: T2MSCs and T4MSCs: n=3). Values are represented as the mean $\pm$ STD. *t* test (unpaired, two-tailed) was used to compare results between two groups. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

2.4.6 Therapeutic UC-MSCs expressing high levels of MHCI have reduced healing potential in experimental BPD

To explore the healing potential of therapeutic UC-MSCs expressing high levels of MHCI, we used fluorescence-activated cell sorting (FACS) to isolate the HLA-ABC^{low} and HLA-ABC^{high} subpopulations of T4MSCs (Suppl. Fig. 2.3A). Therapeutic efficacy of those subpopulations (Suppl. Fig. 2.3B) was then evaluated *in vivo* (Fig. 2.5A). No difference in survival was observed between all groups (Suppl. Fig. 2.3C). At PD4, the BW of hyperoxia-exposed pups treated with T4MSCs was lower than that of those treated with T4MSCs-HLA-ABC^{low} and T4MSCs-HLA-ABC^{high}. No difference in BW was observed between groups at PD14 and PD21 (Suppl. Fig. 2.3D). No differences could be observed in lung structure and MLI between the lungs from hyperoxia-exposed pups treated with full T4MSCs population or T4MSCs-HLA-ABC^{low} (Fig. 2.5B, C). However, T4MSCs-HLA-ABC^{high} treated pups had simplified alveolar lung structure and increased MLI compared to T4MSCs- and T4MSCs-HLA-ABC^{low}-treated animals (Fig. 2.5B, C). No difference in lung function was observed between all groups (Fig. 2.5D-E).

Significant decrease in the PAAT/PAET ratio in hyperoxia-exposed pups injected with T4MSCs-HLA-ABC^{high} subpopulation compared to animals injected with the full T4MSCs population and T4MSCs-HLA-ABC^{low} was observed (Fig. 2.5F, G). Similarly, T4MSCs-HLA-ABC^{high} treated pups had significantly greater RVH compared to pups treated with the full T4MSCs population or T4MSCs-HLA-ABC^{low} (Fig. 2.5H). No difference in RVH was observed between pups treated with the full T4MSCs population and T4MSCs-HLA-ABC^{low} (Fig. 2.5H).

Thus, HLA-ABC^{low} expression marks the therapeutic subset of T4MSCs.

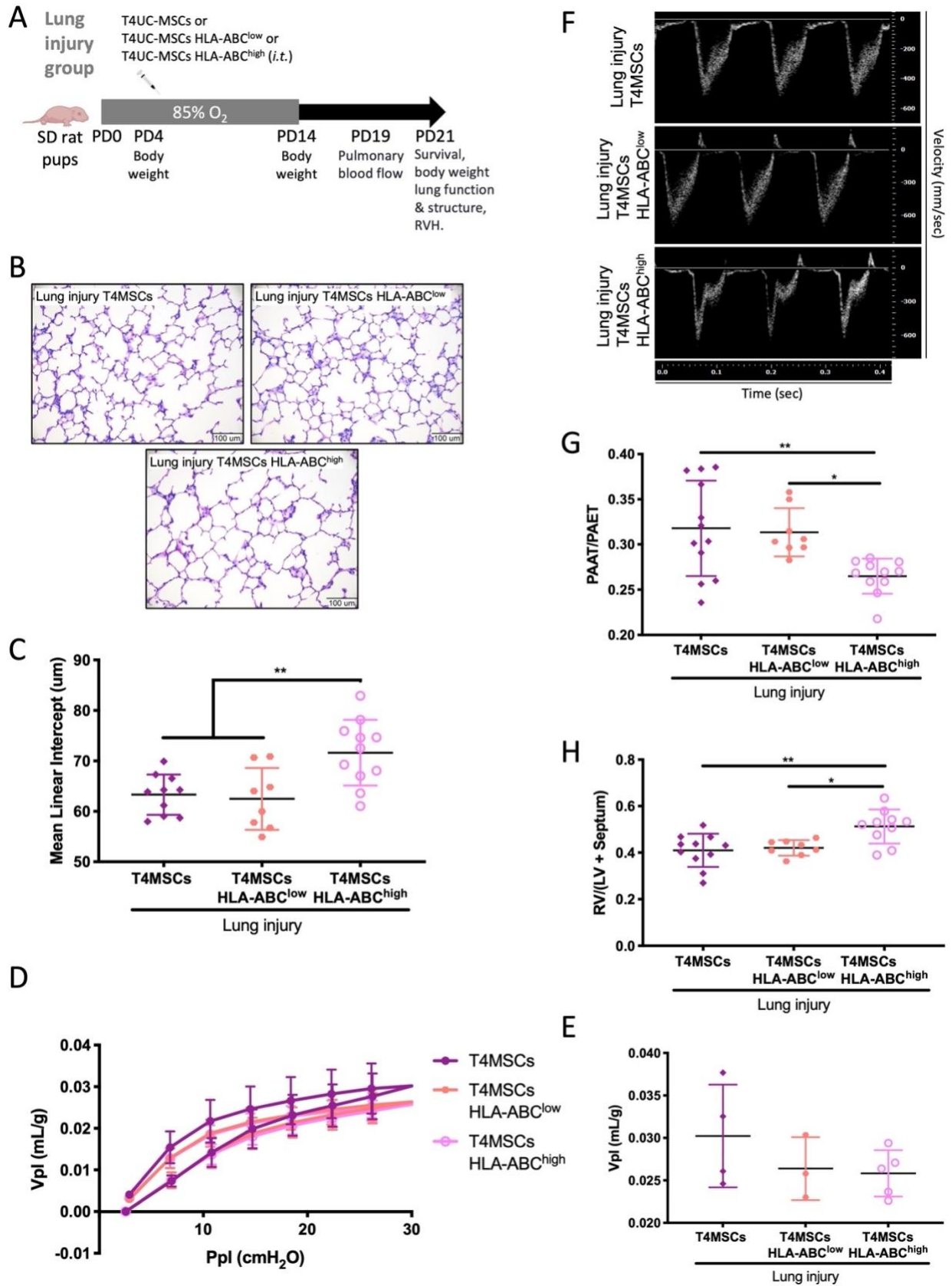


Figure 2.5 Reduced healing potential after administration of a subpopulation of therapeutic UC-MSCs (T4MSCs) expressing high levels of MHCI (HLA-ABC) in a hyperoxia-induced lung injury BPD rat model.

A) Schematic representation of hyperoxia-induced lung injury model in neonatal rodents, as well as injection regimen and endpoints. Newborn SD rat pups were exposed to 85% O₂ until PD14 and kept under normoxic conditions until harvest. At PD4, PBS, T4MSCs, or subpopulations of T4MSCs were administered (*i.t.*). PAAT/PAET was assessed at PD19, while left lung histology, lung function, and Fulton's index (RVH) were assessed at PD21. **B)** Representative images of lung histology stained with H&E from the hyperoxic groups injected with either T4MSCs or the subpopulations of T4MSCs HLA-ABC^{high} or HLA-ABC^{low}. **C)** Graph depicting the MLI (T4MSCs: n=10, T4MSCs HLA-ABC^{low}: n=8, T4MSCs HLA-ABC^{high}: n=11). **D)** BW-adjusted PV-loop. PV-loops are shown as the mean volume of all animals within one group at each pressure point (Vpl: volume plethysmography, Ppl: pressure plethysmography) (T4MSCs: n=4, T4MSCs HLA-ABC^{low}: n=3, T4MSCs HLA-ABC^{high}: n=5). **E)** BW-adjusted lung volume at peak pressure 30 cmH₂O extracted from the PV-loop in **D** (T4MSCs: n=4, T4MSCs HLA-ABC^{low}: n=3, T4MSCs HLA-ABC^{high}: n=5). **F)** Representative images of echocardiography for each experimental group. **G)** Graph depicting PAAT/PAET ratio. PAAT and PAET were measured from echocardiography images, such as the ones in **F** (T4MSCs: n=12, T4MSCs HLA-ABC^{low}: n=8, T4MSCs HLA-ABC^{high}: n=11). **H)** Fulton's index, reflecting RVH (T4MSCs: n=11, T4MSCs HLA-ABC^{low}: n=8, T4MSCs HLA-ABC^{high}: n=10). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001.

2.4.7 Non-therapeutic UC-MSCs expressing high or low levels of MHCI do not provide therapeutic potential in experimental BPD

HLA-ABC^{low} and HLA-ABC^{high} subpopulations of non-therapeutic UC-MSCs from donor T2 were isolated by FACS, and their healing potential was tested *in vivo* (Suppl. Fig. 2.3E-F, Fig. 2.6A). No differences in survival and BW were observed (Suppl. Fig. 2.3G, H). Lung structure in the hyperoxia-exposed pups treated with T2MSCs-HLA-ABC^{high} was similar to that of hyperoxia-exposed pups treated with the full T2MSCs population (Fig. 2.6B, C). However, T2MSCs-HLA-ABC^{low} treated pups showed an improved alveolar lung structure and a significant decrease in MLI compared to T2MSCs-treated pups (Fig. 2.6B, C). Nevertheless, no significant difference in MLI could be observed between T2MSCs-HLA-ABC^{low}- and PBS-treated pups (Fig. 2.6D). Significant increase in the PAAT/PAET ratio in hyperoxia-exposed pups treated with T2MSCs-HLA-ABC^{high}

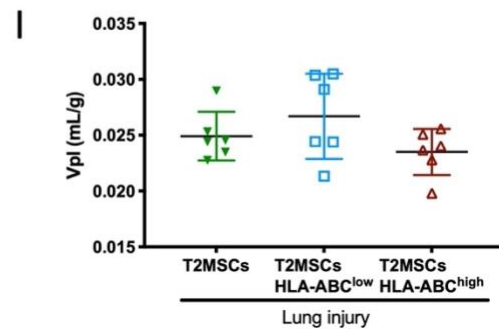
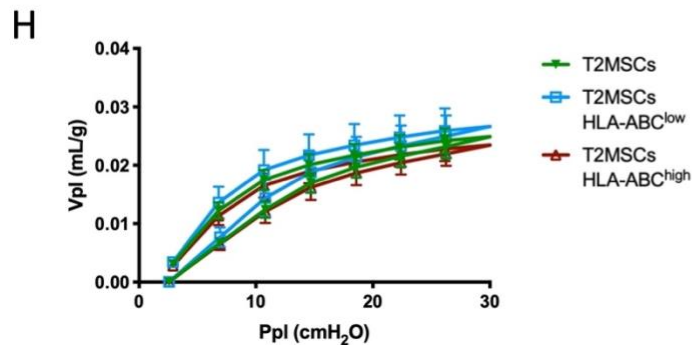
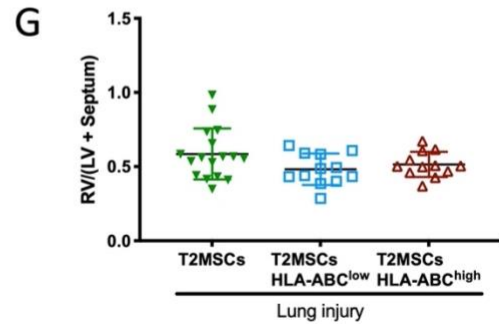
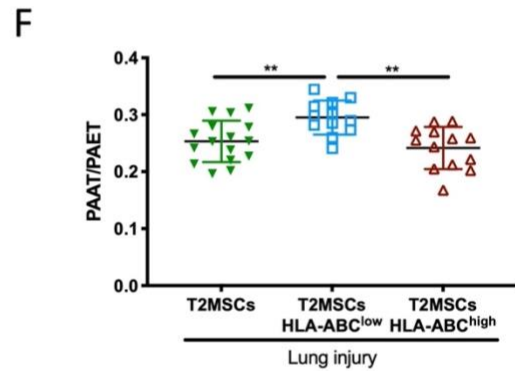
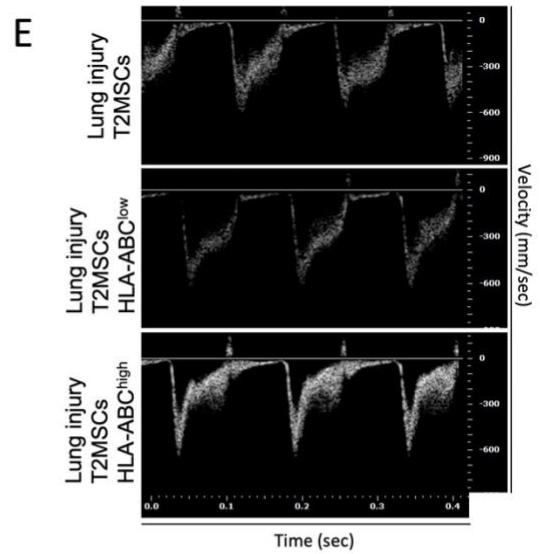
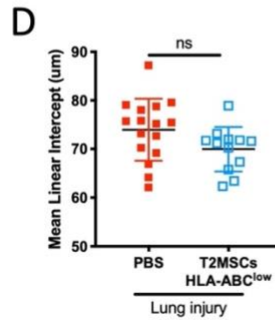
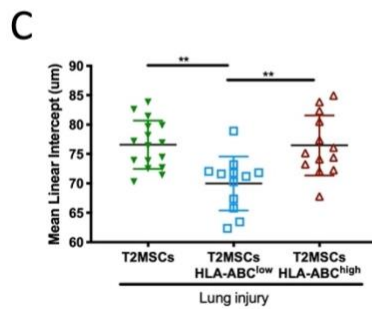
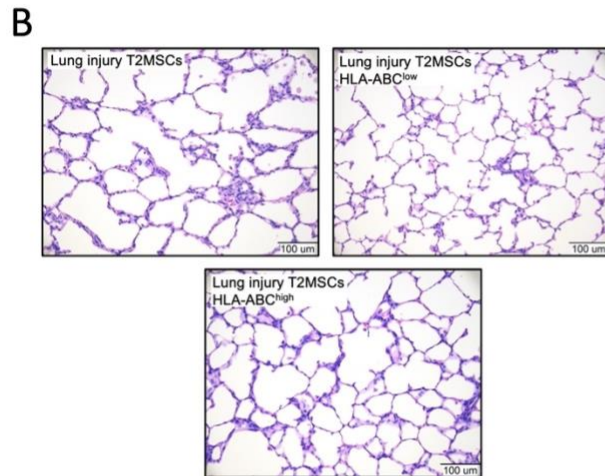
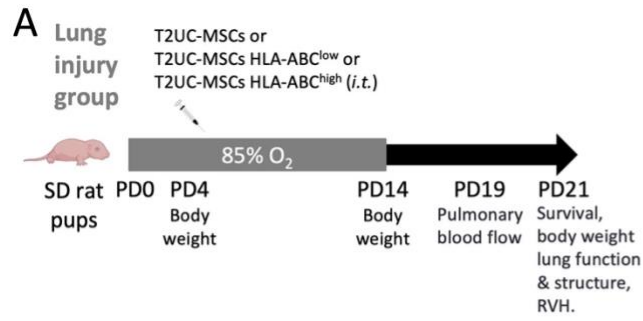


Figure 2.6 No difference in healing potential after administration of a subpopulation of non-therapeutic UC-MSCs (T2MSCs) expressing high or low levels of MHCI (HLA-ABC) in a hyperoxia-induced lung injury BPD rat model.

A) Schematic representation of hyperoxia-induced lung injury model in neonatal rodents, as well as injection regimen and endpoints. Newborn SD rat pups were exposed to 85% O₂ until PD14 and kept under normoxic conditions until harvest. At PD4, T2MSCs or subpopulations of T2MSCs were administered (*i.t.*). PAAT/PAET was assessed at PD19, while left lung histology, lung function, and Fulton's index (RVH) were assessed at PD21. **B)** Representative images of lung histology stained with H&E from the hyperoxic groups injected with either T2MSCs or the subpopulations of T2MSCs HLA-ABC^{high} or HLA-ABC^{low}. **C)** Graph depicting the MLI (T2MSCs: n=16, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=13). **D)** Graph depicting the MLI of PBS group and T2MSCs HLA-ABC^{low} only (PBS: n=16, T2MSCs HLA-ABC^{low}: n=12). **E)** Representative images of echocardiography for each experimental group. **F)** Graph depicting PAAT/PAET ratio. PAAT and PAET were measured from echocardiography images, such as the ones in **E** (T2MSCs: n=16, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=13). **G)** Fulton's index, reflecting RVH (T2MSCs: n=17, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=12). **H)** BW-adjusted PV-loop. PV-loops are shown as the mean volume of all animals within one group at each pressure point (Vpl: volume plethysmography, Ppl: pressure plethysmography) (n=6 for all groups). **I)** BW-adjusted lung volume at peak pressure 30 cmH₂O extracted from the PV-loop in **H** (n=6 for all groups). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test, except for panel D, where *t* test (unpaired, two-tailed) was used. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001. ns: non significant.

subpopulation compared to animals treated with the full T2MSCs population and T2MSCs-HLA-ABC^{low} was observed (Fig. 2.6E, F). No differences in RVH and lung function were observed between groups (Fig. 2.6G-I).

Hence, high expression of MHCI is not the only contributing factor to the lack of therapeutic potential in T2MSCs.

2.5 Discussion

For the first time, to our knowledge, we showed a scRNA-seq study of UC-MSCs with HNDFs as control cells, where we associated their transcriptome with their *in vivo* therapeutic

potential. In addition, we showed that elevated expression of MHC I by UC-MSCs might contribute to negating their therapeutic effect, partly by reducing the time that the cells stay in the host system.

Higher accessibility and fewer ethical issues associated with their use make UC-MSCs a favorable resource for cell therapies and clinical applications. However, the dichotomy of UC-MSC donors, as well as UC-MSC subpopulations with greatest therapeutic potential remain unknown, and are potentially the main reason leading to results' inconsistency in clinical trials (30–32). Previous work has investigated the heterogeneity of MSCs using traditional bulk RNA-seq (33–36). Contrary to bulk RNA-seq, scRNA-seq allows measurement of mRNA expression in individual cells and enables the identification of unknown subpopulations in an unbiased way. Hitherto, eight studies have investigated the heterogeneity of cultured UC-MSCs using scRNA-seq (37–44). These studies include the comparison of mRNA expression between MSCs isolated from different tissue sources (37,40,42,44), or between primary isolates and cultured UC-MSCs (38), as well as between different UC-MSCs stimulations (IFN γ and TNF α) and different PNs (41). The majority of these previous studies lack the correlation between UC-MSC transcriptomes and their functions and all included one to three donors only. To comprehensively investigate UC-MSC heterogeneity and correlate *in silico* data with *in vivo* therapeutic efficacy, we analyzed the transcriptome of cells derived from five donors and included a control cell line of mesenchymal origin (HNDFs).

scRNA-seq analysis revealed low heterogeneity within the UC-MSC populations. Four distinct clusters were identified. HNDFs and UC-MSCs from donor T2 formed independent clusters (clusters 3 and 1, respectively), both with transcriptomes enriched in genes related to collagen and ECM functions. UC-MSCs from donor T1, T3, T4, and T5 were found mostly within clusters 0 and 2 and showed enriched GO terms related to cell division, cell proliferation, cell

cycle, gene expression, cell signaling, and protein folding functions (progenitor-like UC-MSCs). Furthermore, we investigated the effect of administering UC-MSCs having a progenitor-like transcriptome (T4MSCs) compared to UC-MSCs with a fibroblast-like transcriptome (T2MSCs) in neonatal rats exposed to hyperoxia. Our findings indicate that progenitor-like T4MSCs, but not fibroblast-like T2MSCs, improve lung outcomes and attenuate PH. We generated a list of DEGs between the four UC-MSC donors with progenitor-like transcriptomes (including therapeutic T4MSCs) and the remaining non-therapeutic cells (T2MSCs and HNDs). Progenitor-like UC-MSCs were enriched in genes with functions related to cell division, cell cycle, cell proliferation, DNA transcription, and chromatin organization. In contrast, non-therapeutic cells showed high expression of genes related to ECM organization and collagen processes. Interestingly, recent studies evaluating UC-MSC heterogeneity also identified subpopulations of UC-MSCs with DEGs significantly enriched in DNA replication pathway and cell cycle process, as well as high expression levels of several histone genes (39,41). Moreover, CD142⁺ UC-MSC subpopulation exhibited lower proliferation capacity and had superior wound healing potential *in vitro*. Altogether these data support the idea that detailed characterization and adequate pre-selection of UC-MSC subpopulations, based on their functional potential and precisely defined medical intended need, should be achieved for optimal healing effects. However, it is important to note that questions remain regarding how *in vitro* assay testing actually correlates to *in vivo* activity and whether it can predict therapeutic effects (45).

Furthermore, among the identified DEGs, we observed that non-therapeutic cells expressed high levels of MHCI (*HLA-ABC*). Although past evidence pointed toward the immune privileged/evasive character of MSCs, and that MSCs could be transplanted between HLA incompatible patients, some results have shown otherwise. As such, stronger allorecognition

response was seen with natural killer, B, and T cells increasing amount in peripheral blood when MSCs with higher MHCI mismatch between donor and host were injected *in vivo* (46). Similar results were found in a study showing a strong and specific cellular immune response in immunocompetent mice following implantation of MHC-mismatched MSCs (47). Also, human MSCs had limited repair abilities after bleomycin challenge in immunocompetent mice compared to severe combined immunodeficiency mice (48). Nevertheless, although allogeneic MSCs can be recognized by the host immune system, clinical trials have shown that the administration of allogeneic MSCs is safe and feasible (49). Our study found that UC-MSCs with higher MHCI expression exhibit a lower retention time in the host lung than UC-MSCs with lower MHCI expression. We further isolated UC-MSC subpopulations expressing high and low levels of HLA-ABC to determine the impact of MHCI expression on their therapeutic potential. We found that HLA-ABC^{high} subpopulation of UC-MSCs with a progenitor-like transcriptome had a lower therapeutic potential compared to the UC-MSC full, and HLA-ABC^{low} subpopulation. Furthermore, HLA-ABC^{low} subpopulation of fibroblast-like UC-MSCs did not provide therapeutic potential in neonatal rats exposed to hyperoxia. Taken together, these results indicate that the immunogenicity of MSCs must be taken into consideration for clinical applications, and high expression of MHCI (HLA-ABC) is not the only contributing factor to the lack of therapeutic effect in UC-MSCs, and other intrinsic properties must prohibit their healing potential.

We believe there are at least two possible explanations for the difference in transcriptome observed between UC-MSCs isolated from donor T2 compared to the transcriptome of the other UC-MSC donors. Firstly, underlying genetic background variations could be responsible for the heterogeneity between donors (50). Secondly, despite following standardized operating procedures, inter-individual operator variations are possible. Hence, cell-therapy manufacturing

can benefit from automated processes, which could eliminate user-to-user variability and error (51).

Our study presents certain limitations. *In vivo* UC-MSCs' therapeutic potential was investigated only for two of the five UC-MSC donors included in this study. However, it should be noted that the remaining three donors were indistinguishable from a transcriptomic point of view from donor T4 tested *in vivo*. In addition, in a parallel study, we have confirmed the comparable *in vivo* therapeutic effect of donor T3 to donor T4, which will be discussed in **Chapter 3**. Moreover, we have found an association between low expression of HLA-ABC and enhanced therapeutic potential of UC-MSCs in our neonatal hyperoxia-induced lung injury model. Nevertheless, we did not establish whether low expression of HLA-ABC is only a marker or a true mediator. Definitive evidence to establish causality requires additional experiments.

In conclusion, *in vitro* expanded UC-MSCs exhibited limited heterogeneity. However, UC-MSCs should not be considered a homogenous population since different UC-MSC subpopulations manifested different repair abilities in hyperoxia-exposed neonatal rodents. Furthermore, UC-MSCs with a progenitor-like transcriptome (T4MSCs) rather than a fibroblast-like transcriptome (T2MSCs), as well as low expression of MHCI molecules, demonstrated superior therapeutic efficacy in experimental BPD and could serve as markers of therapeutic UC-MSCs for treatment of BPD. Ultimately, future development of cell-therapy products should prioritize detailed characterization of UC-MSCs to select for superior repair cells resulting in improved clinical trial results.

2.6 References

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2.7 Appendix

2.7.1 Supplementary materials and methods

UC-MSCs isolation

UC-MSCs were isolated after parental consent from the UCs of five healthy term pregnancies (T1, T2, T3, T4, and T5) (Table 2.1) using enzymatic digestion. Briefly, the UC was rinsed in 30% [vol/vol] Citrate Phosphate-Dextrose Adenine 1 anticoagulant in PBS. After disinfection in Povidone-Iodine solution (cat#3955-16, Ricca Chemical Company, Arlington, TX, USA), the UC was cut into 2-3 cm pieces and minced with surgical scissors and scalpel. The minced tissue was placed into Dulbecco's PBS (D-PBS) supplemented with Ca^{2+} , Mg^{2+} , glucose, and pyruvate (cat#14287-072, Gibco, Waltham, MA, USA), as well as with 400 U/mL Collagenase I (cat#4196, Worthington, Columbus, OH, USA), 240 U/mL Hyaluronidase (cat#H3884, SIGMA Life Science, St-Louis, MO, USA), and DNase I (cat#D5025, SIGMA Life Science, St-Louis, MO, USA) (3.2 mL of D-PBS solution/cm of UC was used). Following the digestion at 37°C for 3 to 4 h (until complete dissolution of the tissue), the digestion was stopped by adding 250 μL 0.15 M EDTA (cat#15575-038, Invitrogen, Carlsbad, CA, USA) per cm of UC at collection. The solution was filtered through a 100 μm cell strainer (ca#431752, Corning, New York, NY, USA) and 18 mL of Hank's Balanced Salt Solution (cat#14174-079, Gibco, Waltham, MA, USA) per cm of UC at collection was added. The solution was transferred in 15 mL Falcon tubes on the ficoll gradient (Ficoll-PaqueTM PREMIUM 1.073 g/cm³, cat#17-5446-52, GE Healthcare, Chicago, IL, USA) (1 mL of ficoll was used per cm of UC at collection). Centrifugation was performed at 900 RCF, 19°C for 20 minutes with an acceleration speed of five and no deceleration. The interphase was collected and washed with PBS, and centrifugation was performed at 500 RCF, 19°C for 5 minutes. Cells were seeded in minimum essential medium

Eagle, alpha modification (cat#M8042-500ML, SIGMA Life Science, St-Louis, MO, USA) containing 20% fetal bovine serum (cat#1051-500ML, SIGMA Life Science, St-Louis, MO, USA), 10% L-glutamine 200 mM (cat#25030-081, Gibco, Waltham, MA, USA) and 5% standard antibiotics (cat#15240-062, Gibco, Waltham, MA, USA) in tissue culture treated plastic flasks (cat#83.3911.002, Sarstedt, Newton, NC, USA). Culture was maintained in a cell culture incubator at 37°C with 5% CO₂ and 5% O₂ as previously described (1).

UC-MSCs differentiation assays

Osteogenic, chondrogenic, and adipogenic differentiation assays were performed at PN3 as previously described (2) with these modifications: chondrogenic differentiation was induced for 28 days, and differentiation was assessed by Alcian Blue with red counterstain. Tissue dehydration, paraffin embedding, sectioning (4 μ m), and staining were performed by the University of Ottawa Louise Pelletier Histology Core Facility.

Multiplexing individual samples for scRNA-seq

A total of 4,000 UC-MSCs/well from each donor, as well as 4,000 HNDFs/well (non-progenitor control cells) (cat#CC-2509, Lonza, Hayward, CA, USA) were plated at PN3 in a 96-well plate (four technical replicates were used for each UC-MSC donor and for HNDF) and placed in a cell culture incubator at 37°C with 5% CO₂ and 5% O₂ as previously described (1). Twenty-four hours later, cells were resuspended in a solution of TrypLE-Express (cat#12605028, ThermoFisher Scientific, Waltham, MA, USA) and 200 nM Anchor/200 nM Barcode (kindly provided by Prof. Zev Gartner from University of California, San Francisco), with each sample receiving a different barcode, followed by the co-anchor as previously described (3). The samples

were pooled together at 1:1 ratio while maintaining the final concentration of 1,000 cells/ μ l. Viability and cell counts were assessed using a manual hemacytometer (Bright-Line™ Hemacytometer; Sigma-Aldrich, Oakville, ON, Canada). Samples with viability $\geq$ 80% were further processed by 10x Chromium.

scRNA-seq library preparation and sequencing

The multiplexed pool of UC-MSCs and HNDFs was processed for scRNA-seq on the 10x Genomics Chromium Single Cell 3' platform (StemCore Laboratories, OHRI). The pool was processed on a single lane of the Chromium platform. Gene expression libraries were prepared following the standard 10x Genomics protocol and a separate MULTI-seq barcode library was isolated by size selection and PCR amplified as described by McGinnis *et al.* (4). Libraries were sequenced using NextSeq500 (Illumina) high-output 75-cycle runs. Gene expression libraries were sequenced to an average depth of 22,597 reads/cell and MULTI-seq barcode libraries were sequenced to a depth of approximately 1,000 reads per cell. Multiplexing was successful for all technical replicate (Supplementary Fig. 2.4).

Differential gene expression and gene ontology (GO) analysis

Differential gene expression analysis between clusters was performed using the Wilcoxon test implemented in the FindMarkers() function in Seurat. A cut-off of “avg_log2FC” $>$ 0.25 and an adjusted p value $<$ 0.05 was used to identify differentially expressed genes (DEGs) among clusters. In the figures and tables, DEGs are always shown starting from the DEG with the highest avg_log2FC to the DEG with the lowest avg_log2FC.

DAVID was used to identify enriched GO terms (5,6). All GO terms presented in the manuscript are significantly enriched (p value < 0.05) and the number of genes expressed for each GO term is presented in the figures.

Animal model

Pregnant SD rats (Charles Rivers, Saint Constant, QC, Canada) were delivered on embryonic day (E)14-E15. Pups from all litters were randomized on the day of birth (PD0) and allocated to each dam equally in order to obtain a litter size of 12 pups per dam. Hyperoxia-induced neonatal lung injury model was achieved by maintaining half of the cages in hyperoxic environment (85% O₂) from PD0 to PD14, while the other half was maintained in normoxic conditions (21% O₂) as previously described (7,8). From PD14 all cages were maintained in normoxia until harvest. Hyperoxic environment was maintained in sealed plexiglass chambers with continuous oxygen monitoring [BioSpherix, Redfield, NY]. Hyperoxia and normoxia-exposed dams were rotated every 48 h to avoid oxygen toxicity. The number of pups in each litter was kept equal ($\pm$ one) by euthanizing normoxic pups when deceased pups were found in hyperoxia-exposed litters. Such animals, as well as all remaining animals at PD21 (endpoint), were euthanized by *i.p.* injection of pentobarbital sodium (Euthanyl, 0.2 mL, 65 mg/mL).

UC-MSCs and HNDFs preparation for *in vivo* administration

PN3 UC-MSCs or HNDFs were plated at 5,000 cells/cm² in T75 flasks and were placed for 24 h in a cell culture incubator 37°C with 5% CO₂ and 5% O₂ prior to collection as previously described (1). At PD4, hyperoxic pups were anesthetized by inhalation of isoflurane (anaesthesia was induced and maintained with 5% and 3% of isoflurane respectively, in a mixture of 100% O₂

at 1.5 L/min), and vehicle or HNDFs (in 25 µl PBS) or UC-MSCs (in 25 µl PBS) were administered *i.t.* at a dose of 10×10^6 cells/kg BW. The experimental groups were as follows: 1) normoxia control (Ctrl), 2) hyperoxia vehicle control (PBS), 3) hyperoxia control with non-progenitor cells (HNDFs), 4) hyperoxia with donor UC-MSCs (T2MSCs or T4MSCs).

Echocardiography

At PD19, pups were anaesthetized by inhalation of isoflurane (anaesthesia was induced and maintained with 5% and 3% of isoflurane respectively, in a mixture of 100% O₂ at 1.5 L/min). The researcher was blinded and PAAT and PAET were measured as previously described (9,10).

Lung function testing, Fulton's index, histology, and lung structure analysis

At PD21, a tracheotomy was performed and lung function (PV-loop) was assessed using FlexiVent (Scireq, Montreal, QC, Canada) as described earlier (11). In addition, the hearts were collected and RVH was calculated by Fulton's index as described before (12) (before cutting the heart, the researcher was blinded), as well as the lungs for histology as described earlier (13). Blinded analysis of alveolar size was performed using a motorized microscope stage, and the MLI estimation (12).

Fluorescent activated cell sorting (FACS)

PN2 UC-MSCs (from donors T2 and T4) were plated in T75 flasks and incubated as previously described (1). Once 80-90% confluency was reached, cells were lifted with 3 mL TrypLE-Express (cat#12605028, ThermoFisher Scientific, Waltham, MA, USA). UC-MSCs were stained with anti-HLA-ABC-PE-Vio-770-REAffinity antibody (cat#130-120-433, Miltenyi

Biotech, Bergisch Gladbach, Germany). Control antibody was used to avoid any false signals (REA control antibody human IgG1 PE cat#130-113-438). No stain controls were used as needed. Flow cytometry was performed on a MoFlo XDP (BeckmanCoulter, Brea, California, USA) at the flow cytometry and cell sorting facility of the OHRI, and data were analyzed using FlowJo v10 (FlowJo LLC, Ashland, OR). Sorted cells (HLA-ABC^{low} and HLA-ABC^{high}) were plated and incubated under the same conditions as described before. Twenty-four hours later, PN3 cells were prepared as described above for *i.t.* injections. A total of 5,000 UC-MSCs from each population were used to confirm the preservation of HLA-ABC^{low} and HLA-ABC^{high} expression profile by flow cytometry.

UC-MSCs transduction

PN3 UC-MSCs (from donors T2 and T4) were plated in T75 flasks and placed in a cell incubator at 37°C with 5% CO₂ and 5% O₂ as previously described (1). Once the cells reached 50% confluency, Firefly Luciferase Lentivirus (cat#7969-G, BPS Bioscience, San Diego, CA, USA) was added to 15 mL media at multiplicity of infection 10 in the presence of 8 µg/mL polybrene (TR-1003-G, Millipore, Burlington, MA, USA). The cells were incubated at 37°C with 5% CO₂ and 5% O₂ as previously described (1). Forty-eight hours later, the cells were prepared for *i.t.* injections as described above.

In vivo imaging systems (IVIS)

Transduced UC-MSCs (from donors T2 and T4) were administered *i.t.* to hyperoxia-exposed pups at PD4 as described above.

At PD4 (3 h post-injection), PD5, PD6, PD7, and PD11, IVIS images were acquired. D-luciferin, sodium salt (BioVision, Milpitas, CA, USA) (15 mg/mL in PBS) was subcutaneously injected (150 mg/kg) in rat pups 20 minutes before IVIS imaging. Up to five rats were imaged by IVIS simultaneously. For each image, a region of interest (ROI) was defined around the visual boundaries of the bioluminescence as described previously (14). Quantifications of ROIs were generated using the Living Image 3.2 software according to the manufacturer's instructions (Caliper Life Sciences, Hopkinton, MA, USA). For each animal, the total flux (photons per second; p/s), average radiance (photons per second per cm² per solid angle of 1 steradian; p/c/cm²/sr), the STD of radiance, as well as the minimum and maximum radiance were obtained from the tracheal, thorax (lung), and abdominal regions. The total flux (p/s) was used to quantify the bioluminescence signal from IVIS figures in this study. For each animal, the total flux at each time point was first normalized to the control (vehicle-injected) animal total flux. Then, for each animal, the total flux at PD5, PD6, PD7, and PD11 was normalized to the animal's initial total flux at PD4.

Quantification of UC-MSCs Alu sequence by real-time PCR

UC-MSCs (from donors T2 and T4) were administered *i.t.* to hyperoxia-exposed pups at PD4 as described above. At PD4 (3 h post-injection), PD5, PD6, PD7, and PD11, right lungs were weighted, chopped into small pieces, and snap-frozen. Randomly selected small pieces of lung (20-25 mg/sample) were used for DNA extraction. First, tissue was lysed at room temperature by using Qiagen TissueLyser II bead mill and Stainless Steel Beads (5 mm, Qiagen cat#69989, Hilden, Germany) in 180 mL of buffer ATL (cat#69504, Qiagen, Hilden, Germany). Then, genomic DNA was extracted using the DNeasy Blood and Tissue Kit (cat#69504, Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and quality were

assessed by NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Quantitative real-time-PCR (qPCR) analysis was performed using CFX96 Touch Real-Time PCR (Bio-Rad). Reactions were performed in 20 μ L volume using 1 μ M primer concentration, 1 $\times$ SsoAdvancedTM Universal SYBR Green Supermix (cat#172-5271, Bio-Rad, Hercules, CA, USA), and 25 ng of rat genomic DNA. Alu sequence primers were used as; Alu-F: 5'CACCTGTAATCCCAGCACTTT-3', Alu-R: 5'-CCCAGGCTGGAGTGGAGT-3' (15). The PCR was performed under the following conditions: 95 °C for 15 mins, 95 °C for 15 s, 70.9 °C for 30 s, 72 °C for 30 s for 40 cycles. Standard curves were generated by a 10-fold serial dilution of T2MSCs and T4MSCs' genomic DNA with rat lung genomic DNA and plotted with Ct vs Log(ng of UC-MSC DNA). To calculate the number of UC-MSC in the right lung, first, ng UC-MSC DNA/ng rat genomic DNA was estimated based on the standard curve formula as $X = \text{Power}(x)/25$. Next, total rat genomic DNA from each lung was calculated as $D = (\text{whole tissue weight}/\text{part tissue weight used to extract genomic DNA}) \times \text{DNA quantity extracted from the part tissue weight}$. Then, the number of UC-MSCs in each right lung was calculated as $N = \text{total UC-MSC DNA in each right lung} (XD) \times (\text{number of UC-MSC/ng UC-MSC DNA})$.

Statistics

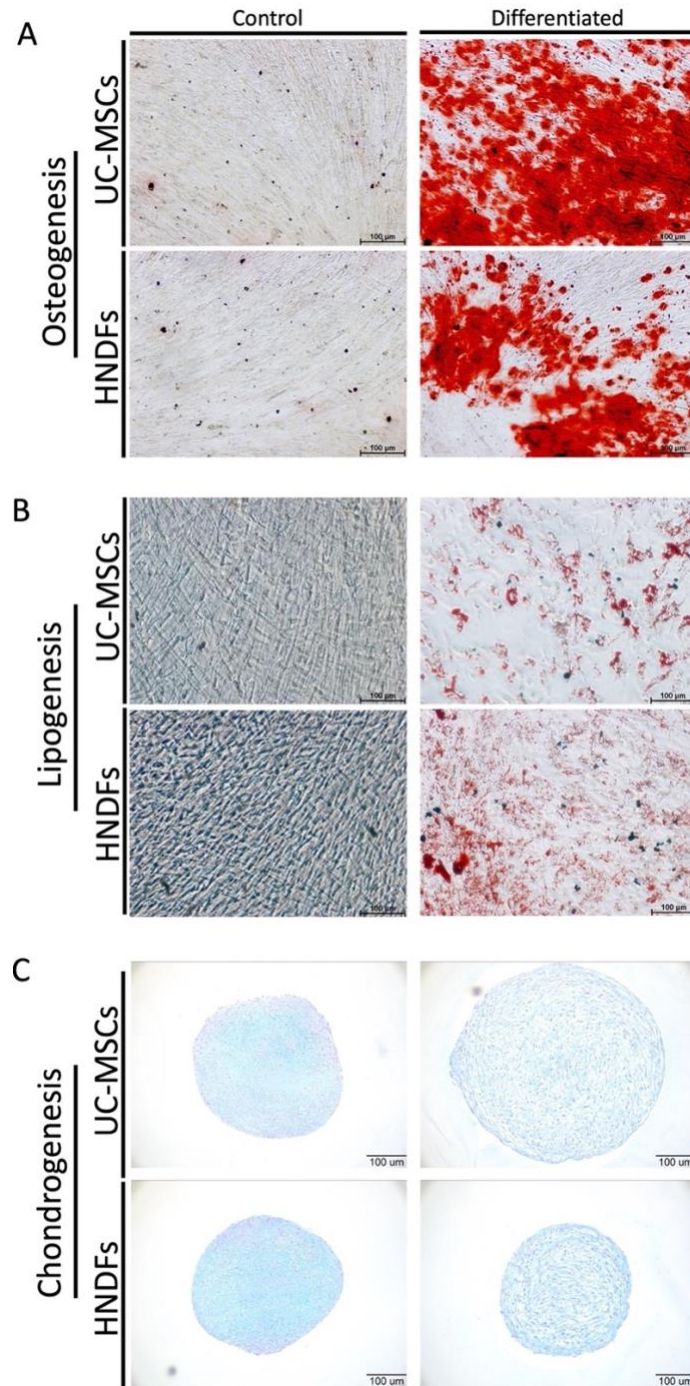
Statistical analyses were performed using GraphPad Prism version 9.0.0 (GraphPad Software, Inc., La Jolla, CA).

References

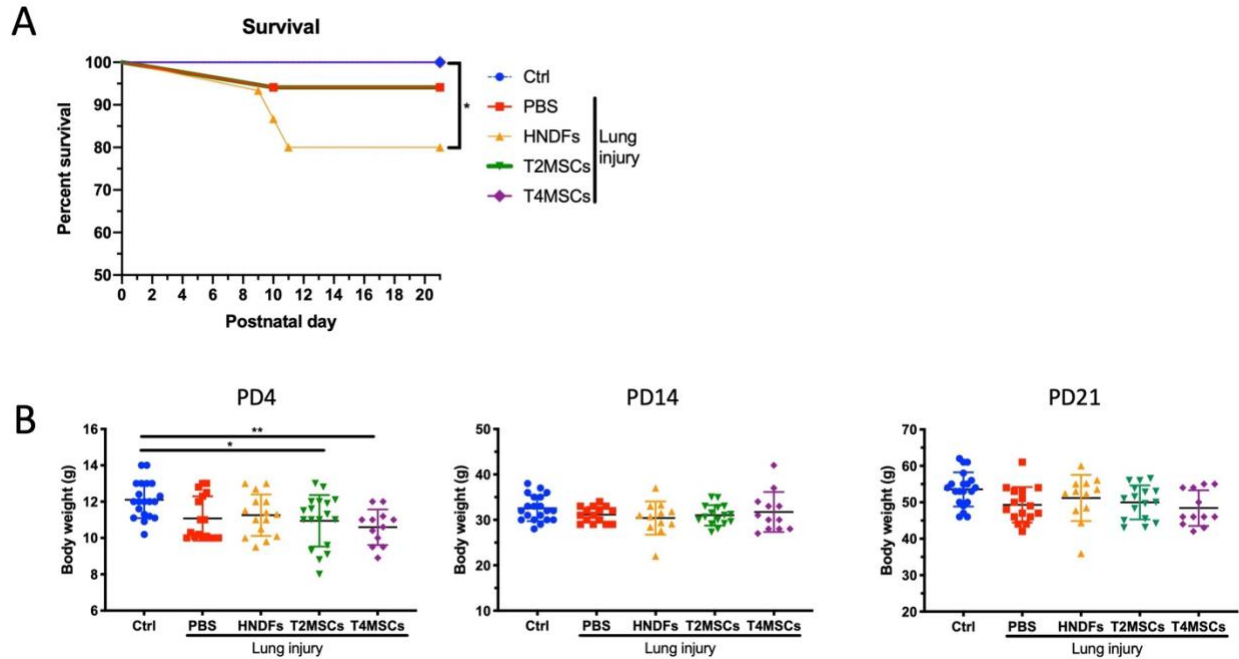
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2.7.2 Supplementary figures

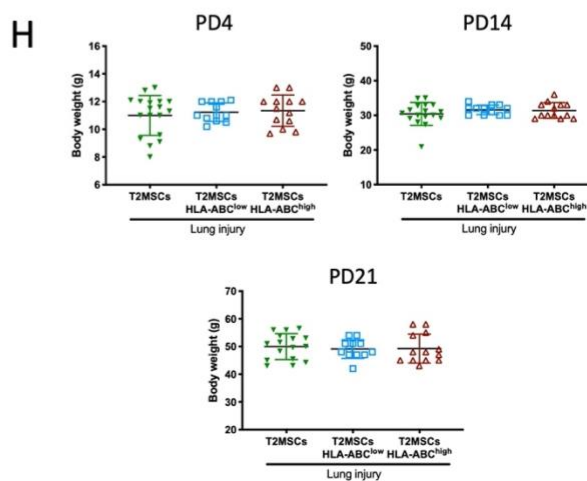
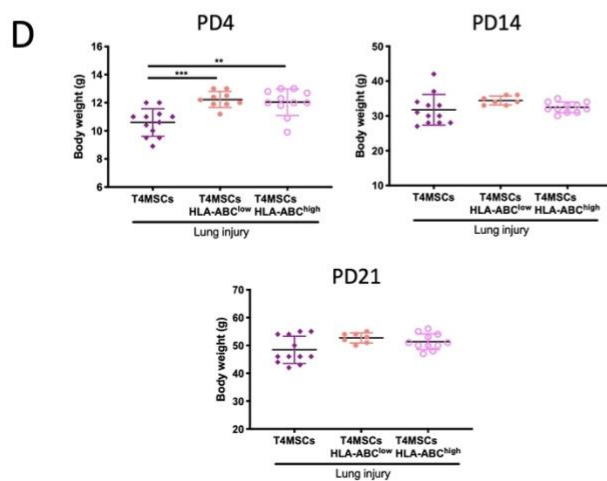
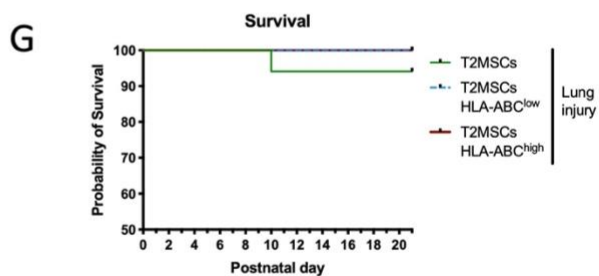
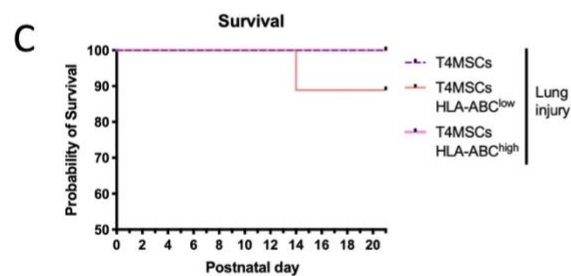
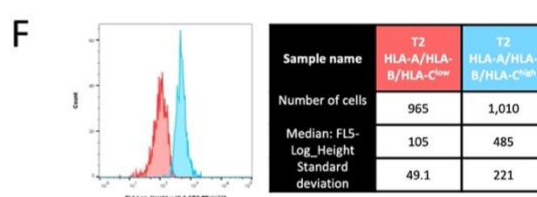
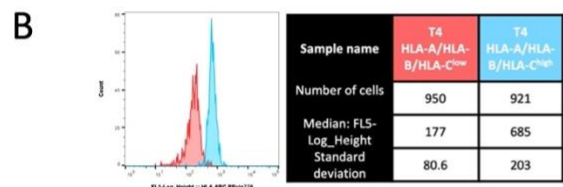
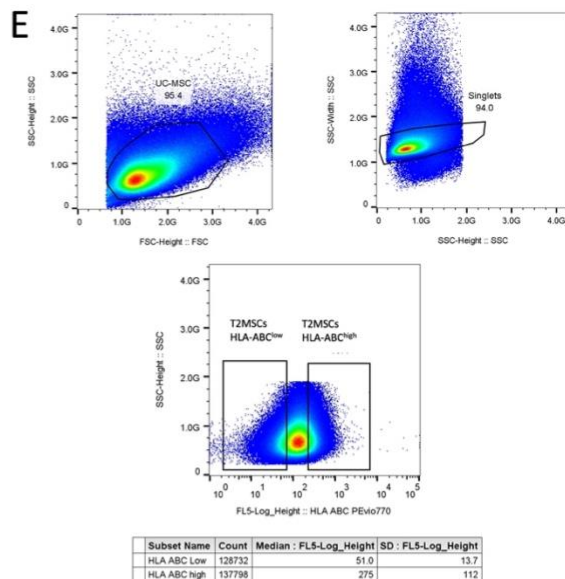
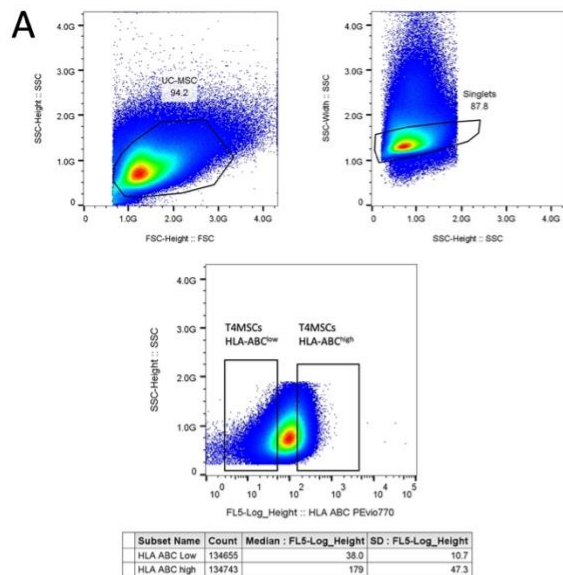


Supplementary Figure 2.1 UC-MSCs and HNDFs show tri-lineage differentiation potential. Representative images of PN3 UC-MSCs and HNDFs stained with **A)** Alizarin Red S staining for osteogenesis in control and differentiated cells, **B)** Oil Red O staining for adipogenesis in control and differentiated cells, and **C)** Alcian Blue staining for chondrogenesis in control and differentiated cells.



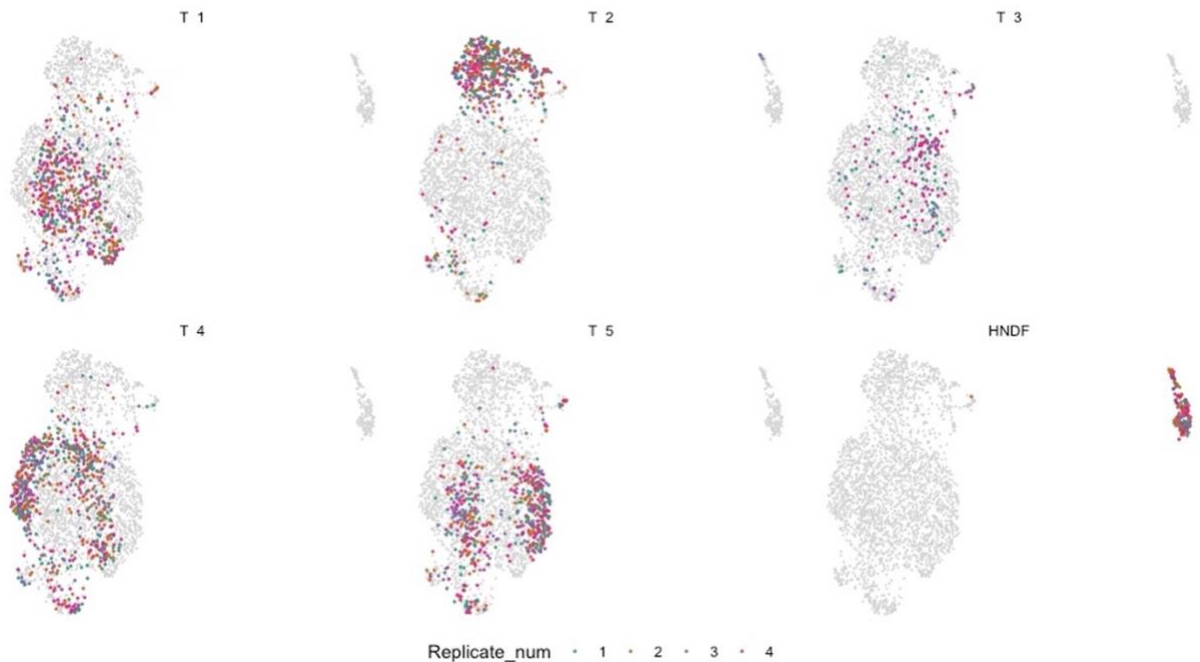
Supplementary Figure 2.2 Survival and body weight of normoxic and hyperoxic pups injected with PBS (vehicle), HNDFs, T2MSCs or T4MSCs.

A) Kaplan-Meier survival curve (at PD4, Ctrl; n=20, PBS; n=17, HNDFs; n=15, T2MSCs; n=18, T4MSCs; n=12, and at PD21, Ctrl: n=20, PBS; n=16, HNDFs: n=12, T2MSCs: n=17, T4MSCs: n=12). **B)** BW of normoxic pups and hyperoxic pups at PD4 (Ctrl: n=20, PBS; n=17, HNDFs: n=15, T2MSCs: n=18, T4MSCs: n=12), PD14 (Ctrl: n=20, PBS: n=16, HNDFs: n=11, T2MSCs: n=17, T4MSCs: n=11) and PD21 (Ctrl: n=20, PBS: n=16, HNDFs: n=11, T2MSCs: n=17, T4MSCs: n=11). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels except panel **A** for which log-rank (Mantel-Cox) test was used. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.



Supplementary Figure 2.3 HLA-A, HLA-B, and HLA-C high and low expression study.

A) HLA-A, HLA-B, and HLA-C high and low populations from donor T4 UC-MSCs were isolated by FACS. 13.36% of all cells were isolated for the HLA-ABC^{low} population and 13.38% of all cells were isolated for the HLA-ABC^{high} population. **B)** Flow cytometry histogram showing the expression of HLA-ABC by both cell populations isolated in **A** following 24 h in culture. In red is HLA-ABC^{low} subpopulation and in blue is HLA-ABC^{high} subpopulation. Number of cells analyzed, median, and STD are shown in the table on the right for both cell populations. **C)** Kaplan-Meier survival curve (at PD4, T4MSCs: n=12, T4MSCs HLA-ABC^{low}: n=9, T4MSCs HLA-ABC^{high}: n=11, at PD21, T4MSCs: n=12, T4MSCs HLA-ABC^{low}: n=8, T4MSCs HLA-ABC^{high}: n=11). **D)** BW of normoxic pups and hyperoxic pups at PD4, PD14 and PD21 (at PD4, T4MSCs: n=11, T4MSCs HLA-ABC^{low}: n=9, T4MSCs HLA-ABC^{high}: n=11, at PD14, T4MSCs: n=11, T4MSCs HLA-ABC^{low}: n=10, T4MSCs HLA-ABC^{high}: n=11, at PD21, T4MSCs: n=11, T4MSCs HLA-ABC^{low}: n=8, T4MSCs HLA-ABC^{high}: n=11). **E)** HLA-A, HLA-B, and HLA-C high and low populations from donor T2 UC-MSCs were isolated by FACS. 12.06% of all cells were isolated for the HLA-ABC^{low} population and 13.29% of all cells were isolated for the HLA-ABC^{high} population. **F)** Flow cytometry histogram showing the expression of HLA-ABC by both cell populations isolated in **E** following 24 h in culture. In red is HLA-ABC^{low} subpopulation and in blue is HLA-ABC^{high} subpopulation. Number of cells analyzed, median, and STD are shown in the table on the right for both cell populations. **G)** Kaplan-Meier survival curve (at PD4, T2MSCs: n=18, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=13, at PD21, T2MSCs: n=17, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=13). **H)** BW of normoxic pups and hyperoxic pups at PD4, PD14 and PD21 (at PD4, T2MSCs: n=18, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=13, at PD14, T2MSCs: n=17, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=13, at PD21, T2MSCs: n=17, T2MSCs HLA-ABC^{low}: n=12, T2MSCs HLA-ABC^{high}: n=13). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels except panels **C** and **G** for which log-rank (Mantel-Cox) test was used. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001.



Supplementary Figure 2.4 Technical replicate of each UC-MSC donor and HNDFs.

UMAPs showing the four technical replicates for each UC-MSC donor and HNDFs. Cells from each technical replicate for each donor and HNDFs are colored as indicated by the legend at the bottom of the figure. The four technical replicates for each donor and HNDFs are shown within the UMAP showing the four clusters that were identified based on expression profiles. Cells that do not belong to that particular donor or to HNDFs are shown in grey.

2.7.3 Supplementary tables

Supplementary Table 2.1 – Number of sequenced cells per donor or HNDFs

Sample	Cell count
T1	617
T2	565
T3	219
T4	689
T5	586
HNDF	158
Average number of cells per sample	472.3
Standard deviation	224.7
Total number of cells analyzed	2,834

Supplementary Table 2.2 – List of the top 50 genes differentially expressed in the four clusters*

Cluster 0	Cluster 1	Cluster 2	Cluster 3
DIRAS3	SERPINE2	LRRC75A	MMP3
XIST	TIMP3	DDIT3	KRT7
STMN1	COL3A1	MTRNR2L12	IGFBP31
PTTG1	TIMP1	AC092069.1	CXCL12
H2AFZ	TFPI2	FTH11	S100A4
KRT18	HLA-C	ATF3	PRRX2
HMGB1	HLA-A	HSPA5	HLA-B1
PTMA	COL1A2	FTL	CRYAB
CCNB1	IGFBP7	EEF1A1	POSTN
HMG2	TM4SF1		THBS1
KRT8	SPARC		OXTR
RANBP1	RPS4Y1		COL6A1
CENPW	NDUFA4L2		ELN
RPL22L1	IGFBP5		IGFBP51
UBE2S	SAT1		PSG5
CENPF	CXCL8		COL6A2
NASP	COL1A1		KISS1
PCLAF	MALAT1		TNFRSF11B
CENPV	TAGLN		MMP1
CDKN3	B2M		ID1
CSRP2	LOX		B2M1
SRSF2	CDKN1A		MYLK
CKS1B	IGFBP3		C12orf75
MAD2L1	BGN		GREM1
TYMS	NUPR1		HLA-A1
COL8A1	IFITM3		APLP2
FABP4	PPIC		RPS4Y11
HSPD1	P4HA2		CTGF1
ANP32B	SPON2		RAB3B
TK1	COL5A1		NEAT11
HMGB3	CLTB		PDLIM1
HMGB2	CDH11		ARID5B
KRT19	NEAT1		COL6A3
ANP32E	IGFBP6		CAV1
PFKP	S100A11		RGMB
JPT1	SQSTM1		PRPS1

HNRNPA2B1	KRTAP2-3		LBH
TUBA1B	RALA		CITED2
HNRNPD	MIR4435-2HG		MGST1
CDC20	CTSB		WNT5B
HSP90B1	DCN		MGLL
TPX2	ACTG2		GAL
SMC2	THY1		TGM2
HSP90AA1	MT2A		ARHGAP18
HNRNPC	P4HA1		AEBP1
NAP1L1	CXCL6		PRRX1
FABP5	PTGS2		TIMP11
DKK 1.00	SCRG1		LDHA
CCNB2	CST3		SERINC2
DIRAS4	HLA-B		PPP1R14A

*Genes are shown from highest avg_logFC. All genes have a *p* value < 0.05.

Supplementary Table 2.3 – List of the top 10 GO terms characteristic of each cluster*

Cluster 0	Cluster 1	Cluster 2	Cluster 3
cell division	negative regulation of cell proliferation	positive regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress	extracellular matrix organization
mitotic nuclear division	cell adhesion	endoplasmic reticulum unfolded protein response	cell adhesion
cell proliferation	extracellular matrix organization	intracellular sequestering of iron ion	negative regulation of apoptotic process
mRNA splicing, via spliceosome	response to drug	ER overload response	immune response
nucleosome assembly	oxidation-reduction process	iron ion transport	positive regulation of gene expression
G2/M transition of mitotic cell cycle	immune response	cellular iron ion homeostasis	response to drug
cell-cell adhesion	angiogenesis		positive regulation of cell proliferation

response to drug	platelet degranulation		response to hypoxia
negative regulation of transcription, DNA-templated	cellular protein metabolic process		collagen catabolic process
gene expression	negative regulation of apoptotic process		extracellular matrix disassembly

*Go terms are shown from highest count of genes number. All GO terms have a *p* value < 0.05.

Supplementary Table 2.4 – List of the top 60 genes differentially expressed in progenitor-like UC-MSCs and non-therapeutic cells*

Progenitor-like UC-MSCs (T1, T3, T4 and T5)	Non-therapeutic cells (T2 and HNDs)
DIRAS3	SERPINE2
XIST	IGFBP3
HMG2	TIMP3
UBE2S	TIMP1
STMN1	HLA-A
KRT18	IGFBP5
CENPF	MMP3
KRT8	HLA-C
PTTG1	RPS4Y1
MKI67	COL3A1
HMGB1	IGFBP7
PTMA	COL1A2
H2AFZ	MALAT1
RPL22L1	HLA-B
HIST1H4C	B2M
HMGB2	SPARC
CCNB1	NEAT1
CDKN3	COL1A1
CSRP2	TFPI2
PCLAF	TM4SF1
ANP32E	KRT7
TPX2	SAT1
NR2F2	LOX
COL8A1	BGN
CENPW	POSTN
CENPV	NDUFA4L2
HNRNPC	IFITM3
ASPM	MIR4435-2HG
TK1	CXCL12
CKS1B	CXCL8
RANBP1	COL6A1
ALCAM	COL6A2
BIRC5	CLTB

SRSF2	TAGLN
PHF19	NUPR1
HMGN1	CTGF
SMC4	CTSB
MAD2L1	CDKN1A
NAP1L1	MT-CYB
HMGB3	SPON2
TMPO	MT-ND3
MAP1B	PPIC
HSP90B1	P4HA2
TYMS	SQSTM1
FABP4	COL5A1
NASP	PRRX2
CENPE	PTGS2
DLGAP5	CDH11
RRM2	FTH1
CDC20	IGFBP6
DEK	EIF1AY
ACTG1	CST3
PFKP	ATP6AP2
CLDN11	CRYAB
ANP32B	S100A11
SET	DCN
JPT1	BRI3
EIF1AX	PSAP
HNRNPH3	SERF2
CRIM1	CYSTM1

*Genes are shown from highest avg_logFC. All genes have a p value < 0.05 .

CHAPTER 3: Single-cell transcriptome of term and preterm umbilical cord mesenchymal stromal cells highlights a neonatal lung injury treatment

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3.1 Abstract

Rationale: Bronchopulmonary dysplasia (BPD), a chronic lung disease, is the most severe and common complication of extreme prematurity (<28 weeks of gestation) responsible for infant death. Umbilical cord-derived MSCs (UC-MSCs) represent the opportunity for an autologous BPD treatment as UC-MSCs have been shown to improve lung function and structure in experimental BPD. However, characterization and repair capacity evaluation of UC-MSCs derived from donors with pregnancy-related complications associated with prematurity remain absent. **Objectives:** Investigate the phenotypic diversity of UC-MSCs and determine if pregnancy-related complications (preeclampsia and chorioamnionitis) alter therapeutic effects of UC-MSCs in experimental BPD. **Methods:** Transcriptome of UC-MSCs derived from 21 term, preterm donors, and human neonatal dermal fibroblasts (HNDFs, control), were investigated by single-cell RNA sequencing, and correlated to their therapeutic effect in experimental BPD. Using publicly available neonatal lung single-nuclei RNA sequencing data, we determined putative communication networks between UC-MSCs and resident lung cell populations. **Measurements and main results:** Most UC-MSCs displayed a similar transcriptome despite their pregnancy-related conditions, and mitigated hyperoxia-induced lung injury in newborn rats. Conversely, HNDFs and two preeclampsia preterm UC-MSC donors showed distinct fibroblast-like transcriptomes and did not improve hyperoxia-induced BPD. Therapeutic UC-MSCs displayed a unique transcriptome active in cell proliferation, and distinct cell-cell interactions with neonatal lung cell populations including via NEGR and NRNX pathways. **Conclusions:** Therapeutic UC-MSCs show a distinct transcriptome from non-therapeutic cells, low in ECM activity and with cell proliferation characteristics, and a unique cellular communication network. Our transcriptomic findings identified genes that may predict UC-MSCs' potency in clinical settings.

3.2 Introduction

Mesenchymal stromal cells (MSCs) have been identified as putative repair cells with pleiotropic effects. They represent a promising treatment for many diseases, including bronchopulmonary dysplasia (BPD) (1), the most common neonatal chronic respiratory disease affecting extreme preterm infants (2,3). BPD occurs following ventilator and oxygen treatment for acute failure to breathe and is characterized by impaired vascular and alveolar growth. While advances in perinatal care have contributed to the increase in survival of extreme preterm infants, limited progress has been made in reducing the incidence of BPD, and the disease still lacks a cure (3). In experimental BPD, MSC therapy improves alveolar architecture and reduces lung inflammation, pulmonary fibrosis, and pulmonary hypertension (PH) (4–8). Early phase clinical trials indicate safety and feasibility of MSC administration in preterm infants at high risk of developing BPD (9–11). However, MSC heterogeneity characteristics remain unclear, limiting clinical application (12).

MSCs can be isolated from various tissues, including the umbilical cord (UC), with an attractive advantage of non-invasive harvesting, and the resulting UC-MSCs demonstrate excellent expansion potential and superior repair capabilities (12–14). MSCs used in the clinic can be either allogeneic or autologous. To discern if autologous UC-MSC treatment should be considered for BPD, it is necessary to explore how perinatal conditions affect their repair potential. In addition, to achieve successful translation of UC-MSCs, greater molecular characterization and improved standardized methods of UC-MSCs' selection are needed. We hypothesized that UC-MSCs isolated from different donors consist of a heterogeneous population with various therapeutic potentials.

Single-cell RNA sequencing (scRNA-seq) is a powerful technique to investigate cellular heterogeneity by providing the transcriptome of individual cells in an unbiased way (15). In this study, we compared the transcriptomic profile of UC-MSCs isolated from 21 donors with various pregnancy-related complications, including preeclampsia and chorioamnionitis. We report that most UC-MSCs isolated from preterm donors with underlying complications are not transcriptionally distinct from term and preterm UC-MSCs, and display a transcriptome enriched in translation and transcription activity. Three UC-MSC donors were characterized by a fibroblast-like transcriptome. In addition, we showed that administration of preterm UC-MSCs with pregnancy-related complications and with a transcriptome related to translation and transcription activity improves lung structure, function, and signs of PH in neonatal hyperoxia-exposed rats. We confirmed the lack of therapeutic potential by preterm UC-MSCs with a fibroblast-like transcriptome. Moreover, we established a molecular signature of the therapeutic term and preterm UC-MSCs, which was enriched in genes related to proliferation and contained potential novel markers for UC-MSCs' purification for cell-therapy utilization. Finally, we identified possible cell-cell interactions between UC-MSCs and neonatal lung cell populations (NLCPs).

3.3 Materials and methods

Please refer to section 3.7.1 “Supplementary materials and methods” for detailed methods in addition to the following information.

Ethics approval

Human Research Ethics Board (Ottawa, Canada) and “Ethikkommission der Technischen Universität Dresden” (Dresden, Germany) approved human samples collection. Animal Care Committee of the University of Ottawa (OHRI-1696) approved animal experiments.

UC-MSCs isolation

UC-MSCs were isolated from five healthy term, five preterm, nine preterm with preeclampsia, one preterm with chorioamnionitis, and one small for gestational age (GA) donors (Table 3.1) as previously described (16).

scRNA-seq library preparation and sequencing

Passage number (PN) three UC-MSCs and human neonatal dermal fibroblasts (HNDfFs) (cat#CC-2509, Lonza, Hayward, CA, USA) were processed for scRNA-seq on the 10x Genomics Chromium Single Cell 3' platform (StemCore Laboratories, OHRI). Gene expression libraries were prepared and sequenced using NextSeq500 (Illumina) high-output 75-cycle runs.

scRNA-seq data processing and pipeline

Following sequencing, fastq files were generated using Cell Ranger mkfastq tool (10x Genomics). Gene expression libraries were processed using the Cell Ranger count function, aligning to the human transcriptome (GRCh38) with default parameters other than explicitly setting *--expect-cells 15000*. MULTI-seq barcode fastq files were processed using the deMULTiplex tool developed for MULTI-seq (<https://github.com/hris-mcginnis-ucsf/MULTI-seq>), providing a sample annotation for each cell barcode.

Gene expression libraries were imported into R and processed with Seurat v.4.0.1 (17). Cells containing high proportions of mitochondrial reads (>20% of the transcripts) were removed, resulting in a final total of 9,646 cells. Normalization was performed using SCTransform (18). This was also used to regress out cell cycle effects and stress to ensure that downstream clustering would not be influenced by cell cycle activity. The data were processed with principal component analysis and clustered using the Louvain algorithm (resolution=0.25) on a nearest neighbor graph derived from first 30 principal components. UMAP embeddings were generated using the RunUMAP() function on the first 30 principal components.

Animal model

Hyperoxia-induced lung injury was achieved by maintaining a hyperoxic environment (85% O₂) for neonatal Sprague Dawley (SD) rats (Charles Rivers, Saint-Constant, QC, Canada) from postnatal day (PD) zero to PD14 (5,8). At PD21 or PD35, the rats were euthanized by intraperitoneal (*i.p.*) injection of pentobarbital sodium (Euthanyl, 0.2 mL, 65 mg/mL) (cat#00141704, Bimeda-MTC, Cambridge, ON, Canada).

Administration of UC-MSCs

At PD4, hyperoxic pups received intratracheal (*i.t.*) vehicle (1× phosphate buffered saline [PBS], 25 µL) or PN3 UC-MSCs (in 25 µL PBS) at a dose of 10×10⁶ cells/kg body weight (BW).

Lung morphology

Lungs were prepared as previously described and mean linear intercept (MLI) was used for morphometry analysis (5,19).

Statistics

Values are represented as the mean $\pm$ standard deviation (STD). Outliers were identified using Grubb's test and normal distribution using Shapiro-Wilk normality test. *P* values ($P \leq 0.05$ was considered significant) were calculated with *t* test (unpaired, two-tailed), one-way ANOVA with Tukey's multiple comparison post hoc test, or log-rank (Mantel-Cox) test. All group assignments were blinded prior to analysis.

3.4 Results

3.4.1 UC-MSCs show limited transcriptomic heterogeneity

To determine if clinical features of UC-MSCs are associated with a distinct transcriptome, UC-MSCs from five term donors (abbreviated as T1 to T5), five preterm donors (P1 to P5), five preterm with preeclampsia donors (PP1 to PP5), four late preterm (36 weeks to 36 weeks + 4 days gestational age [GA]) with preeclampsia donors (LPP1 to LPP4), one preterm with chorioamnionitis donor (PC1), and one small for gestational age donor (SGA1) were analyzed by scRNA-seq (Table 3.1). To determine transcriptomic differences between UC-MSCs and other plastic-adherent mesenchymal cells without therapeutic effect in experimental BPD (8), HNDFs were also analyzed. After quality control, 9,646 cells were sequenced (Suppl. Table 3.1).

Seven cell populations (clusters 0 to 6) were identified based on mRNA expression profiles (Fig. 3.1A, B). Most UC-MSCs clustered together in clusters 0, 1, and 5 (referred to as "main UC-MSCs clusters"), showing that UC-MSCs from donors with different clinical features have only limited transcriptomic heterogeneity (Fig. 3.1B, C). Also, three subpopulations (clusters 2, 3, and

Table 3.1 – Characteristics of UC-MSC donors

Donor	Gestational age (weeks + days)	Birth weight (g)	Sex	Maternal age (years)	Delivery	Associated perinatal condition	BPD (y/n/unk)*
T1	39 + 5	3470	Male	24	C-section	None	n
T2	39 + 3	4490	Male	34	C-section	None	n
T3	39 + 1	2635	Female	39	C-section	None	n
T4	39 + 4	3455	Female	38	C-section	None	n
T5	37 + 2	2385	Female	23	C-section	None	n
P1	25 + 2	655	Female	17	C-section	None	y
P2	27 + 0	1210	Female	25	C-section	None	n
P3	28 + 1	1150	Male	34	C-section	None	n
P4	31 + 3	1895	Male	40	C-section	None	n
P5	24 + 4	595	Male	31	C-section	None	y
PP1	28 + 5	985	Male	26	C-section	Preeclampsia	n
PP2	24 + 5	465	Female	28	C-section	Preeclampsia	n
PP3	31 + 1	1310	Male	39	C-section	Preeclampsia	n
PP4	31 + 4	1310	Female	30	C-section	Preeclampsia	n
PP5	30 + 0	1010	Male	28	C-section	Preeclampsia	unk
LPP1	36 + 0	2860	Female	37	C-section	Preeclampsia, placental abruption	n
LPP2	36 + 0	2155	Male	26	C-section	Preeclampsia	unk
LPP3	36 + 4	2445	Female	38	Vaginal delivery	Preeclampsia	unk
LPP4	36 + 1	3010	Female	33	C-section	Preeclampsia	unk
PC1	25 + 5	1110	Male	38	C-section	Chorio-amnionitis	n
SGA1	36 + 4	2120	Male	19	Vaginal delivery	None	unk

*y = yes, n = no, unk = unknown

4) were mostly composed of cells originating from one donor only (LPP3, T2, and PP1, respectively) (Fig. 3.1B, C). HNDfFs, control cells, formed a unique population (cluster 6) (Fig. 3.1B, C).

By analyzing the expression of endothelial (*PECAMI*), epithelial (*EPCAM*), mesenchymal (*COL1A1*), pericyte (*RGS5*), immune (*PTPRC*), and red blood cell (*HBA-A1*) markers, we determined that only the mesenchymal marker *COL1A1* was expressed (Fig. 3.1D). In line with the cell surface markers for MSCs (20), we confirmed that both UC-MSCs and HNDfFs expressed

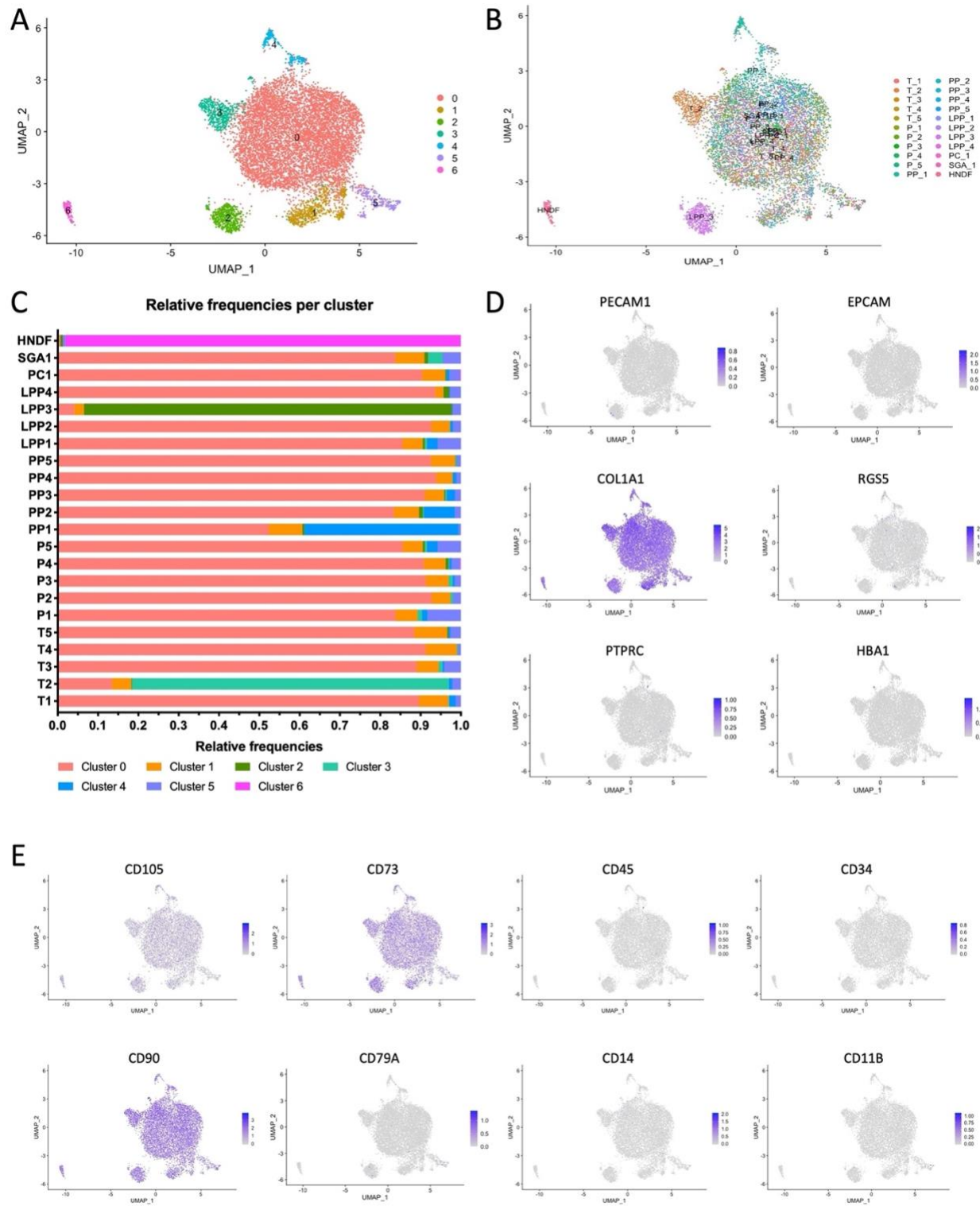


Figure 3.1 scRNA-seq analysis of UC-MSCs isolated from term and preterm donors, as well as HNDFs.

A) UMAP plot of scRNA-seq data showing the seven clusters identified based on mRNA expression profiles. Cell clusters are colored as indicated by the legend. **B)** UMAP plot showing the distribution of cells from each donor and HNDFs within the clusters. Cells from each donor and HNDFs are colored as indicated by the legend. **C)** Relative contribution of cells to the seven clusters for each donor and HNDFs. Clusters are colored as indicated by the legend on the right of the graph. **D)** UMAP plot showing expression levels of canonical markers for endothelial (*PECAMI*), epithelial (*EPCAM*), mesenchymal (*COL1A1*), pericyte (*RGS5*), immune (*PTPRC*), and red blood cell (*HBA-A1*) populations. The intensity of expression is indicated by the purple color. **E)** UMAP plot showing the expression levels of ISCT MSC markers *CD105*, *CD73*, *CD90*, *CD79A*, *CD45*, *CD34*, *CD14*, and *CD11B* (no graphs available for *CD19* and *HLA-DR*, as the cells completely lacked the expression). Expression levels in UMAP plots are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to log-transformed UMIs per 10K.

CD73, *CD90*, and *CD105*, and that *CD14*, *CD11B*, *CD34*, *CD45*, *CD79A*, *CD19*, and *HLA-DR* expression was absent (Fig. 3.1E). Also, all UC-MSCs and HNDFs differentiated into adipocytes, chondrocytes, and osteoblasts (representative images shown in **Chapter 2**, Suppl. Fig. 2.1A-C).

Based on differentially expressed genes (DEGs), the transcriptomic profile specific for each cluster (Suppl. Table 3.2, Fig. 3.2A) and associated gene ontology (GO) terms (Suppl. Table 3.3, Fig. 3.2B) were identified. Among enriched GO terms in the main UC-MSC clusters were those associated with sarcomere organization, cell division, and histone exchange (cluster 0), translational processes (cluster 1), and transcription regulation (cluster 5) (Fig. 3.2B). Interestingly, clusters 2, 3, and 4 shared a number of terms with HNDFs in cluster 6, including ECM organization, collagen catabolic process, antigen processing, and MHC class I exogenous peptide antigen presentation and other fibroblast-specific terms (Fig. 3.2B).

Additional investigation of UC-MSC heterogeneity isolated from term compared to preterm donors without other underlying pathologies was achieved by re-clustering UC-MSCs from donors T1 to T5 and P1 to P5 (Suppl. Fig. 3.1A-C). Only the transcriptome of UC-MSCs from donor T2 was significantly different, and UC-MSCs from all other term and preterm donors

showed a similar transcriptomic profile (Suppl. Fig. 3.1D, E). Further investigation concerning the difference in transcriptome of donor T2 compared to the other term donors is discussed in **Chapter 2** of this thesis and revealed a lack of therapeutic effect by T2 UC-MSCs in experimental BPD.

To further investigate the differences of UC-MSCs isolated from preterm compared to preterm donors with preeclampsia or chorioamnionitis, expression profiles of UC-MSCs from donors P1 to P5, PP1 to PP5, and PC1 were combined and re-clustered (Suppl. Fig. 3.2A-C). Only the UC-MSCs transcriptome from donor PP1 was significantly different, and UC-MSCs from all the other donors showed a similar transcriptome (Suppl. Fig. 3.2D, E).

We showed that cultured UC-MSCs from various donors have limited heterogeneity and form distinct subpopulations from HNDFs.

3.4.2 UC-MSCs isolated from term and preterm donors improve lung function, structure, and PH

To compare the lung protective effect of UC-MSCs isolated from term and preterm donors, *i.t.* administration was performed in hyperoxia-exposed rat pups at PD4 with either PBS (vehicle control) or UC-MSCs derived from donor T3 or P5, localizing primarily in the main UC-MSC clusters (Fig. 3.3A). Survival was significantly decreased (74.3%) at PD21 in control hyperoxia-exposed pups compared to the normoxia control group. Treatment with UC-MSCs from donors T3 and P5 increased survival to 93.3% and 88.9%, respectively (Fig. 3.3B). The lungs of control hyperoxia-exposed pups displayed larger and fewer alveoli than the normoxia-exposed pups (Fig. 3.3C, D). Treatment of hyperoxia-exposed pups with UC-MSCs from both donors decreased alveolar size compared to those injected with PBS (Fig. 3.3C, D).

Figure 3.2 Differentially expressed genes (DEGs) and enriched gene ontology (GO) terms for each cell cluster.

A) Dot plot depicting relative expression levels of the top 30 DEGs for clusters 2 to 7 or the full list of DEGs for clusters 0 and 1. The intensity of expression is indicated by the purple color, and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. **B)** Selected enriched GO terms for each cluster. All terms are significantly enriched (p value < 0.05). Expression levels in Dot plots are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to log-transformed UMIs per 10K.

Pressure-volume (PV)-loop FlexiVent measurement was used to assess lung function (Fig. 3.3E). Significant differences in delivery volume at 30 cmH₂O, and in static compliance, inspiratory capacity, and hysteresis, between the normoxia and hyperoxia PBS group were observed, and T3 and P5 UC-MSCs treatment of hyperoxia-exposed pups improved lung function (Fig. 3.3E-G, Suppl. Fig. 3.3A-C).

Hyperoxia exposure significantly decreased pulmonary arterial acceleration time/pulmonary arterial ejection time (PAAT/PAET) ratio and right ventricular hypertrophy (RVH), whereas T3 and P5 UC-MSC treatment of hyperoxia-exposed pups increased PAAT/PAET ratios and prevented RVH (Fig. 3.3H, I and Suppl. Fig. 3.3D).

Pathologic features of BPD include arrest in pulmonary vascular development. Lung Ct scans of barium-injected arteries showed decreased pulmonary vessel volume in control hyperoxia-exposed pups (Fig. 3.3J). T3 and P5 UC-MSC treated pups showed increased pulmonary vessel volume (Fig. 3.3J).

UC-MSCs isolated from both term and preterm donors prevent neonatal hyperoxia-induced lung injury and PH.

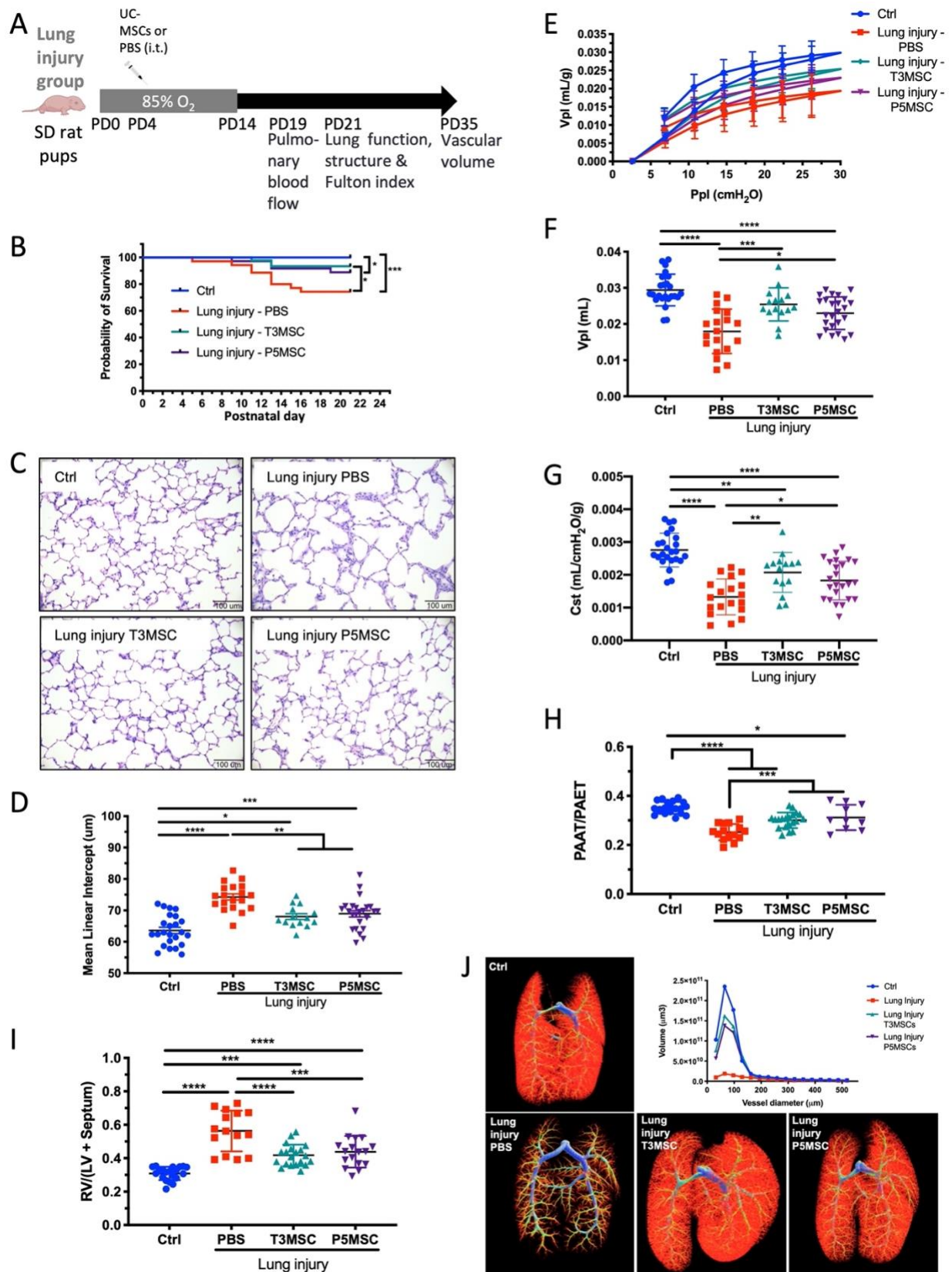


Figure 3.3 Treatment with preterm UC-MSCs reversed the hyperoxia-induced effects on the developing lung in a BPD rat model.

A) Schematic representation of the hyperoxia-induced lung injury model in neonatal rodents, as well as injection regimen and endpoints. Newborn SD rat pups were exposed to 85% O₂ until PD14 and kept under normoxic conditions until harvest. At PD4, PBS or UC-MSCs were administered (*i.t.*). PAAT/PAET was assessed at PD19. Left lung histology, lung function, and Fulton's index were assessed at PD21, and vascular volume was assessed at P35. **B)** Kaplan-Meier survival curve (at PD4 Ctrl: n=48, PBS: n=49, T3MSC: n=29, P5MSC: n=36, at PD21 Ctrl: n=48, PBS: n=36, T3MSC: n=26, P5MSC: n=32). **C)** Representative images of lung histology stained with H&E from the normoxic group (Ctrl) and lung injury (hyperoxic) groups injected with either vehicle (PBS) or UC-MSCs from donor T3 (T3MSC) or P5 (P5MSC). **D)** Graph depicting the MLI (Ctrl: n=24, PBS: n=19, T3MSC: n=14, P5MSC: n=24). **E)** BW-adjusted PV-loops are shown as the mean volume of all animals within one group at each pressure point (Vpl: volume plethysmography, Ppl: pressure plethysmography) (Ctrl: n=24, PBS: n=19, T3MSC: n=15, P5MSC: n=25). **F)** BW-adjusted lung volume at peak pressure 30 cmH₂O extracted from the PV-loop in **E** (Ctrl: n=24, PBS: n=19, T3MSC: n=15, P5MSC: n=25). **G)** BW-adjusted static compliance (Cst) extracted from the Salazar-Knowles equation (Ctrl: n=24, PBS: n=19, T3MSC: n=15, P5MSC: n=25). **H)** Graph depicting PAAT/PAET ratio. PAAT and PAET were measured from echocardiography images, such as the ones shown in Suppl. Figure 3.3D (Ctrl: n=20, PBS: n=15, T3MSC: n=20, P5MSC: n=10). **I)** Fulton's index reflecting RVH (Ctrl: n=20, PBS: n=15, T3MSC: n=20, P5MSC: n=17). **J)** Micro-CT images for one animal of each experimental group, as well as quantification of vascular volume in function of vessel diameter at PD35. Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels except panel **B** for which log-rank (Mantel-Cox) test was used. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001.

3.4.3 UC-MSCs isolated from preterm donors with underlying pathologies localizing in the main UC-MSC clusters improve lung structure and function and reduce PH

To determine if transcriptionally similar UC-MSCs isolated from preterm donors $\pm$ pregnancy-related complications provide a similar therapeutic potential, the therapeutic effect of UC-MSCs from donors P5, PP2 and PC1 was assessed *in vivo*. While vehicle control hyperoxia-exposed pups' survival was significantly lower (87.5%) than normoxia-exposed pups' survival, survival of P5 and PP2 UC-MSC treated hyperoxia-exposed pups was 100%, and survival of PC1 UC-MSC treated hyperoxia-exposed pups was 92.9% (Fig. 3.4A). All UC-MSCs improved lung morphology and significantly decreased the MLI (Fig. 3.4B, C). A significant improvement in

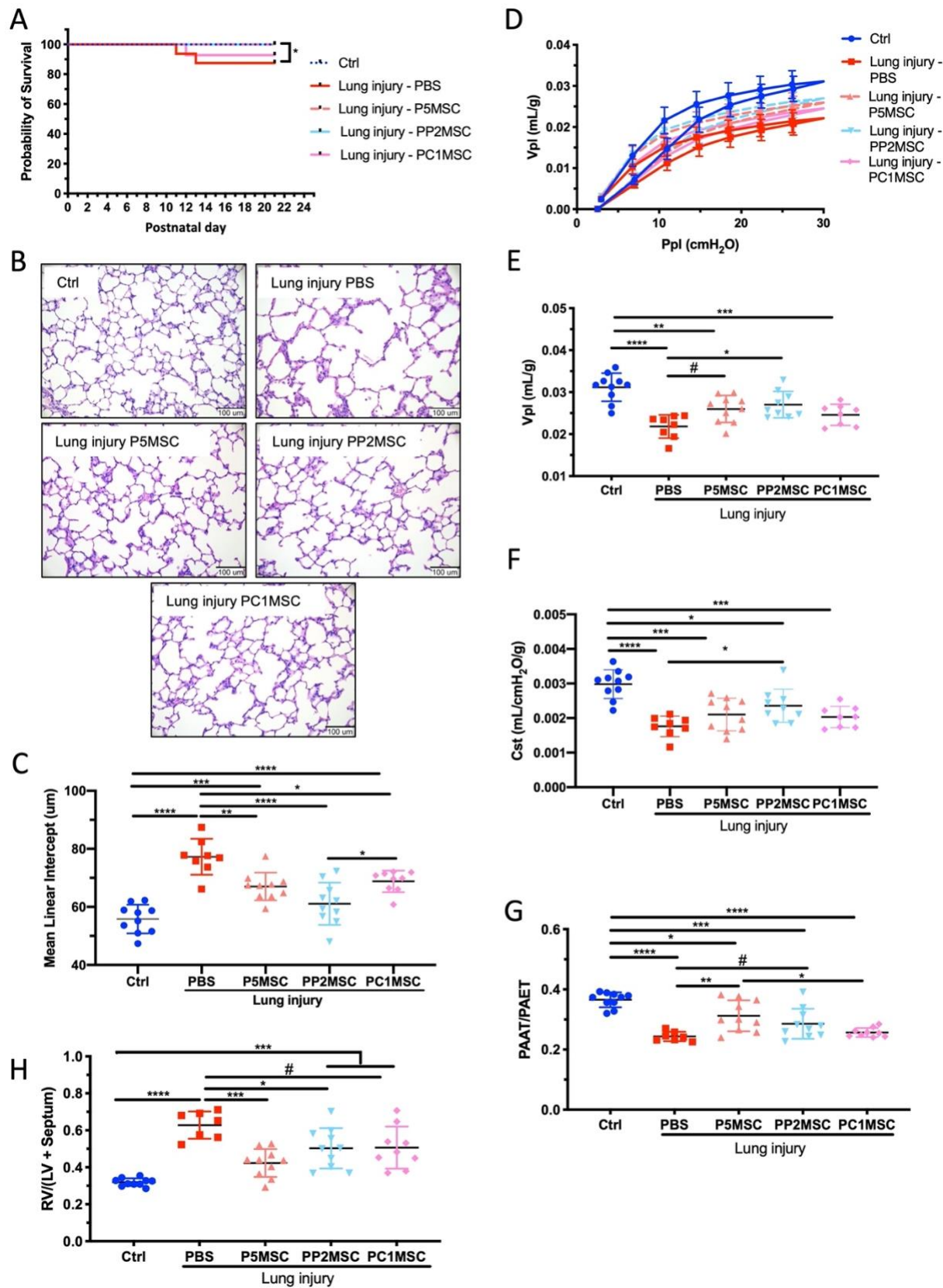


Figure 3.4 Treatment with UC-MSCs isolated from preterm donors with preeclampsia or chorioamnionitis reversed the hyperoxia-induced effects on the developing lung in a BPD rat model.

A) Kaplan-Meier survival curve (at PD4 Ctrl: n=24, PBS: n=14, P5MSC: n=10, PP2MSC: n=10, PC1MSC: n=10, at PD21 Ctrl: n=24, PBS: n=10, P5MSC: n=10, PP2MSC: n=10, PC1MSC: n=9). **B)** Representative images of lung histology stained with H&E from the normoxic group (Ctrl) and lung injury (hyperoxic) groups injected with either vehicle (PBS), or UC-MSCs from donor P5 (P5MSC) or PP2 (PPMSC) or PC1 (PC1MSC). **C)** Graph depicting the MLI (Ctrl: n=10, PBS: n=8, P5MSC: n=10, PP2MSC: n=10, PC1MSC: n=9). **D)** BW-adjusted PV-loops are shown as the mean volume of all animals within one group at each pressure point (Vpl: volume plethysmography, Ppl: pressure plethysmography) (Ctrl: n=10, PBS: n=8, P5MSC: n=10, PP2MSC: n=9, PC1MSC: n=8). **E)** BW-adjusted lung volume at peak pressure 30 cmH₂O extracted from the PV-loop in **D** (Ctrl: n=10, PBS: n=8, P5MSC: n=10, PP2MSC: n=9, PC1MSC: n=8). **F)** BW-adjusted static compliance (Cst) extracted from the Salazar-Knowles equation (Ctrl: n=10, PBS: n=8, P5MSC: n=10, PP2MSC: n=9, PC1MSC: n=8). **G)** Graph depicting PAAT/PAET ratio measured from echocardiography images (Ctrl: n=10, PBS: n=7, P5MSC: n=10, PP2MSC: n=10, PC1MSC: n=9). **H)** Fulton's index reflecting RVH (Ctrl: n=10, PBS: n=7, P5MSC: n=10, PP2MSC: n=9, PC1MSC: n=9). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels except panel **A** for which log-rank (Mantel-Cox) test was used. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001. For panels, **E**, **G**, and **H** *t* test (unpaired, two-tailed) was used to compare results between two groups only, and significant results are shown with # (*P* $\leq$ 0.05 was considered significant).

lung function and PAAT/PAET was observed with P5 and PP2 UC-MSCs (Fig. 3.4D-G, Suppl. Fig. 3.4A-C). RVH was significantly improved in hyperoxia-exposed pups treated with P5, PP2, and PC1 UC-MSCs (Fig. 3.4H).

Despite being isolated from preterm donors with pregnancy-related complications, UC-MSCs that localize to the main UC-MSC clusters provide therapeutic benefits.

3.4.4 UC-MSCs that cluster separately from the main UC-MSC clusters do not provide optimal therapeutic potential in experimental BPD

Next, we assessed the therapeutic potential of transcriptionally distinct UC-MSCs (PP1 and LPP3). No difference in survival was observed for all groups (Fig. 3.5A). Treatment with LPP3 cells improved lung morphology and significantly decreased the MLI, but no significant

difference was observed with PP1 treatment, compared to the hyperoxia control group (Fig. 3.5B, C). Treatment with neither UC-MSC donors improved lung function (Fig. 3.5D-F, Suppl. Fig. 3.5A-C) nor signs of PH (Fig. 3.5G, H).

Transcriptionally distinct UC-MSCs provide less or no protection against lung injury and development of PH in experimental BPD.

3.4.5 Therapeutic UC-MSCs are associated with a proliferative phenotype, while non-therapeutic cells express markers of senescence

To determine the molecular signature of therapeutic UC-MSCs, we performed differential expression between therapeutic UC-MSCs (main clusters) and non-therapeutic cells (clusters 2, 3, 4, and 6; composed of LPP3, T2, PP1, and HNDFs, respectively) (Fig. 3.6A). We found 98 DEGs characteristic for therapeutic cells and 100 for non-therapeutic cells (Fig. 3.6B, C). Among the genes, the MSCs marker *CD73* (*NT5E*) was characteristic of non-therapeutic cells, and a lower expression of the other MSC markers *CD90* and *CD105* was observed in therapeutic compared to non-therapeutic cells (Fig. 3.6C, Suppl. Fig.3.6). Using flow cytometry, we confirmed the expression of selected DEGs as defined by higher percentage of expressing cells among therapeutic UC-MSC donors (T3, P5, PP2, and PC1) compared to non-therapeutic cells (T2, PP1, LPP3, and HNDFs) (PTX3 and IDH2), or by higher mean fluorescence intensity (MARCKS) (Fig. 3.6D, E). In addition, therapeutic UC-MSCs had multiple enriched GO terms related to cell proliferation, cell cycle regulation, and organ regeneration, including *MKI67* (proliferation marker). Non-therapeutic cells showed terms enriched in ECM activity, aging, and negative regulation of cell growth and proliferation, including the well-established senescence marker *CDKN1A*, and the osteoblast differentiation marker *IGFBP5* (Fig. 3.6F, G). Additionally, using a

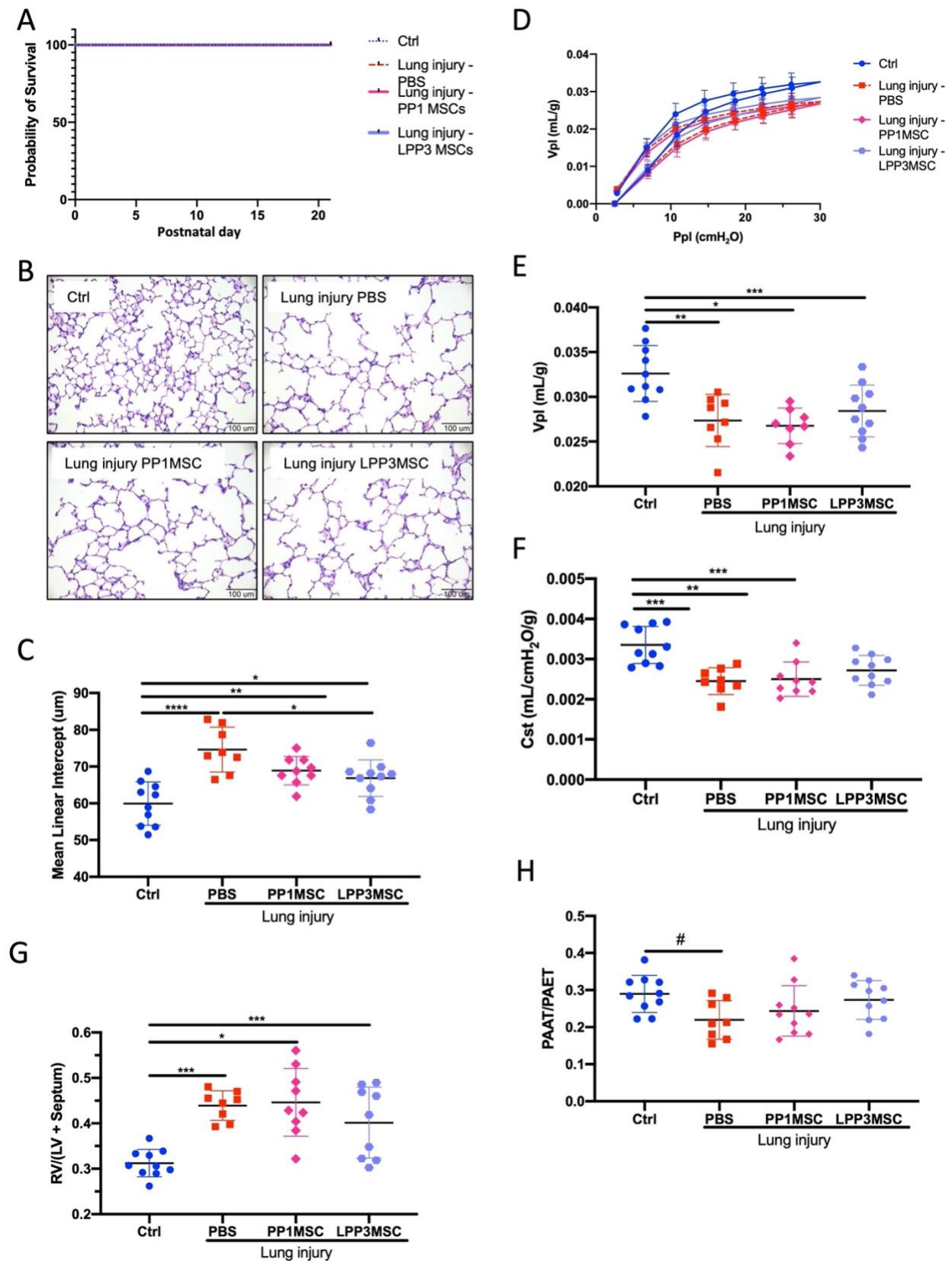


Figure 3.5 Treatment with UC-MSCs that cluster away from the main UC-MSC clusters did not provide optimal therapeutic potential in hyperoxia-induced lung injury in neonatal rats. **A)** Kaplan-Meier survival curve (Ctrl: n=12, PBS: n=8, PP1MSC: n=10, LPP3MSC: n=10). **B)** Representative images of lung histology stained with H&E from the normoxic group (Ctrl) and lung injury (hyperoxic) groups injected with either vehicle (PBS) or UC-MSCs from donor PP1 (PP1MSC) or LPP3 (LPP3). **C)** Graph depicting the MLI (Ctrl: n=10, PBS: n=8, PP1MSC: n=9, LPP3MSC: n=10). **D)** BW-adjusted PV-loops are shown as the mean volume of all animals within one group at each pressure point (Vpl: volume plethysmography, Ppl: pressure plethysmography) (Ctrl: n=10, PBS: n=8, PP1MSC: n=10, LPP3MSC: n=8). **E)** BW-adjusted lung volume at peak pressure 30 cmH₂O extracted from the PV-loop in **D** (Ctrl: n=10, PBS: n=8, PP1MSC: n=10, LPP3MSC: n=8). **F)** BW-adjusted static compliance (Cst) extracted from the Salazar-Knowles equation (Ctrl: n=10, PBS: n=8, PP1MSC: n=10, LPP3MSC: n=8). **G)** Fulton's index reflecting RVH (Ctrl: n=10, PBS: n=8, PP1MSC: n=9, LPP3MSC: n=9). **H)** Graph depicting PAAT/PAET ratio measured from echocardiography images (Ctrl: n=10, PBS: n=7, PP1MSC: n=10, LPP3MSC: n=10). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels except panel **A** for which log-rank (Mantel-Cox) test was used. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001. For panel **G**, *t* test (unpaired, two-tailed) was used to compare results between two groups only and significant results are shown with # (*P* $\leq$ 0.05 was considered significant).

combination of senescence biomarkers alongside each other, we showed their upregulation in non-therapeutic cells, suggesting a senescent phenotype (Fig. 3.6H).

We propose a novel therapeutic UC-MSC molecular signature, which is enriched in genes related to proliferative capacities, while the molecular signature of non-therapeutic cells is enriched in genes related to cellular senescence.

3.4.6 Therapeutic UC-MSCs show distinct communication networks with lung cell populations in neonates

Using CellChat and published human neonatal lung single-nuclei RNA-seq dataset (21), we predicted possible intercellular communication between UC-MSCs and NLCPs. Communication probabilities were the highest between UC-MSCs and alveolar epithelial type 1 cells (AT1s). Higher probability of interaction with all NLCPs was observed for the non-therapeutic cells, except for pulmonary neuroendocrine cells (PNECs) (Fig. 3.7A).

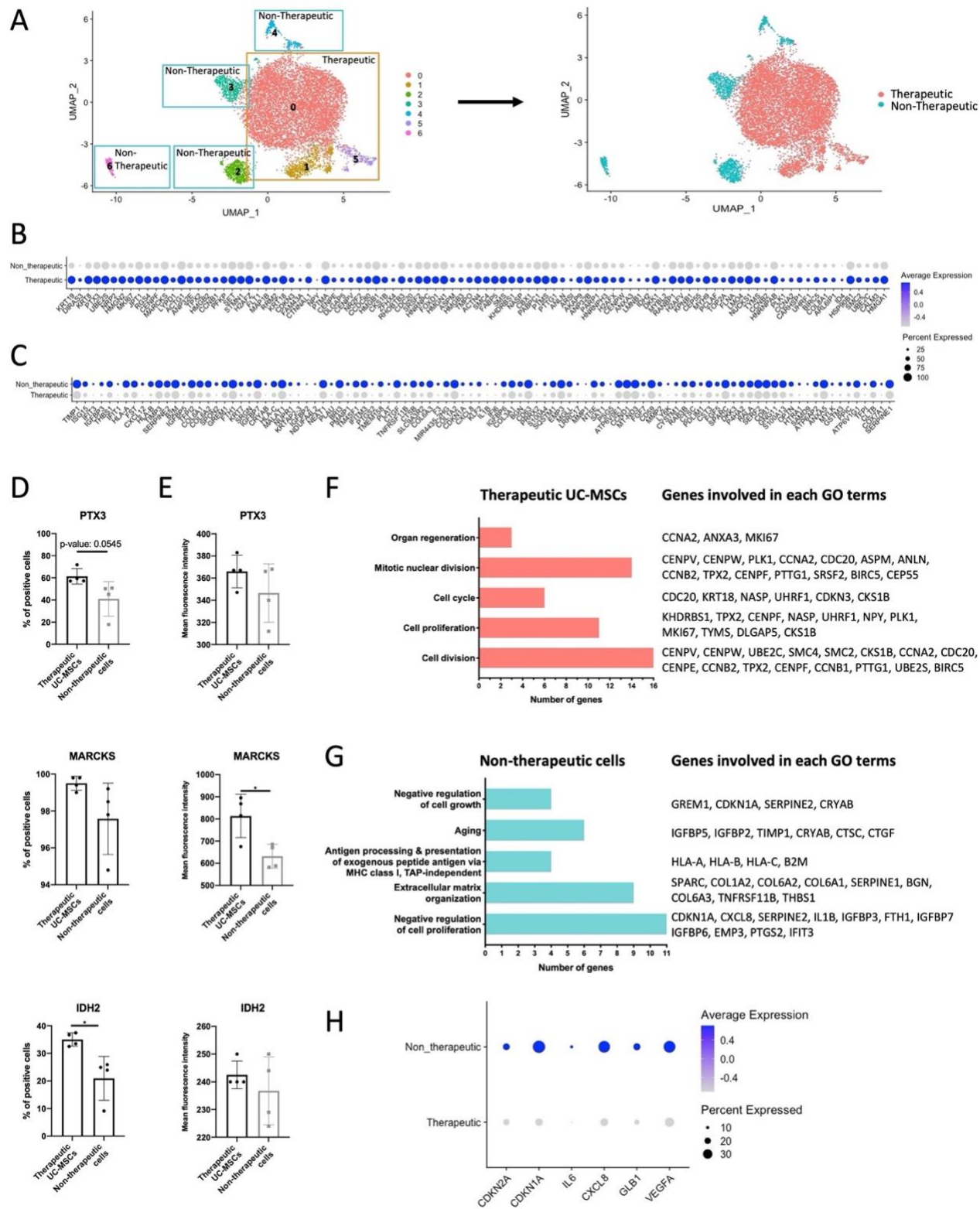


Figure 3.6 Molecular signature of therapeutic UC-MSCs and of non-therapeutic cells.

A) UMAP plot on the left showing the localization of cells that showed therapeutic potential in our BPD model (T3, P5, PP2, and PC1, localized in clusters 0, 1, and 5), as well as cells that did not show therapeutic potential (LPP3, T2, PP1, and HNDFs, localized in cluster 2, 3, 4, and 6). These cells were given a new identity, “therapeutic” or “non-therapeutic,” as shown in the UMAP plot on the right. In the UMAP plot on the left, cells from each donor and HNDFs are colored as indicated by the legend. In the UMAP plot on the right, cells are colored based on their new identity, as indicated in the legend. **B)** Dot plot depicting relative mRNA expression levels of the DEGs for the therapeutic UC-MSCs. **C)** Dot plot depicting relative mRNA expression levels of the DEGs for non-therapeutic cells. For panels **B** and **C**, the intensity of expression is indicated by the purple color and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. **D)** Percentage of positive cells expressing some of the top DEGs (PTX3, MARCKS, and IDH2) in therapeutic UC-MSCs (n=4: T3, P5, PP2, and PC1) and non-therapeutic cells (n=4: T2, PP1, LPP3, and HNDFs) detected by flow cytometry. **E)** Mean fluorescence intensity of some of the top DEGs (PTX3, MARCKS, and IDH2) in therapeutic UC-MSCs (n=4: T3, P5, PP2, and PC1) and non-therapeutic cells (n=4: T2, PP1, LPP3, and HNDFs) detected by flow cytometry. **F)** Representative GO terms of therapeutic UC-MSCs using the list of DEGs. **G)** Representative GO terms of non-therapeutic cells using the list of DEGs. For panels **F** and **G**: all terms are significantly enriched (p value < 0.05). **H)** Dot plot depicting relative expression levels of senescent markers for therapeutic and non-therapeutic cell populations. The intensity of expression is indicated by the purple color and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. Expression levels in Dot plots are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to $\log$ -transformed UMIs per 10K. For panels **D** and **E**: values are represented as the mean $\pm$ STD. P values were calculated with t test (unpaired, two-tailed). Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

CellChat detected 1,098 significant ligand-receptor pairs between therapeutic UC-MSCs and NLCPs, and 1,101 between non-therapeutic cells and NLCPs, which were further categorized into 38 signalling pathways for the therapeutic UC-MSCs and 36 for the non-therapeutic cells, with neuronal growth regulator (NEGR) and neurexin (NRXN) pathways specific to therapeutic cells only. Interactions via key pathways through which MSCs have been reported to exert their therapeutic functions, including collagen-related (Fig. 3.7B), vascular endothelial growth factor (VEGF) (Fig. 3.7C), transforming growth factor-beta (TGF β) (Fig. 3.7D) and fibroblast growth factor (FGF) (not shown) pathways were identified between therapeutic and non-therapeutic cells and various NLCPs. Multiple differences in significant ligand-receptor pairs identified for

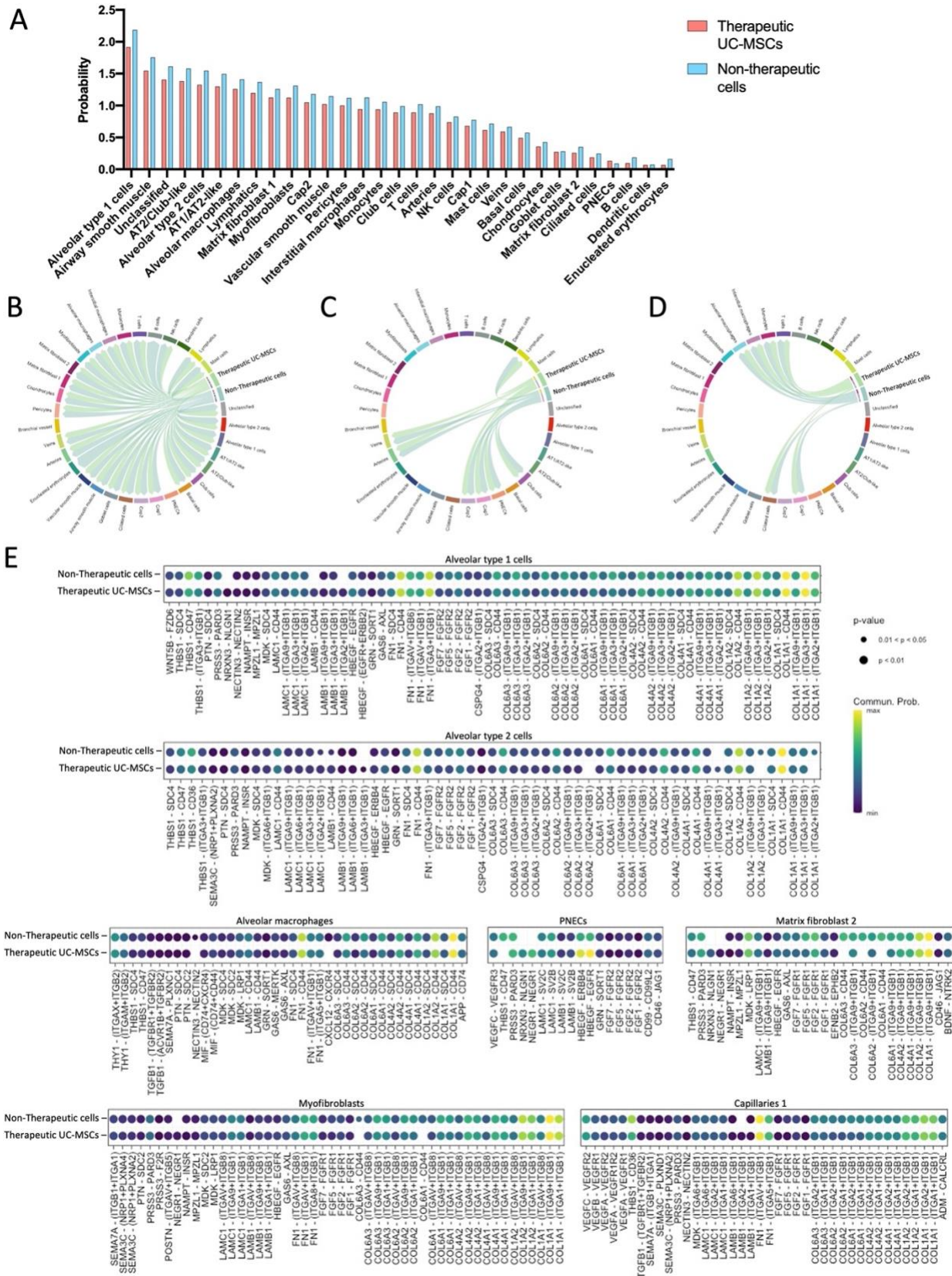


Figure 3.7 Cellchat analysis of the communication between UC-MSCs and neonatal lung cells.

A) Probability of communication between neonatal lung cell populations (NLCPs) and therapeutic UC-MSCs or non-therapeutic cells. **B)** Chord diagram of collagen pathway ligand-receptor interactions between therapeutic UC-MSCs, as well as non-therapeutic cells and NLCPs. **C)** Chord diagram of VEGF pathway interactions between therapeutic UC-MSCs, as well as non-therapeutic cells and NLCPs. **D)** Chord diagram of TGF β pathway interactions between therapeutic UC-MSCs, as well as non-therapeutic cells and NLCPs. For panels **B**, **C**, and **D**, the thickness of the line indicates the intensity of the signal. **E)** Dot plot showing all the significant ligand-receptor pairs that contribute to the signaling sending from the therapeutic UC-MSCs and non-therapeutic cells to AT1s, alveolar epithelial type 2 cells (AT2s), alveolar macrophages, PNECs, matrix fibroblasts 2, myofibroblasts and capillaries 1 cells. The dot color and size represent the calculated communication probability and p values. P values are computed from a one-sided permutation test (a cut-off of p values < 0.05 was used).

therapeutic and non-therapeutic cells were observed. Many ligand-receptor pairs were specific to therapeutic UC-MSCs and NLCPs of most relevance to BPD pathogenesis, including ligand-receptor pairs NRXN3-NLGN1, NEGR1-NEGR1, LAMB1-CD44, LAMB1-(ITGA2+ITGB1) and COL4A1-ITGA3+ITGB1 (Fig. 3.7E). Furthermore, many interactions specific to non-therapeutic cells included collagen signaling, such as COL6A1-CD44, COL6A2-CD44, and COL6A3-CD44 (Fig. 3.7E).

These data suggest multiple interactions occurring specifically between therapeutic UC-MSCs and NLCPs, potentially contributing to their therapeutic effect.

3.5 Discussion

In this study, we explored the heterogeneity and therapeutic potential of UC-MSCs isolated from multiple donors, including preterm donors with pregnancy-related complications. We determined that UC-MSCs from preterm donors with preeclampsia or chorioamnionitis, which have a transcriptome enriched in cell proliferation activity, but low in ECM processes, provide

healing effects in experimental BPD. Contrastingly, preterm UC-MSCs with a transcriptome enriched in genes related to ECM organization and senescence lacked therapeutic effects. Finally, we identified specific cellular interactions between UC-MSCs and NLCPs and identified potential DEGs, which may serve as markers for UC-MSCs isolation for clinical application.

Recently, many studies have investigated MSC heterogeneity using scRNA-seq (22–36). Most of them compared the transcriptome between MSCs isolated from different tissues. Significant differences in expression were found in genes involved in ECM, immune, matrisome, angiogenesis, antigen processing/presentation, senescence, and cell cycle activity (22,23,32,33). It was highlighted that within MSC subpopulations isolated from a specific tissue, gene expression was highly variable from cell to cell, suggesting that MSCs isolated from one source comprise a heterogenous population (22,33). Hence, studies of cell-to-cell variability in transcriptome on MSCs remain needed.

More importantly, little is known about the intrinsic characteristics and therapeutic potential of UC-MSCs isolated from preterm donors with pregnancy-related complications for BPD treatment. We performed high-throughput scRNA-seq of UC-MSCs isolated from five term donors, five preterm donors, and eleven preterm donors with underlying pathologies and non-progenitor control cells, HNDFs, and associated their transcriptome with their therapeutic potential in neonatal rat hyperoxia-mediated lung injury model.

Clusters 0, 1, and 5 (main UC-MSC clusters) were composed of most UC-MSCs, while clusters 2, 3, 4, and 6 were formed almost exclusively of single donor: LPP3, T2, PP1, and HNDFs, respectively. Thus, regardless of clinical features, following cell culture, most UC-MSCs represent a relatively homogenous cell population, with a minority of donors possessing a distinct transcriptome. The main UC-MSC clusters were significantly enriched in genes involved in cell

division, transcriptional and translational processes. Conversely, UC-MSC donors, which formed their own cluster and HNDFs, were enriched in genes related to ECM organization and collagen deposition. ECM remodeling is crucial for regulating morphogenesis of many organs including the lungs, and dysregulation of ECM content, composition, structure, and stiffness is associated with disorders such as fibrosis (37). Thus, the significant difference in expression of ECM-related genes may be key to the differences in function of cells that cluster together within the main UC-MSC clusters and those that cluster separately. In addition, UC-MSCs cannot be distinguished from HNDFs based on the minimal criteria for defining MSCs (20), showing the importance of characterizing MSCs for novel marker expression.

Previously, the conditioned media and extracellular vesicles derived from preterm UC-MSC donors were shown to improve lung structure and inflammation in hyperoxia-induced neonatal mouse lung injury (38). To our knowledge, we are the first to correlate the therapeutic effect of UC-MSCs isolated from preterm donors with underlying pathologies in experimental BPD with their transcriptome. Using a well-characterized neonatal rat lung injury model, we confirmed that administration of UC-MSCs isolated from preterm donors $\pm$ pregnancy-related complications (grouped together in the main UC-MSC clusters) protect against hyperoxia-induced injury by preserving the lung architecture and function and preventing PH. Conversely, administration of UC-MSCs isolated from preterm donors forming donor-specific subpopulations (PP1 and LPP3) and with high ECM activity reminiscent of the fibroblasts' transcriptome were unable to confer optimal protection against hyperoxia-induced injury. These data confirm our previous findings in **Chapter 2** that “progenitor-like” but not “fibroblast-like” UC-MSCs, provide an optimal therapeutic potential. This is important knowledge to consider for UC-MSC clinical translation for treatment of BPD.

Identification of DEGs between therapeutic and non-therapeutic cell populations and GO analysis confirmed enrichment of terms related to cell cycle, cell division, and cell proliferation in therapeutic cells, and enrichment of a fibroblast-like phenotype and senescence in non-therapeutic cell populations. Since all UC-MSCs were sequencing at PN3, non-therapeutic cells might enter a senescent state at an earlier PN than therapeutic UC-MSCs affecting their healing efficacy. Thus, even in early PNs, monitoring senescence appears to be particularly important.

Up- and downregulated genes within the therapeutic UC-MSC subpopulations might play critical roles in their therapeutic function. Interestingly, lower expression of MSC marker *CD105* in therapeutic cells was observed, which was previously associated with higher immune modulatory capacities, and thus favorable for MSCs to be used to treat inflammatory diseases such as BPD (39). MARCKS, a marker of our therapeutic UC-MSCs, was previously shown to play a role in multiple regenerative processes, and its expression is upregulated in optic nerve regeneration and during neuron regeneration and cardiac tissue regeneration (40). Another therapeutic UC-MSCs marker, IDH2, plays an essential role in protecting cells against oxidative stress, an important factor contributing to the pathophysiology of BPD and which impact MSC's function (41–44). MSC-derived PTX3, previously shown to help protect from hyperoxia-induced lung damage in neonatal rats through regulation of macrophage polarization (45), is also a marker of our therapeutic UC-MSCs. Thus, therapeutic UC-MSC markers might contribute to UC-MSCs' therapeutic effect.

Additionally, DEGs may serve as candidate markers for the expansion and manufacture of UC-MSC products. Flow cytometry represents a method of choice for the identification of MSCs, and using this approach, we confirmed the expression of therapeutic cell markers PTX3,

MARCKS, and IDH2. Prospectively, selecting optimal cell donors for large-scale expansion could be done by screening for a list of therapeutic markers.

Finally, UC-MSCs and NLCPs interactions were analyzed. Higher probability of interactions between PNECs and therapeutic UC-MSCs compared to non-therapeutic cells was observed. PNECs represent a rare multi-functional epithelial cell population, known for their sentinel role in the lung. PNECs have been linked to many chronic-lung diseases, including BPD. In particular, PNEC hyperplasia and increased PNEC secretory products were displayed in BPD patients (46,47). Moreover, PNECs have stem cell properties and play a role in the regenerative response to severe airway injury.

In line with their fibroblast-like phenotype, many collagen-related pathways were specific to non-therapeutic cells and NLCPs. NEGR1/NRXN pathways were specific to therapeutic UC-MSCs and NLCPs. Although the biological functions of NEGR1/NRXN pathways remain elusive, data indicate that these genes are involved in neurogenic tissue development (48). The respiratory system development seems to be closely associated with the formation of a complex neuronal network generating considerable interest in the role of neurogenic tissue in lung development. Interestingly, NEGR1 and NRXN1 were previously shown to be expressed by PNECs in human fetal lungs (49,50). Other important pathways included VEGF, critical for normal vascular development during alveolarization, and TGF β , necessary for elastogenesis in the developing lung, and AT1s flattening. However, no difference in intercellular communication within the pathways mentioned above was observed between therapeutic and non-therapeutic cells.

This study has some limitations. First, not all UC-MSC donors were tested for their therapeutic efficacy *in vivo*. Second, our study included only a single donor with chorioamnionitis.

Finally, presently we are not able to explain with certainty the cause behind the different transcriptomic profiles between donors.

In summary, to our knowledge, our study is the first to extensively dissect the transcriptome of UC-MSCs derived from preterm, and preterm donors with pregnancy-related complications at the single-cell resolution, revealing an insight into the correlation between UC-MSC molecular signature and lung repair function. Therapeutic UC-MSC includes UC-MSCs derived from preterm donors with preeclampsia and chorioamnionitis. These findings are especially important to foresee in clinical trial settings, as the proposed therapeutic molecular signature could serve as candidate markers for UC-MSCs' screening and might lead to a UC-MSC autologous treatments approach.

3.6 References

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3.7 Appendix

3.7.1 Supplementary materials and methods

UC-MSCs isolation

Using enzymatic digestion UC-MSCs were isolated after parental consent from the UC of 21 donors: five healthy term pregnancies (T1, T2, T3, T4, and T5), five preterm pregnancies (P1, P2, P3, P4, and P5), five preterm with preeclampsia pregnancies (PP1, PP2, PP3, PP4, and PP5), four late preterm (36 weeks to 36 weeks + 4 days GA) with preeclampsia pregnancies (LPP1, LPP2, LPP3, and LPP4), one preterm with chorioamnionitis pregnancy (PC1) and one SGA1 (Suppl. Table 3.1). Please refer to the isolation process described in **Chapter 2**, section 2.7.1. Culture was maintained in a cell culture incubator at 37°C with 5% CO₂ and 5% O₂ as previously described (1).

UC-MSCs differentiation assays

Please refer to **Chapter 2**, section 2.7.1.

Multiplexing individual samples for scRNA-seq

Please refer to **Chapter 2**, section 2.7.1.

scRNA-seq library preparation and sequencing

The multiplexed pool of UC-MSCs and HNDs was processed for scRNA-seq on the 10x Genomics Chromium Single Cell 3' platform (StemCore Laboratories, OHRI). The pool was processed on a single lane of the Chromium platform to target 15,000 cell yield. Gene expression libraries were prepared following the standard 10x Genomics protocol and a separate MULTI-seq

barcode library was isolated by size selection and PCR amplified as described by McGinnis *et al.* (2). Libraries were sequenced using NextSeq500 (Illumina) high-output 75-cycle runs. Gene expression libraries were sequenced to an average depth of 22,597 reads/cell and MULTI-seq barcode libraries were sequenced to a depth of approximately 1,000 reads/cell. Multiplexing was successful except for one technical replicate for samples LPP3 and SGA1 (Suppl. Fig.3.7).

Differential gene expression and gene ontology (GO) analysis

Please refer to **Chapter 2**, section 2.7.1.

Cell-cell communication analysis of UC-MSCs and neonatal lung cell populations

To perform cell-cell communication between UC-MSCs and neonatal lung cell populations, single-nucleus RNA-seq data (3) performed on non-diseases lung tissue isolated from three preterm donors were downloaded from <https://lungmap.net/>. Cell-cell communication analyses were performed using CellChat software (4) on therapeutic and non-therapeutic groups, and on all neonatal lung cell populations. Ligand-receptor interactions were identified and measured between either therapeutic or non-therapeutic cells and all lung cell types. Intercellular communication is based on the projected ligand and receptor profiles where the expression level of ligand and receptor is approximated by their geometrical mean across individual cell types. Interaction strengths between ligands and receptors are referred to as “probability” in CellChat. A cut-off of p value < 0.05 (permutation test) was used.

Animal model

Please refer to **Chapter 2**, section 2.7.1.

***In vivo* administration of UC-MSCs**

PN3 UC-MSCs were plated at 5,000 cells/cm² in T75 flask and were placed for 24 h in a cell culture incubator 37°C with 5% CO₂ and 5% O₂ prior to collection as previously described (1). At PD4, hyperoxia-exposed pups were anesthetized by inhalation of isoflurane (anaesthesia was induced and maintained with 5% and 3% of isoflurane respectively, in a mixture of 100% O₂ at 1.5 L/min), and vehicle or UC-MSCs (in 25 µl PBS) were administered *i.t.* at a dose of 10×10⁶ cells/kg BW. The experimental groups were as follows: 1) normoxia control (Ctrl), 2) hyperoxia vehicle control (PBS), 3) hyperoxia with donor UC-MSCs (T3MSCs or P5MSCs or PP2MSCs or PC1MSCs or PP1MSCs or LPP3MSCs).

Echocardiography

Please refer to **Chapter 2**, section 2.7.1.

Lung function testing, Fulton's index, histology and lung structure analysis

Please refer to **Chapter 2**, section 2.7.1.

Micro-computed tomography (micro-CT) lung vasculature analysis.

The lungs of normoxia control, hyperoxia vehicle control and hyperoxia injected with UC-MSCs from donor T3 or donor P5 rat pups were perfused at PD35 with a gelatin-barium sulfate solution to visualize the pulmonary vasculature as previously described (5). The lungs were fixed with 10% buffered formalin under constant hydrostatic pressure of 20 cmH₂O before micro-CT scanning. The lungs were imaged using the Bruker Skyscan 1272 system (Bruker micro-CT, Kontich, Belgium). Scanning was done using a Cu 0.11 m filter (100 kV, 100 mA) at a pixel size

of 16 μm . The lungs were reconstructed from the raw files using NRecon (version 1.7.3.1), and lung vascular volume measurements were obtained using CTAn (version 1.14.4.1+) (all from Bruker).

Flow cytometry

PN2 UC-MSCs (T2, T3, P5, PP1, PP2, LPP3, and PC1) and PN2 HNDFs were plated in T75 flasks and incubated as previously described (1). Twenty-four hours later, cells were lifted with 3 mL TrypLE-Express. UC-MSCs and HNDFs were fixed with 4% paraformaldehyde and permeabilized in 0.25% saponin buffer, and stained with anti-PTX3 (cat#AF1826, R&D system, Minneapolis, Minnesota, USA) conjugated with Alexa fluor 488, and anti-IDH2-PE (cat#sc-374476, Santa Cruz Biotechnology, Dallas, Texas, USA) or anti-MARCKS-Coralight 488 (cat#CL488-20661, Proteintech, Rosemont, Illinois, USA) alone. Single and unstained controls were used as needed. Flow cytometry was performed on a BD LSRFortessa cell analyzer (Beckton Dickinson Biosciences, Franklin Lakes, NJ) at the flow cytometry and cell sorting facility of the OHRI. Samples compensation was performed with BD FACSDIVA software, and data were analyzed using FlowJo v10 (FlowJo LLC, Ashland, OR).

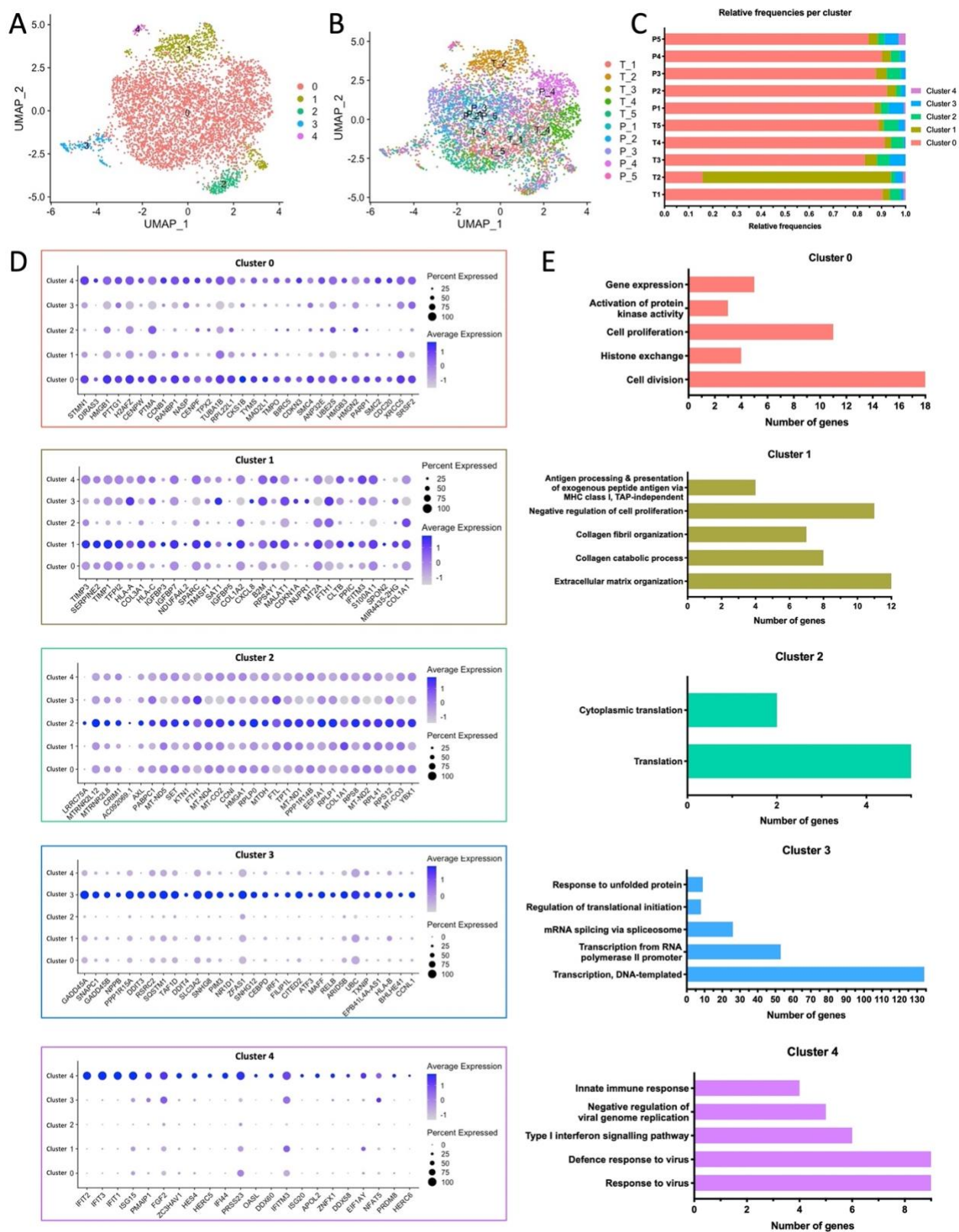
Statistics

Statistical analyses were performed using GraphPad Prism version 9.0.0 (GraphPad Software, Inc., La Jolla, CA).

References

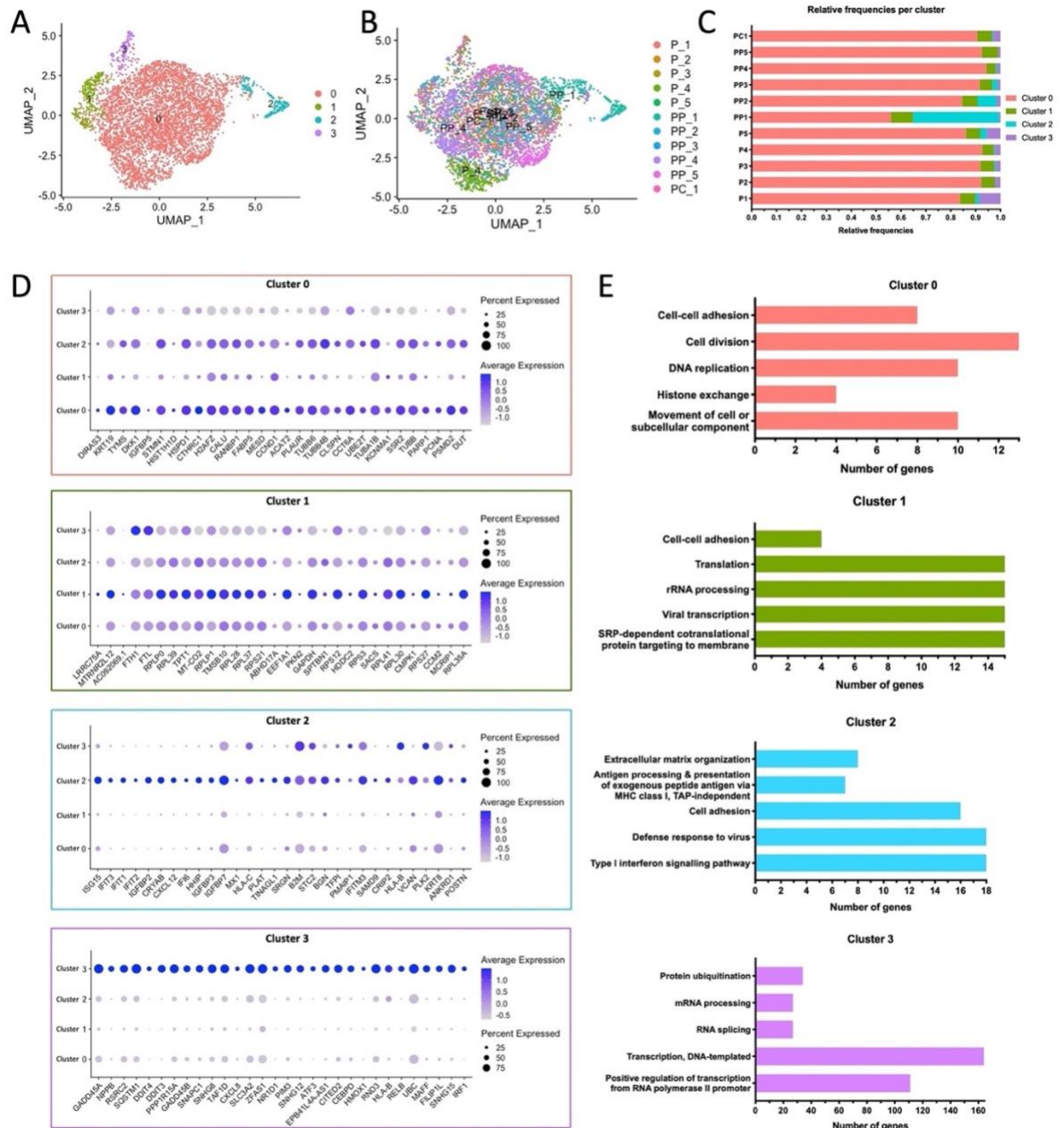
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3.7.2 Supplementary figures



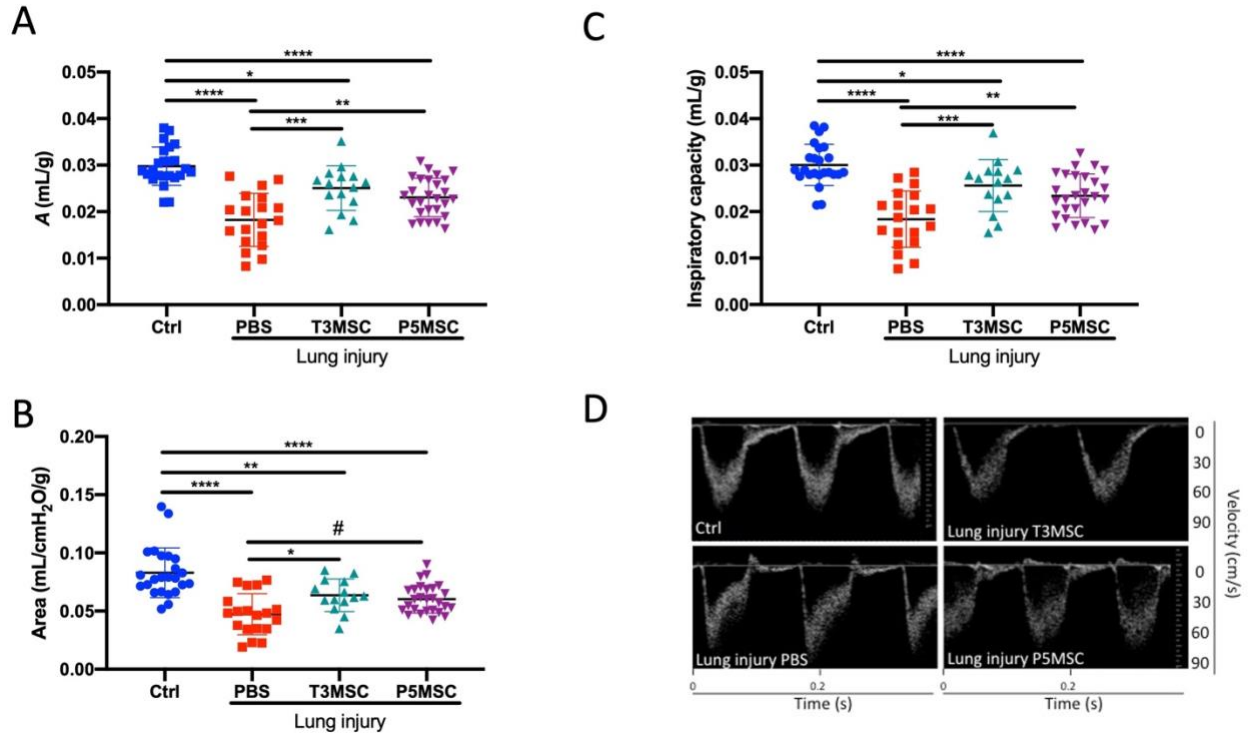
Supplementary Figure 3.1 scRNA-seq analysis of UC-MSCs isolated from term and preterm donors without other pregnancy-related complications.

A) UMAP plot showing the five clusters that were identified based on mRNA expression profiles. Cell clusters are colored as indicated by the legend. **B)** UMAP plot showing the localization of cells from each donor within the clusters. Cells from each donor are colored as indicated by the legend. **C)** Relative contribution to the five clusters for each UC-MSC donor. Clusters are colored as indicated by the legend on the right of the graph. **D)** Dot plot depicting expression level of the top 30 DEGs for each cluster. The intensity of expression is indicated by the purple color and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. **E)** Selected enriched GO terms for each cluster. All terms are significantly enriched (p value < 0.05). Expression levels in Dot plots are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to log-transformed UMIs per 10K.



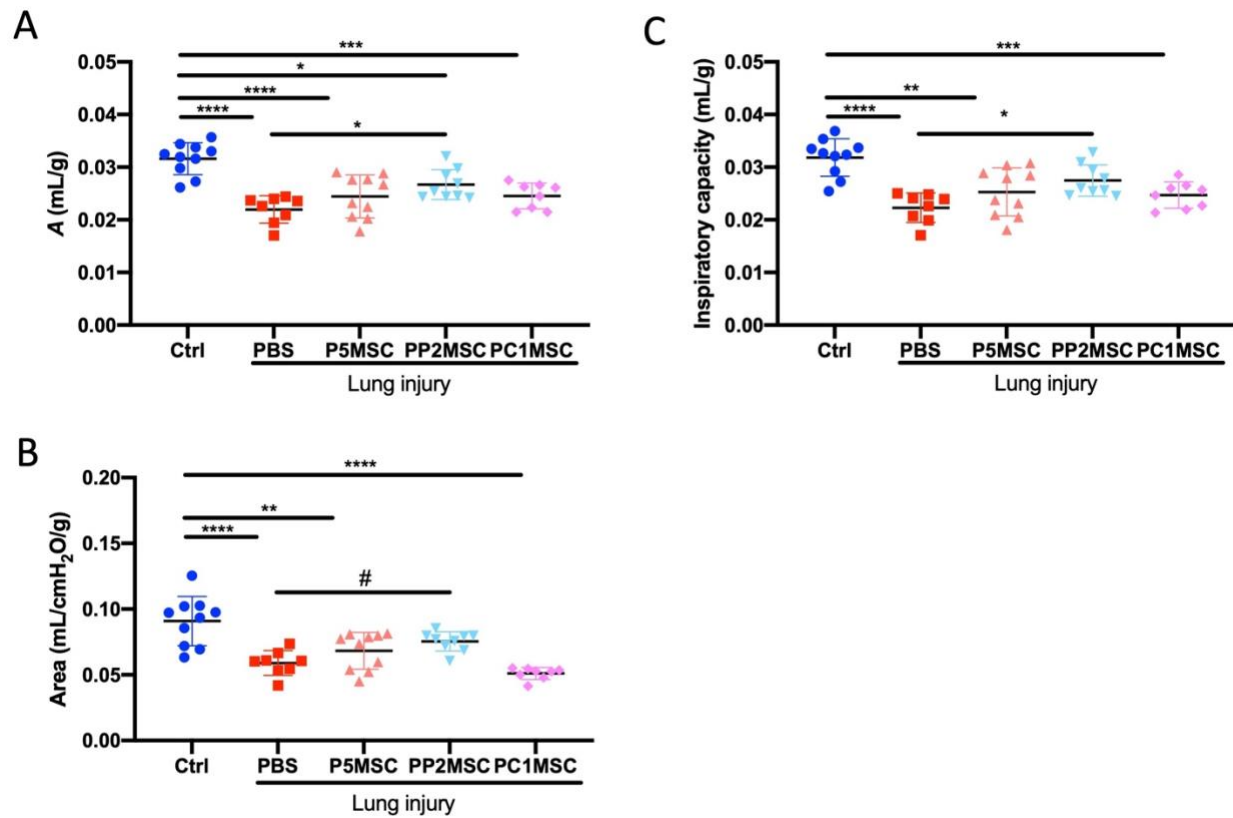
Supplementary Figure 3.2 scRNA-seq analysis of UC-MSCs isolated from preterm, preterm with preeclampsia and preterm with chorioamnionitis donors.

A) UMAP plot showing the four clusters that were identified based on mRNA expression profiles. Cell clusters are colored as indicated by the legend. **B)** UMAP plot showing the localization of cells from each donor within the clusters. Cells from each donor are colored as indicated by the legend. **C)** Relative contribution to the four clusters for each UC-MSC donor. Clusters are colored as indicated by the legend on the right of the graph. **D)** Dot plot depicting expression level of the top 30 DEGs for each cluster. The intensity of expression is indicated by the purple color and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. **E)** Selected enriched GO terms for each cluster. All terms are significantly enriched (p value < 0.05). Expression levels in Dot plots are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to log-transformed UMIs per 10K.



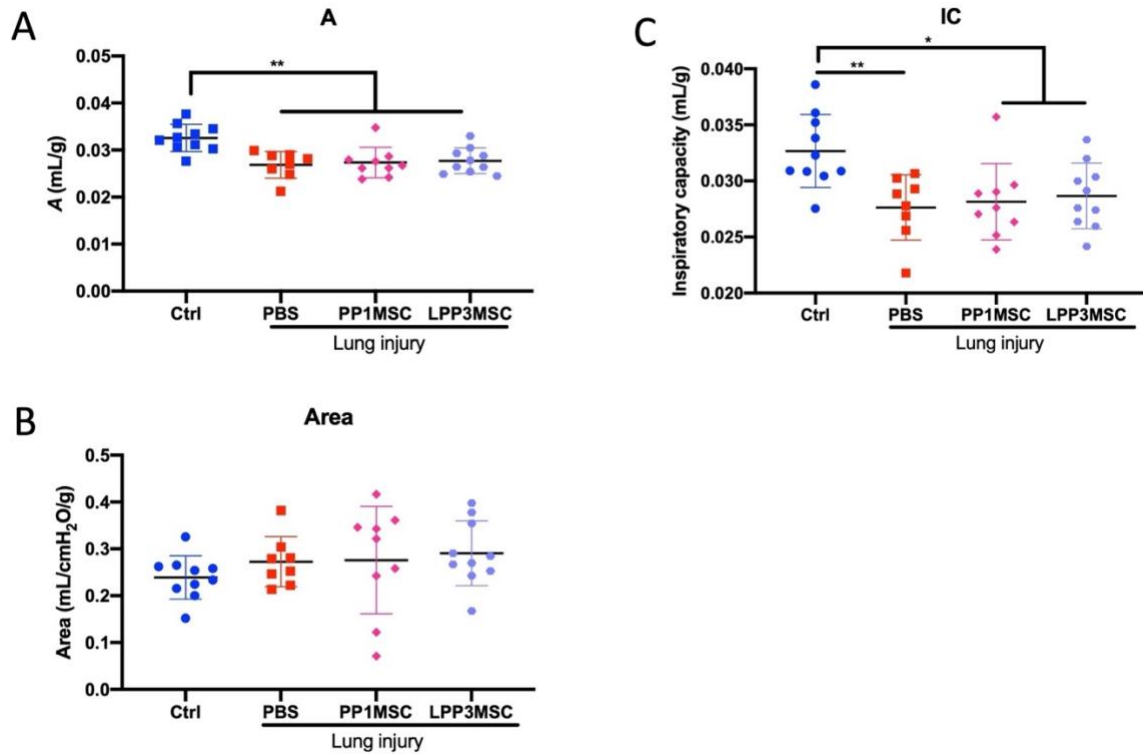
Supplementary Figure 3.3 Lung function and echocardiography of term and preterm UC-MSCs.

A) BW-adjusted inspiratory capacity (**A**) and **B)** BW-adjusted hysteresis (Area) (area under PV-loop) extracted from the Salazar-Knowles equation for each experimental group. **C)** BW-adjusted inspiratory capacity for each experimental group (Ctrl: n=24, PBS: n=19, T3MSCs: n=15, P5MSCs: n=25). **D)** Representative images of echocardiography for each experimental group. Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001. For panel **B**, *t* test (unpaired, two-tailed) was used to compare results between two groups only and significant results are shown with # (*P* < 0.05 was considered significant).



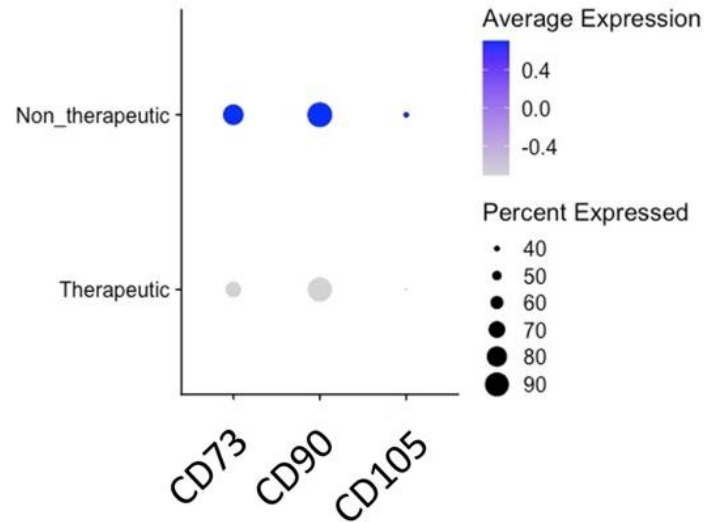
Supplementary Figure 3.4 Lung function of preterm UC-MSCs with underlying complications.

A) BW-adjusted inspiratory capacity (A) and **B**) BW-adjusted hysteresis (Area) (area under PV-loop) extracted from the Salazar-Knowles equation for each experimental group. **C**) BW-adjusted inspiratory capacity for each experimental group (Ctrl: n=10, PBS: n=8, P5MSC: n=10, PP2MSC: n=9, PC1MSC: n=8). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001. *t* test (unpaired, two-tailed) was used to compare results between two groups only in panel **B** and significant results are shown with # (*P* < 0.05 was considered significant).



Supplementary Figure 3.5 Lung function of preterm UC-MSCs that cluster away from the main UC-MSC clusters.

A) BW-adjusted inspiratory capacity (A) and **B)** BW-adjusted hysteresis (Area) (area under PV-loop) extracted from the Salazar-Knowles equation for each experimental group. **C)** BW-adjusted inspiratory capacity for each experimental group (Ctrl: n=10, PBS: n=8, PP1MSC: n=10, LPP3MSC: n=8). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.



Supplementary Figure 3.6 Expression of MSC markers.

Dot plot depicting relative mRNA expression levels of the MSCs markers *CD73*, *CD90*, and *CD105* for the therapeutic and non-therapeutic cells. The intensity of expression is indicated by the purple color and the size of the cell population expressing the gene is indicated by the circle size as indicated by the legend. Expression levels in Dot plot are presented as $\log(\text{TP10K} + 1)$ values. $\log(\text{TP10K} + 1)$ corresponds to log-transformed UMIs per 10K.



Supplementary Figure 3.7 Multiplexing technical replicates.

UMAP plots featuring the localization of the technical replicates of each donor and HNDFs separately. For each donor and the HNDFs, the cells are colored based on which technical replicate they come from, as indicated by the legend at the bottom of the figure. For every donor and for the HNDFs, cells come from all four technical replicates, except for donor LPP3 and donor SGA1 for which cells come from three technical replicates.

3.7.3 Supplementary tables

Supplementary Table 3.1 – Number of sequenced cells per donor or HNDFs

Sample	Cell count
T1	617
T2	565
T3	219
T4	689
T5	586
P1	290

P2	468
P3	333
P4	522
P5	484
LPP1	501
LPP2	408
LPP3	537
LPP4	486
PP1	348
PP2	403
PP3	336
PP4	440
PP5	619
PC1	416
SGA1	221
HNDF	158
Average number of cells per sample	438
Standard deviation	141.33
Total number of cells analyzed	9,646

Supplementary Table 3.2 – List of the top 50 differentially expressed genes in the 7 clusters*

Cluster 0	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
KRT19	LRR75A	THBS1	TIMP3	IFIT1	GADD45A	MMP3
DIRAS3	MTRNR2L12	FST	SERPINE2	IFIT3	CXCL81	IGFBP32
PTX3	AC092069.1	GREM1	TIMP1	IFIT2	GADD45B	S100A4
STMN1	C1orf56	SRGN	TFPI2	ISG151	RSRC2	CXCL122
KRT8	FTH1	PLAU	COL3A1	CRYAB	SNAPC1	KRT72
H2AFZ	SET	SPON2	HLA-A	IGFBP2	PPP1R15A	PRRX2
CCNB1	MT-ND4L	KRTAP2-3	CXCL8	CXCL121	SQSTM11	HLA-B4
PFKP	RPLP0	FTH11	HLA-C	IFI6	DDIT3	COL6A12
JPT1	LPP	KRT7	COL1A2	PMAIP11	NPPB	COL6A22
RANBP1	MBNL1	TMEM158	NDUFA4L2	MX1	TAF1D	MMP1
DKK1	FTL	SLC9A3R2	TM4SF1	HHIP	DDIT4	ELN
FABP5	TPT1	LRR17	MALAT1	IGFBP71	PIM3	PSG5
HMGB1	AKT1	COL6A3	SPARC	SRGN1	SLC3A2	IGFBP52
TYMS	DAP	IL1B	IGFBP7	IGFBP31	SNHG8	KISS1
ACTG1	METRNL	FGF5	IGFBP51	IFITM31	ATF3	CRYAB1

FLT1	BRD4	CXCL12	SAT1	TINAGL1	NR1D1	OXTR
TPX2	PPP1CB	COL6A2	CXCL1	HLA-C1	IRF1	B2M3
PTMA	EEF1A1	COL6A1	IGFBP3	ANKRD1	SNHG12	POSTN1
TUBA1B	CREB3L1	PMAIP1	B2M	KRT81	CEBPD	HLA-A2
UBE2S	RPLP1	PLAT	COL1A1	SAMD9	ZFAS1	ID11
NASP	PEA15	DKK3	CDKN1A	BGN1	MAFF	THBS11
		LY6K	NEAT1	B2M1	FILIP1L1	C12orf751
		CTSC	RPS4Y1	PLAT1	CITED2	TNFRSF11B1
		TNFRSF11B	P4HA2	POSTN	RND31	MYLK1
		F3	FTH12	KRT18	RELB	TIMP11
		RAB3B	MT-ND3	HES4	HMOX1	APLP2
		BRI3	NUPR11	CRIP2	EPB41L4A-AS1	RAB3B1
		NUPR1	MT2A	STC21	ARID5B	TGM2
		IGFBP5	SPON21	PRSS23	UBC	CAV1
		POU2F2	HLA-B1	VCAN	RRAD	COL6A31
		HLA-B	CTSB	TFPI	SNHG15	MGST1
		S100A13	PTGS2	HAPLN1	CCNL1	NEAT12
		FGF7	SQSTM1	IGFBP61	TXNIP	RPS4Y12
		XIST	MIR4435-2HG	PLK2	HLA-B3	PRPS1
		WNT5B	CLTB	DSP	BHLHE41	ARHGAP18
		PDLIM1	RALA	HLA-B2	PLK21	CTGF
		LMO7	LUM	PARP14	ARID4B	LBH
		KLF2	PPIC	LMO2	SAT11	RGMB
		NT5E	LOX	NFIB	ATF4	MGLL1
		MGLL	TAGLN	RPS4Y11	ADM	AEBP1
		C12orf75	IGFBP6	STAT1	CXCL31	GAL
		AKAP12	P4HA1	EFEMP1	PRPF38B	PRRX1
		LAMC2	CXCL6	COL4A1	PMAIP12	PDLIM11
		HMGA2	INHBA	IFI44	IFRD1	MIR44352HG1
		IL6	IFITM3	HERC5	EIF4A2	CITED21
		SERPINE1	COL5A1	ZC3HAV1	BTG1	WNT5B1
		NR2F1	CST3	DDX58	CEBPB	ARID5B1
		IL11	MT-CYB	MCAM	NR1D2	STMN2
		RGS10	CDH11	MALL	NFKBIA	SERINC2
		KHDRBS3	DCN	IFI16	NUPR12	LDHA

*Genes are shown from highest avg_logFC. All genes have a *p* value < 0.05.

Supplementary Table 3.3 – List of the top 10 GO terms characteristics of each cluster*

Cluster 0	Cluster 1	Cluster 2	Cluster 3
sarcomere organization	positive regulation of actin filament polymerization	signal transduction	cell adhesion
cell division	intracellular sequestering of iron ion	positive regulation of transcription from RNA polymerase II promoter	extracellular matrix organization
cell differentiation involved in embryonic placenta development	regulation of glycogen biosynthetic process	immune response	negative regulation of cell proliferation
histone exchange	iron ion transport	positive regulation of cell proliferation	signal transduction
mitotic spindle organization	positive regulation of pseudopodium assembly	extracellular matrix organization	immune response
viral process	nucleosome disassembly	cell adhesion	oxidation-reduction process
activation of protein kinase activity	translational elongation	positive regulation of cell migration	collagen catabolic process
cell proliferation	cell motility	inflammatory response	cellular protein metabolic process
response to glucocorticoid	cytoplasmic translation	positive regulation of angiogenesis	collagen fibril organization
vascular endothelial growth factor receptor signaling pathway		response to hypoxia	angiogenesis
Cluster 4	Cluster 5	Cluster 6	
defense response to virus	transcription, DNA-templated	signal transduction	

type I interferon signaling pathway	positive regulation of transcription from RNA polymerase II promoter	extracellular matrix organization
response to virus	regulation of transcription, DNA-templated	negative regulation of apoptotic process
cell adhesion	negative regulation of transcription from RNA polymerase II promoter	cell adhesion
negative regulation of viral genome replication	transcription from RNA polymerase II promoter	negative regulation of transcription from RNA polymerase II promoter
innate immune response	apoptotic process	response to hypoxia
positive regulation of transcription from RNA polymerase II promoter	positive regulation of transcription, DNA-templated	positive regulation of gene expression
immune response	negative regulation of apoptotic process	response to drug
viral process	regulation of transcription from RNA polymerase II promoter	immune response
negative regulation of apoptotic process	negative regulation of transcription, DNA-templated	positive regulation of cell proliferation

CHAPTER 4: Characterization of the innate immune response in a novel murine model mimicking bronchopulmonary dysplasia

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Bernard Thébaud

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BASIC SCIENCE ARTICLE

Characterization of the innate immune response in a novel murine model mimicking bronchopulmonary dysplasia

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BACKGROUND: Bronchopulmonary dysplasia (BPD), the most common complication of prematurity, arises from various factors that compromise lung development, including oxygen and inflammation. Hyperoxia has been used to mimic the disease in newborn rodents. The use of a second hit to induce systemic inflammation has been suggested as an added strategy to better mimic the inflammatory aspect of BPD. Here we report a novel 2 hit (2HIT) BPD model with in-depth characterization of the innate immune response, enabling mechanistic studies of therapies with an immunomodulatory component.

METHODS: C57BL/6N mice were exposed to 85% O₂ from postnatal day (P)1 to P7, and received postnatally (P3) *Escherichia coli* LPS. At various timepoints, immune activation in the lung and at the systemic level was analyzed by fluorescence-activated cell sorting (FACS), and gene and protein expressions.

RESULTS: 2HIT mice showed fewer alveoli, increased lung compliance, and right ventricular hypertrophy. A transient proinflammatory cytokine response was observed locally and systemically. Type 2 anti-inflammatory cytokine expression was decreased in the lung together with the number of mature alveolar macrophages. Simultaneously, a Siglec-F intermediate macrophage population emerged.

CONCLUSION: This study provides long-term analysis of the 2HIT model, suggesting impairment of type 2 cytokine environment and altered alveolar macrophage profile in the lung.

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IMPACT:

- We have developed a novel 2HIT mouse BPD model with postnatal LPS and hyperoxia exposure, which enables mechanistic studies of potential therapeutic strategies with an immunomodulatory component.
- This is the first report of in-depth characterization of the lung injury and recovery describing the evolution of the innate immune response in a standardized mouse model for experimental BPD with postnatal LPS and hyperoxia exposure.
- The 2HIT model has the potential to help understand the link between inflammation and impaired lung development, and will enable testing of new therapies in a short and more robust manner.

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is the most common chronic lung disorder of infancy. Among neonatal morbidities, BPD has the highest cost in initial neonatal intensive care unit stay.¹ BPD was first described nearly 50 years ago by Northway et al.² as a chronic respiratory disease that developed in preterm newborns exposed to mechanical ventilation and oxygen supplementation. With improved survival, infants at risk of developing BPD are now born at earlier gestational ages (23–26 weeks). The hallmarks of the lung pathology are being described as arrested lung development, including fewer and larger alveoli with less septation, thickening of alveolar septa, and impaired development of capillary network.³ These changes may lead to life-long respiratory morbidity, including asthma, reduced exercise capacity, early-onset emphysema, and pulmonary hypertension (PH).⁴

Studies of disease mechanism in BPD have been restricted to animal models, due to limited access to patient lung samples. The most common models include the exposure of term rodents to hyperoxia of varying concentration (40–100%) and length (3 days–4 weeks).^{5,6} Bonikos et al.⁷ were the first to report that exposure of mice to hyperoxia results in chronic lung injury characterized by alveolar simplification reminiscent of BPD. Subsequently, many groups have studied in more detail the physiological and molecular effects of hyperoxia on newborn rodent lungs. Hyperoxia induces innate immune response by stimulating the secretion of several cytokines and chemokines, and subsequent recruitment of neutrophils, monocytes, and macrophages to the lungs.⁸ Recent evidence suggest that during normal mouse lung development, the first breath induces differentiation of alveolar macrophages into mature M2 (anti-inflammatory)

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macrophages.^{9,10} Conversely, neonatal hyperoxic exposure induces a proinflammatory M1 phenotype in the alveolar macrophages.^{11,12} However, the inflammatory injury in rodent hyperoxia models is only moderate.¹³ Considering that perinatal inflammation and episodes of sepsis,¹⁴ as well as pneumonia,¹⁵ are important risk factors for the development of BPD, the use of a second hit to induce systemic inflammation has been suggested and described as an improved strategy to mimic the pathogenesis of clinical BPD.^{16–18}

Translation of potentially life-saving therapies into the clinic is unacceptably slow. It has been argued that one of the underlying reasons is the lack of methodological rigor in the development of preclinical animal models.¹⁹ Here, we present the 2HIT model, where the mice receive two independent hits, including 85% hyperoxia from P1 to P7 and low-dose systemic *Escherichia coli* LPS at P3 to mimic perinatal inflammation in premature newborn infants at risk of developing BPD. We hypothesized that the 2HIT model would provide a short, robust, and standardized mouse model for experimental BPD with impaired innate immunity, which would allow both understanding disease pathogenesis, with a focus on inflammation and testing of new therapies, and establishing mode of action. We demonstrate the evolution of the inflammatory response over the course of both the injury and repair phases during the first month of life. Furthermore, we show that the number of anti-inflammatory homeostatic alveolar macrophages decreases significantly after injury, accompanied by a simultaneous increase in a Siglec-F intermediate population of lung macrophages. Our findings suggest that the 2HIT model causes both local and systemic type 1 cytokine responses, and, importantly, disturbs the homeostatic type 2 cytokine environment of the lung during the crucial stages of lung development, which may in turn contribute to the altered lung architecture. To our knowledge, this is the first report of in-depth characterization of the lung injury and recovery describing the evolution of the innate immune response in a 2HIT mouse model with postnatal LPS and hyperoxia exposure.

MATERIALS AND METHODS

Animals

All procedures were approved by the Animal Care Committee of the University of Ottawa and animal care was performed in accordance with institutional guidelines. Pregnant C57BL/6N mice were delivered on embryonic days (E)15–E17 from Charles Rivers, Saint Constant, QC, Canada. Pups from all litters were randomized at P1 (P0 is the day of birth) and allocated to one dam to obtain a litter size of six to seven pups per dam. The number of pups in each litter was maintained to an equal number (± 1) by euthanizing control pups when deceased pups were found in 2HIT litters. Two experimental hits were used in this study: hyperoxia (85% O₂) from P1 until P7 (sealed plexiglass chambers with continuous oxygen monitoring [BioSpherix, Redfield, NY]) and intraperitoneal (i.p.) injection of 1 mg/kg LPS (*E. coli* O55:B5, Sigma-Aldrich L2880, St. Louis, MO) at P3 (Fig. 1a). Hyperoxic dams were exchanged with normoxic dams every 48 h to avoid oxygen toxicity. The experimental groups were as follows: (1) hyperoxia and LPS (2HIT), (2) room air (RA) control, (3) RA and LPS (RA + LPS), and (4) hyperoxia (O₂). Animals were sacrificed by i.p. injection of pentobarbital sodium (Euthanyl, 0.2 mL, 65 mg/mL).

Histology

Lungs were harvested for histology as described earlier at P8, P15, 4 weeks, and 3 months.²⁰ Spleen, liver, and kidney samples (P15, 4 weeks, and 3 months) were collected from both RA and 2HIT animals and fixed for 48 h in 10% formalin at room temperature (RT). After fixation, all organs were embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin (H&E) by the University of Ottawa, Louise Pelletier Histology Core

Facility. Alveolar size was quantified by a motorized microscope stage, using the mean linear intercept (MLI) estimation.²¹

Lung function testing

At 4 weeks and 3 months timepoints, a tracheostomy was performed and lung function (pressure volume [PV] loop) was assessed using flexiVent (SCIREQ, Montreal, QC, Canada) as described earlier.²⁰ Average of three measurements per mouse was used.

Flow cytometry

Lung or spleen cells were stained with lung immune cell or with spleen macrophage antibodies, respectively (Supplemental Table S1a [online]). In addition, P2–P5 lungs were stained with lung macrophage M1/M2 panel and P8–4 week lungs with the lung immune cell panel with M1/M2 markers. After staining, samples were fixed with 4% paraformaldehyde (PFA). Fluorescence minus one (FMO), single, and no stain controls were used as necessary. Flow cytometry was performed by Ottawa Hospital Research Institute core facility using BD LSRFortessa (Beckton Dickinson Biosciences, Franklin Lakes, NJ). Sample compensation was performed with BD FACSDIVA software and data were analyzed using FlowJo v10 (FlowJo LLC, Ashland, OR).

Quantitative RT-PCR

Complementary DNA (cDNA) was reverse transcribed from 1 μ g of total RNA using iScriptTM Reverse Transcription Supermix (Bio-Rad cat#1708841). Quantitative RT-PCR (qRT-PCR) analysis was performed using CFX96 or CFX384 Touch Real-Time PCR (Bio-Rad). Reactions were performed in 20 μ L volume using 1.2 nM primer concentration, 10 μ L iQTM SYBR Green Supermix (Bio-Rad, cat#1708882) and 5 μ L of diluted cDNA (1:5 in ddH₂O). Primer sequences are described in Supplementary Table S1b (online). The qRT-PCR program consisted of 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C (15 s), annealing at 57 °C (for 30 s), and extension at 72 °C (30 s). Melting curves were obtained by increasing temperature by 0.5 °C every 5 s from 65 °C to 95 °C after the last extension phase to validate primer specificity. Optimal annealing temperature was determined by testing a range of eight different temperatures between 54 °C and 66 °C. Efficiency was calculated using the formula $10^{(-1/\text{slope of standardization})} - 1$. The normalized expression level of the target genes for each condition was calculated using the average threshold cycle (Ct) value from triplicate measurements by the Pfaffl method.²² *Hprt* was identified as a stable housekeeping gene using geNorm and BestKeeper.

Multiplex assay

Lung tissue homogenates were diluted to equal amount of protein (8690 μ g/mL) and serum was collected from whole blood samples (cardiac puncture), and then diluted 1:2 with phosphate-buffered saline. Cytokines/chemokines were quantified simultaneously by Eve Technologies Corp., Calgary, AB, Canada using a Discovery Assay (Mouse Cytokine Array/Chemokine Array 31-Plex [Lung P3, P8, and P15 and Serum P2, P3, P5, and P8] or Mouse High Sensitivity T Cell Discovery Array 18-plex [Lung P29]) (Supplementary Table S2 [online]). The minimum detectable concentration starts at 0.3 pg/mL.

Statistics

Statistical analyses were performed using GraphPad Prism version 7.0c (GraphPad Software, Inc., La Jolla, CA). Outliers were identified using Grubbs' test and normal distribution using Shapiro–Wilk normality test. *P* values (*P* < 0.05 was considered significant) were obtained with *t* test (unpaired, two-tailed) or log-rank (Mantel–Cox) test for Kaplan–Meier curves or one-way ANOVA with Tukey's multiple comparison post hoc testing to compare results between four groups. Data are represented as the mean with standard deviation (SD).

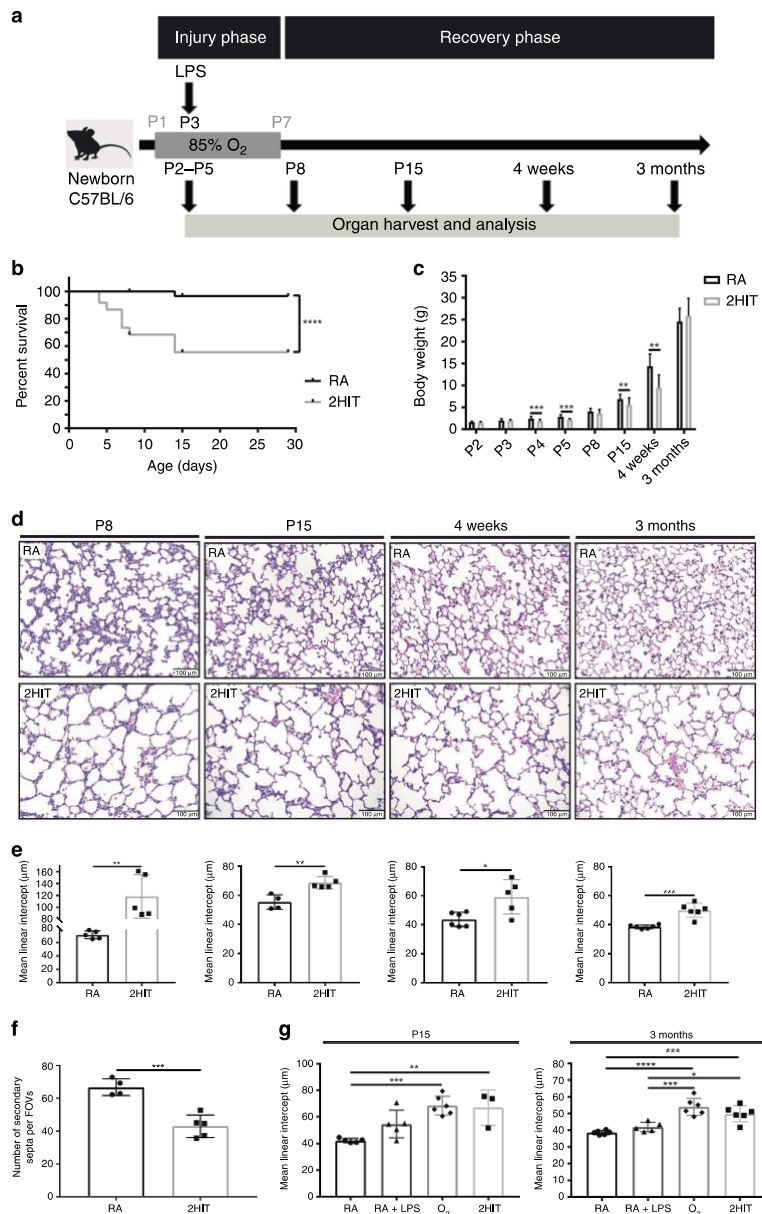


Fig. 1 Survival, body weight, and morphometric analysis of RA, RA + LPS, O₂, and 2HIT lungs. **a** Experimental design and timelines. **b** Kaplan-Meier survival curve ($n = 73$ [RA] and $n = 60$ [2HIT]). **c** Body weight gain in RA animals compared to 2HIT animals (P2, P4, and P5, $n = 12$ [RA and 2HIT]; P3, $n = 38$ [RA], $n = 43$ [2HIT]; P8, $n = 14$ [RA], $n = 11$ [2HIT]; P15, $n = 22$ [RA], $n = 15$ [2HIT]; 4 weeks, $n = 18$ [RA], $n = 13$ [2HIT]; 3 months, $n = 6$ [RA and 2HIT]). **d** Representative images of H&E-stained lung sections of RA and 2HIT animals at P8, P15, 4 weeks, and 3 months. **e** Quantification of alveolar size using the MLI method in RA and 2HIT lung tissue sections at P8, P15, 4 weeks, and 3 months ($n = 4-6$ /group). **f** Quantification of secondary septa at P15 ($n = 4$ [RA] and $n = 5$ [2HIT]). **g** Quantification of alveolar size using the MLI method in RA, RA + LPS, O₂, and 2HIT lung tissue sections at P15 and 3 months ($n = 5-6$ /group, except for 2HIT group at P15, where $n = 3$). Individual data points shown for each bar graph represent individual mice from multiple litters. Values are represented as the mean $\pm$ SD. P value < 0.05 was considered significant. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

RESULTS

2HIT model causes increase in mortality and decrease in body weight of experimental animals

Exposure of mouse pups to hyperoxia and LPS (2HIT) induced a significant increase in mortality compared to pups kept in normoxia (RA) (Fig. 1b). In addition, 2HIT animals had a significantly lower body weight from P4 until 4 weeks of age as compared to RA animals (Fig. 1c). No significant body weight differences were observed at 3 months. To compare the extent of mortality and reduction in body weight in the 2HIT model with single hits (O₂, 85% or LPS), the survival and body weight were assessed at P15 and 3 months. Survival of 2HIT animals was decreased compared to RA + LPS and O₂ animals, but this was not significant (Supplementary Fig. S1a [online]). At P15, RA + LPS, O₂, and 2HIT animals had a significantly lower body weight compared to RA animals, but no significant difference between all injury groups was observed (Supplementary Fig. S1b [online]). However, *t* test analysis did show significant decrease in body weight of 2HIT animals compared to RA + LPS animals. No significant difference in body weight was observed between all groups at 3 months of age.

2HIT animals show altered lung structure and function, as well as right ventricular hypertrophy

Lungs of 2HIT animals had larger and fewer alveoli at P8, P15, 4 weeks, and 3 months (Fig. 1d). Moreover, lungs of 2HIT animals had a significantly higher MLI compared to the lungs of RA animals at all timepoints, indicating that the alveoli were larger in size (Fig. 1e). The number of secondary septa in the 2HIT group was significantly decreased compared to the RA group at P15 (Fig. 1f). To compare the extent of lung injury in the 2HIT model with single hits (O₂, 85% or LPS), the MLI was studied at P15, and 3 months. No significant difference in MLI was observed in the 2HIT group compared to the O₂ group. MLI of the O₂ group was significantly increased compared to the RA group. No significant increase in MLI was observed in the RA + LPS group compared to the RA group. However, MLI of O₂ and 2HIT groups were significantly higher compared to the RA + LPS group at 3 months, but not at P15. (Fig. 1g).

Lung function showed that the 2HIT model induced an upward shift of the PV loop and a significant increase in volume at 30 cmH₂O compared to the RA group at 4 weeks and 3 months suggesting increased lung compliance (Fig. 2a, b). Salazar-Knowles equation parameters were extracted from the PV loop. Static compliance (C_{st}) was significantly increased in the 2HIT group at 4 weeks and 3 months compared to the RA group, as well as inspiratory capacity (A) (Fig. 2c, d). Hysteresis (area under the PV loop) in the 2HIT group, representative of the work of breathing, was also significantly increased compared to the RA group at 4 weeks and 3 months (Fig. 2e).

To compare 2HIT long-term lung function with single hits (O₂, 85% or LPS), the PV loop was studied at 3 months. The PV loop of O₂ and 2HIT animals was shifted upwards. Significant increase in volume at 30 cmH₂O was observed in O₂ animals when compared to RA, RA + LPS, and 2HIT animals, and in 2HIT animals when compared to RA animals (Fig. 2f, g).

Finally, we observed right ventricular hypertrophy (RVH) in 2HIT animals, but not in single hit animals, compared to RA animals at 3 months of age, as measured by the Fulton's index (Fig. 2h).

2HIT model does not induce long-term pulmonary fibrotic changes

Lung fibrosis was assessed by quantifying hydroxyproline content by colorimetry and by collagen staining using the Masson's trichrome in 3-month-old animals. No difference in hydroxyproline content was observed between RA and 2HIT animals (Supplementary Fig. S1c [online]). Moreover, no visible increase in the amount of parenchymal collagen was observed in 2HIT animals (Supplementary Fig. S1d [online]).

2HIT model activates a local pulmonary and systemic type 1 cytokine response

At the messenger RNA (mRNA) level, gene expression levels of *Il1b* and *Tnfa* were significantly increased 3 h after LPS injection at P3 (Fig. 3a, b). While *Il1b* was found to be one of the earliest proinflammatory markers during the injury phase (increased by P3), its expression in 2HIT animals had rapidly decreased in the recovery phase at P8 and P14, when it was even lower than in lungs of RA animals. The expression levels of *Il6*, *Tgfb1*, and *Tnfa* showed a second peak in the 2HIT group at the beginning of the recovery phase at P8, and dropped to comparable levels as in the RA group by P14.

At the protein level, we observed a significant increase of several proinflammatory cytokines and chemokines in lung tissue consistent with the gene expression levels. The cytokines interleukin-17 (IL-17), IL-1 α , IL-1 β , IL-3, tumor necrosis factor- α (TNF α), and IL-6 were highly upregulated in 2HIT animals during the early injury phase at P3 3 h after LPS injection. However, at the beginning of the recovery phase by P8, these cytokine levels had significantly dropped, with only IL-17 and IL-6, together with IL-2, IL-7, interferon- γ (IFN γ), and IL-1 α remaining higher than control levels. All chemokines included in the panel were highly upregulated in the injury phase at P3, but not any longer in the beginning of the repair phase at P8, except for macrophage colony-stimulating factor (M-CSF) and granulocyte CSF (G-CSF) (Fig. 4a). Increase in lung chemokine expression was accompanied by recruitment of innate immune cells (macrophages, neutrophils, and monocytes) to the site of inflammation, the lung (Fig. 4b). By the age of 4 weeks, no difference in the level of expression was found between the 2HIT and the RA group for any studied cytokines, chemokines, or immune cells.

In addition to the local pulmonary immune response, we observed a fast transient systemic proinflammatory response. Already early during the injury phase at P2, a significant increase in eotaxin could be detected in the serum of 2HIT when compared to RA animals (Fig. 4c). At P3, only 3 h after the administration of LPS, a peak in proinflammatory cytokine/chemokine expression was observed. The most highly expressed cytokines in the serum were IL-6 and TNF α , followed by the chemokines monocyte chemoattractant protein-1 (MCP-1), macrophage-inflammatory protein 2 (MIP-2), MIP-1 α , G-CSF, and MIP-1 β . At P5, the cytokine/chemokine levels dropped significantly as compared to P2, with only MCP-1, G-CSF, and MIP-1 β remaining higher than the control. In the beginning of the repair phase at P8, we observed no significant differences between cytokine or chemokine levels between the RA and the 2HIT groups, except for a minor increase in MIG (monokine induced by IFN- γ) and IP-10 (IFN- γ -inducible protein-10), and a decrease in keratinocyte-derived chemokine (KC) and interferon- γ (IFN γ).

Overall, we show that the 2HIT model exhibits a fast pulmonary and systemic proinflammatory response, without signs of chronic inflammation.

2HIT model shows decreased pulmonary type 2 cytokine expression

In the lungs of 2HIT animals, we observed a transient decrease in the mRNA expression levels of type 2 cytokine genes *Il4*, *Il10*, and *Il13* both during the injury and beginning of the recovery phases at P3 and P8 (Fig. 5a, b). Also, the expression of M2 macrophage marker *Arg1* was transiently decreased in the beginning of the recovery phase at P8. At the protein level, we observed slightly increased protein levels of IL-4, and IL-5 at P3 and IL-10 and leukemia inhibitory factor (LIF) at both P3 and P8 (Fig. 5c).

In addition, we observed an early transient systemic anti-inflammatory cytokine response in the serum with a peak just after injection with LPS, with increased levels of IL-5, LIF, IL-13, IL-15, and IL-10 and the growth factor VEGF (vascular endothelial growth factor) for a short period of time at P3 (Fig. 5d). The

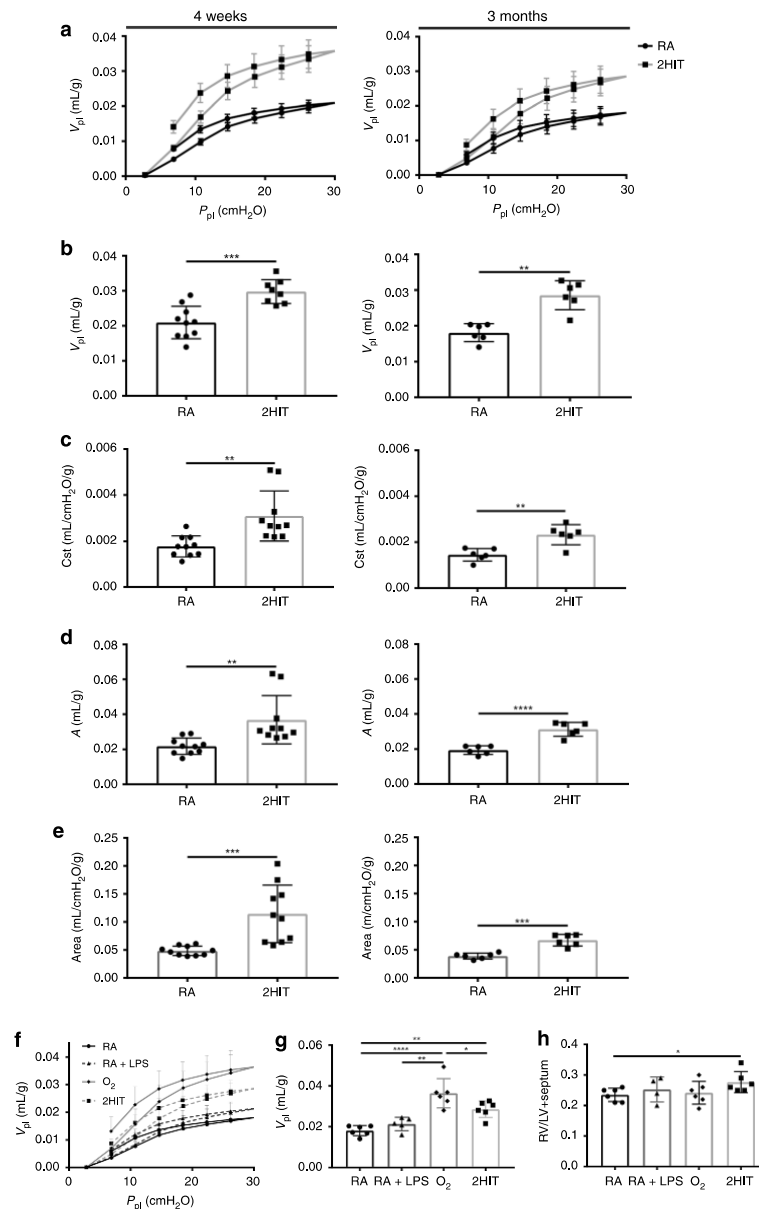


Fig. 2 The effect of LPS, O₂, and 2HIT injuries on lung function and right ventricular hypertrophy. **a** Body weight-adjusted PV curves for RA and 2HIT animals at 4 weeks and 3 months of age and **b** body weight-adjusted lung volume at peak Ppl 30. **c** Body weight-adjusted static compliance (Cst), **d** body weight-adjusted inspiratory capacity (A) and **e** body weight-adjusted hysteresis (area under PV loop) of RA and 2HIT animals at 4 weeks and 3 months of age. **f** Body weight-adjusted PV curves for RA, RA + LPS, O₂, and 2HIT animals at 3 months of age and **g** body weight-adjusted lung volume at peak Ppl 30. **h** Fulton's index quantifying right ventricular hypertrophy of RA, RA + LPS, O₂, and 2HIT animals at 3 months of age. PV curves are shown as the mean volume of all animals within one group at each pressure point. Individual data points shown for each bar graph represent individual mice from multiple litters. Values are represented as the mean $\pm$ SD ($n = 4-10$ /group). P value < 0.05 was considered significant. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

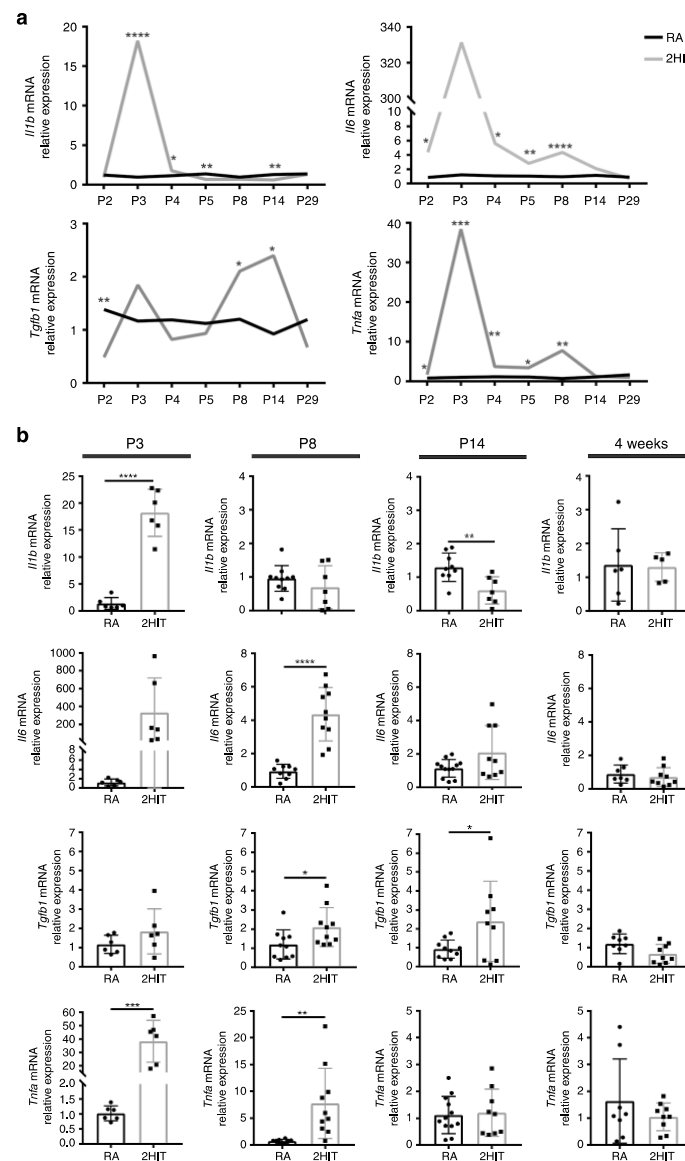


Fig. 3 mRNA expression level of type 1 proinflammatory cytokines in the lung of RA and 2HIT animals. Relative mRNA expression of proinflammatory cytokines *Il1b*, *Il6*, *Tgfb1*, and *Tnfa* in 2HIT animals as compared to RA animals at P2, P3 (3 h after LPS injection), P4, P5, P8, P14, and 4 weeks as **a** a timeline, and **b** individually. Individual data points shown for each bar graph represent individual mice from multiple litters. Values are the means $\pm$ SD of two to three independent experiments ($n = 4-12$ /group). P value < 0.05 was considered significant. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

systemic type 2 cytokine response was no longer detected at P5 (Fig. 5d). Interestingly, the gene expression levels of *Il4* and *Il10* in the lung tissue were simultaneously downregulated, indicating that detected proteins were derived from the circulation and not

from the lung resident or recruited cells (Fig. 5b). In conclusion, the results indicate that lung resident and recruited cells do not produce increased quantities of type 2 cytokines after injury in the 2HIT model.

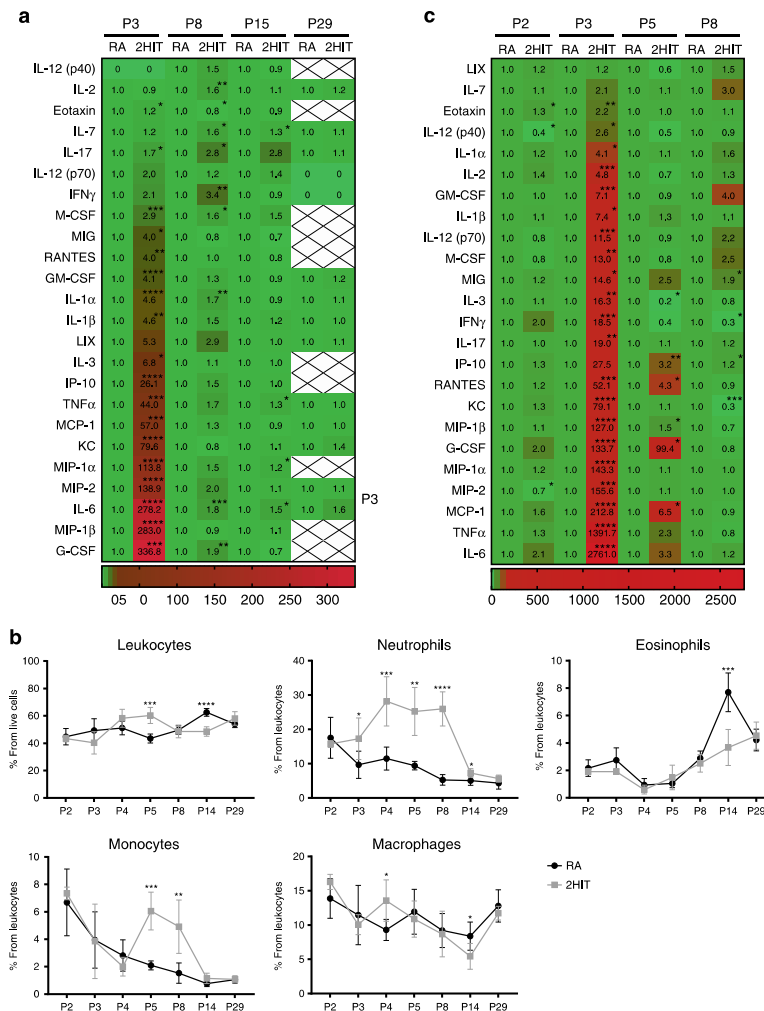


Fig. 4 Innate immune cell counts and proinflammatory cytokine and chemokine protein expression of RA and 2HIT animals. **a** The protein expression pattern of proinflammatory cytokines and chemokines in the lung (P3 [3 h after LPS injection], $n = 4$ [RA and 2HIT]; P8, $n = 4$ –8 [RA and 2HIT]; P15, $n = 6$ [RA] and $n = 5$ –6 [2HIT]; P29, $n = 5$ [RA and 2HIT]). **b** Relative quantities of innate immune cells in the lungs detected by FACS ($n = 4$ –6/group). Immune cell populations were analyzed by FACS from dissociated lungs. **c** The protein expression pattern of proinflammatory cytokines and chemokines in the serum (P2, P3 [3 h after LPS injection] and P5, $n = 3$ [RA and 2HIT]; P8, $n = 5$ [RA and 2HIT]). Values for multiplex panel are given as fold change (see Supplementary Table S2 [online] for details). Values are represented as the means $\pm$ SD. P value < 0.05 was considered significant. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

The number of mature anti-inflammatory lung resident alveolar macrophages decreases in response to the 2HIT injuries. A FACS gating strategy modified from Misharin et al.²³ was used to distinguish between the alveolar (Siglec-F^{high}) and interstitial macrophages (Siglec-F^{low}) as a time course between P8 and 4 weeks timepoints (Supplementary Fig. S2a [online]). We noticed a dramatic reduction in the number of alveolar macrophages in 2HIT animals from P8 onwards (Fig. 6a). This was accompanied by the rise of a Siglec-F^{low} macrophage population (Supplementary Fig. S2b [online]). In contrast, the only significant change—an

increase—in the number of interstitial macrophages was observed at P14 (Fig. 6a).

In concordance with the presence of type 1 cytokines such as IFN γ , we observed macrophage polarization towards proinflammatory M1 phenotype in 2HIT animals early during the injury phase. Alveolar macrophages were analyzed using gating strategies described in Supplementary Fig. S2a, c (online), and were found to upregulate the expression of CD86, a commonly used M1 marker, between P3 and P8 (Supplementary Fig. S3a [online] and Fig. 6a). At P8 (beginning of the recovery phase), we

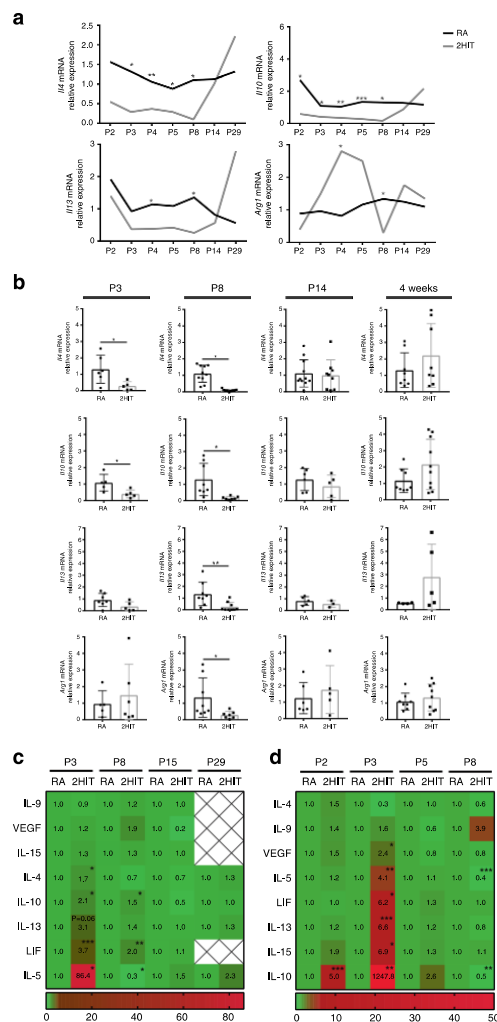


Fig. 5 mRNA expression level of type 2 anti-inflammatory cytokines and arginase-1 in the lung of RA and 2HIT animals, and protein expression level of anti-inflammatory cytokines and chemokines of RA and 2HIT animals. Relative mRNA expression of anti-inflammatory cytokines *Il4*, *Il10*, *Il13*, and *Arg1* in 2HIT animals as compared to RA animals at P2, P3 (3 h after LPS injection), P4, P5, P8, P14, and 4 weeks as **a** a timeline, and **b** individually. Values are the means $\pm$ SD of two to three independent experiments ($n = 3-11$ /group). **c** The expression pattern of anti-inflammatory cytokines and chemokines in the lung (P3 [3 h after LPS injection], $n = 4$ [RA and 2HIT]; P8, $n = 6-8$ [RA and 2HIT]; P15, $n = 6$ [RA and 2HIT]; P29, $n = 6$ [RA] and $n = 5$ [2HIT]) and **d** in the serum (P2, P3 [3 h after LPS injection] and P5, $n = 3$ [RA and 2HIT]; P8, $n = 5$ [RA and 2HIT]). Values for multiplex panel are given as fold change (see Supplementary Table S2 for details [online]). Individual data points shown for each bar graph represent individual mice from multiple litters. P value < 0.05 was considered significant. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

observed upregulation of the anti-inflammatory M2 marker CD206 in the alveolar macrophages. Interestingly, the Siglec-F intermediate macrophage population was not expressing either CD86 or CD206 at P8, but by P15 the upregulation of both receptors was observed (Fig. 6a and Supplementary Fig. S4a, b [online]). On the other hand, in the interstitial macrophage population, we were not able to detect either CD86 or CD206 upregulation after injury at any timepoints (Fig. 6a).

In summary, we detected a decrease in the anti-inflammatory homeostatic macrophages and an increase in a Siglec-F intermediate population of macrophages in lungs of developing 2HIT mice. However, by the age of 4 weeks, macrophage populations were similar in lungs of both RA and 2HIT animals.

Extrapulmonary manifestations in the 2HIT model

2HIT animals appeared to have transient atrophy in the spleen; mild to moderate atrophy, as well as the general structure disorganization, was observed at P14, but this was normalized by 3 months of age (data not shown). In the spleen, resident red pulp macrophages were analyzed using FACS gating strategy as described in Supplementary Fig. S2d (online) and were found to upregulate the expression of CD86 and MHCII, typical markers for M1 macrophage phenotype between P4 and P14 (Supplementary Fig. S3b [online] and Fig. 6b). Additionally, at P4–P8 we observed downregulation of CD206, indicating decrease in M2 macrophage phenotype in the spleen (Supplementary Fig. S3b [online] and Fig. 6b). However, by the age of 4 weeks the changes in spleen macrophages were reverted. No significant toxicities to the liver or the kidney were observed. In conclusion, the immune response in the 2HIT model of BPD is not limited to the lung, but involves also the spleen. No significant extrapulmonary long-term changes were observed.

DISCUSSION

The 2HIT model represents a novel platform for studying BPD pathogenesis and testing potential novel anti-inflammatory therapies. In addition, we demonstrate for the first time the innate immune response evolution in this model as a function of age, characterized by a distinct cytokine pattern and macrophage phenotype after injury.

2HIT animals showed simplified alveolar architecture and enhanced lung compliance, consistent with previous reports in murine models of BPD, as well as findings in BPD patients.^{1,24} The reduced gas exchange surface area was caused by altered alveolar development characterized by decrease in secondary septation. 2HIT animals showed emphysematous functional changes, including increased compliance and an upward shift in the PV loop, reflecting decreased elastic recoil. We found no signs of long-term lung fibrosis. This to our knowledge is consistent with the current literature, as long-term fibrosis has not been reported in C57Bl/6 mouse strain after 6 days of exposure to hyperoxia.²⁵ BPD patients are at higher risk of developing PH, which contributes to significant morbidity and early mortality. It has been reported that 25–35% of infants with moderate-to-severe BPD will develop PH and RVH.²⁶ Similarly, 2HIT mice displayed RVH at 3 months of age as measured by Fulton's index, which is most likely secondary due to PH,²⁷ and therefore enabling studies into PH mechanism and novel experimental therapies.

Inflammation is known to be an important risk factor for the development of BPD.²⁸ Epidemiological data on the role of chorioamnionitis as a risk factor for BPD are inconsistent,^{29,30} but perinatal inflammation and episodes of sepsis,¹⁴ as well as pneumonia¹⁵ seem to be among risk factors for BPD. While other two hit models of BPD using antenatal LPS exist,^{17,31,32} the current 2HIT model is novel and unique, as it combines exposure to high

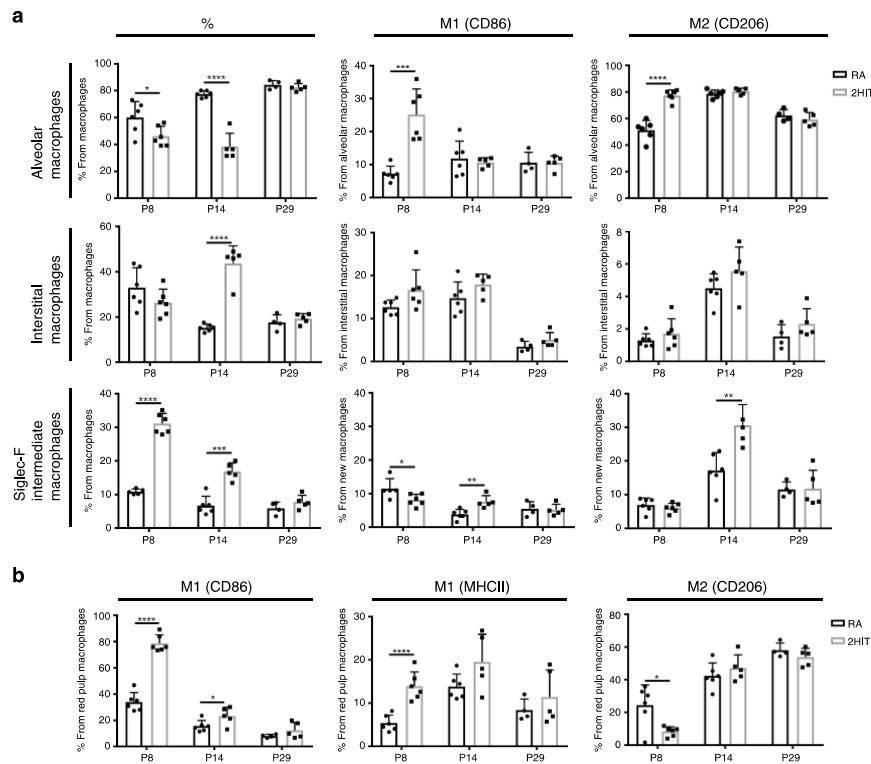


Fig. 6 Time course of lung and red pulp macrophage phenotype in the 2HIT compared to RA animals. **a** Lung macrophages. **b** Spleen red pulp macrophages. The values are given as relative percentages from parent populations and are represented as the means $\pm$ SD ($n = 4-6$ /group). Individual data points shown for each bar graph represent individual mice from multiple litters. P value < 0.05 was considered significant. Significant results are shown as $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $****P \leq 0.0001$.

level of oxygen and to postnatal inflammation. 2HIT animals demonstrated a robust inflammatory response, which in the lung is typically a cascade of events starting with activation of the coagulation and complement systems and stimulation of resident cells to promote recruitment and activation of other immune cells.³³ The hyperoxic tissue damage causes the release of intracellular proteins acting as damage associated molecular patterns. Additionally, LPS acts as a pathogen-associated molecular pattern. Both these molecular patterns are ligands to pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) on alveolar macrophages and epithelial type 2 cells, as well as on the circulating immune cells. The PRRs activated by hyperoxic injury have not been described in detail, but LPS is known to activate TLR4 through nuclear factor- κ B signal transduction.³⁴ In our model, we show a robust initiation of inflammatory response by production of chemokines G-CSF, GM-CSF, M-CSF, KC, LIX (C-X-C motif chemokine ligand 5 [CXCL5]), MCP-1 (C-C motif chemokine ligand 2 [CCL2]), MIP-1 α (CCL3), MIP-2 (CXCL2), as well as high expression of cytokines IL-1 α and IL-1 β , which are typically found in the initial phase of the inflammatory cascade. In the 2HIT model, the expression of IL-1 β peaked in the lung at P3, indicating an early proinflammatory immune response with high levels of cytokine and chemokine expression. Interestingly, recent studies in transgenic mice have shown that early postnatal IL-1 β overexpression leads to chronic lung injury similar to that seen

in the hyperoxia model. This points to the important role of this cytokine in the pathogenesis of BPD.³⁵

Along the increased chemokine levels, we observed recruitment of phagocytes, such as neutrophils, macrophages, and monocytes to the lungs. This correlated with a significant increase in the secretion of type 1 proinflammatory cytokines, such as IL-2, IL-6, IL-7, IL-12, IL-17, LIF, TNF α , and IFN γ . Interestingly, the gene expression levels of type 1 cytokines *Il6*, *Tnfa*, and *Tgfb1* reached a second peak in the beginning of the recovery phase one day after the pups returned to normoxia at P8. The reason for this is not yet apparent, but may indicate that the change in oxygen concentration initiates a second inflammatory response in the lungs. In addition, expression of chemokines required for the recruitment of cells of the adaptive immune system to the site of inflammation, such as RANTES (regulated upon activation, normal T cell expressed and presumably secreted) (CCL5) and MIG (CXCL9) was also upregulated.

Perturbations observed in 2HIT animals were associated with a robust change in pulmonary macrophage phenotype. We observed a decrease in alveolar macrophages and a simultaneous increase in a population of Siglec-F^{low} macrophages. The Siglec-F^{low} macrophages have previously been reported to be recruited to the lungs in an experimental fibrosis model, and lineage tracing linked the origin of these macrophages to circulating monocytes.²³ In addition to the changes in the number of macrophages,

we observed a shift from homeostatic M2 phenotype to more proinflammatory M1 phenotype in the early phases of the disease, whereas this was no longer the case at 1 month of age. This may be due to the maturation of a Siglec-F intermediate emerging macrophage population to the homeostatic phenotype. The shift in macrophage phenotype was observed in both the lung and spleen, emphasizing the fact that there is systemic involvement of the immune system in our 2HIT model. Macrophage in vivo phenotype is known to represent a spectrum of functional phenotypes rather than a simplified division to M1 and M2 categories,³⁶ but on the other hand, the simplified view can provide a more practical approach in terms of studying mechanism of action of novel therapies.¹²

In contrast to the increase in type 1 cytokines, we observed a significant decrease in expression of type 2 cytokines in the lungs of 2HIT animals. Type 2 cytokines are critical for resolution of inflammation and promotion of new tissue growth eventually leading to healing.³⁷ Transgenic mouse models have not been studied to explore the effect of type 2 cytokines in the hyperoxic lung injury of the newborn, except for IL-13, which did not protect newborn mice from hyperoxia-induced lung damage.³⁸ It is important to note that the type 2 response needs to be carefully balanced, as it can cause type 2 inflammation, which causes extensive pulmonary scarring and fibrosis, as well as asthma. In fact, Cheon et al.³⁹ reported increased expression of the type 2 cytokine genes *IL-13* and *IL-5* in the lungs of hyperoxia-exposed mice at 4 weeks of age, suggesting that hyperoxic injury results in late type 2 inflammation, which can explain the observed airway hyperreactivity. However, in our model no significant increase in type 2 cytokines was detected at 1 month of age, although we observed a trend towards increase in both the gene expression and protein levels of IL-4, IL-10, and IL-13 at this time in 2HIT animals. In fact, we detected only very early systemic type 2 cytokine production. One of the crucial functions of type 2 cytokines is to activate the adaptive immune system. The low expression level of type 2 cytokines may therefore indicate an insufficient adaptive immune response in response to the injury in the 2HIT model. However, putative changes in the adaptive immune response were beyond the scope of our study.

Our research has certain limitations. Ideally, single hit groups, RA + LPS and O₂, would be studied for all endpoints. However, neonatal LPS model has recently been characterized, and single dose of LPS was not found to cause permanent lung damage.⁴⁰ Furthermore, the neonatal hyperoxia model has been described over 30 years ago, and since then, the cytokine and immune cell changes have been characterized carefully.^{7,8,11–13} Also, recently, cell atlas of single-cell transcriptome of hyperoxic neonatal lung was characterized describing the sequential (P3–P7–P14) immune cell changes in hyperoxic conditions.⁴¹

Our study provides the basis for unraveling the complexity of the inflammatory response in experimental BPD, which is the first and critical step in investigating the link between inflammation and impaired lung development. Future steps in this direction involve the quickly evolving field of single-cell analysis, which will represent a next level in our understanding of the molecular pathogenesis of complex diseases such as BPD on a tissue level.^{10,41,42} Understanding the disease mechanism in cellular and molecular level will be essential in the clinical translation of novel therapies for BPD.

In this study, we have developed and characterized a novel 2HIT BPD model using a combination of short hyperoxia exposure (6 days) and postnatal LPS administration at P3, which more closely mimics the clinical conditions of BPD. The 2HIT model showed transient inflammation and dysplastic alveolarization with altered lung function. This model is robust and short, therefore allowing efficient preclinical testing for therapeutic interventions and exploring the disease pathogenesis, as well as mechanism of action of novel therapies.

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AUTHOR CONTRIBUTIONS

C.C.-D., M.H., A.V. performed experiments; C.C.-D., M.H., I.M., F.L. analyzed data; C.C.-D., M.H. interpreted results of experiments; C.C.-D., M.H. prepared figures; C.C.-D., M.H. drafted manuscript; B.T. approved final version of manuscript; C.C.-D., M.H., A.V., I.M., F.L., B.T. edited and revised manuscript; C.C.-D., M.H., F.L., B.T. conceived and designed research.

ADDITIONAL INFORMATION

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4.7 Appendix

4.7.1 Supplementary materials and methods

4.7.2 Supplementary figures

Supplementary Figure 4.1 Survival and body weight analysis of RA, RA+LPS, O₂ and 2HIT animals at P15 and 3 months of age, as well as collagen accumulation analysis in the RA and 2HIT animals.

Supplementary Figure 4.2 The FACS gating strategies.

Supplementary Figure 4.3 M1 and M2 polarization.

Supplementary Figure 4.4 Representative FACS histogram of Siglec-F intermediate macrophages.

4.7.3 Supplementary tables

Supplementary Table 4.1a – Antibody details

Supplementary Table 4.1b – Primer details

Supplementary Table 4.2a – The absolute detected cytokine protein quantities and the fold change from lungs of the RA compared with 2HIT animals

Supplementary Table 4.2b – The absolute detected cytokine protein quantities and the fold change from serum of the RA compared with 2HIT animals

All supplementary material can be found online on the website of *Pediatric Research*:

<https://www-nature-com.proxy.bib.uottawa.ca/articles/s41390-020-0967-6#Sec19>

CHAPTER 5: Complementary studies of UC-MSCs using the 2HIT model

5.1 Context

Neonatal management of preterm infants often leads to damage in the developing lung and subsequent morbidity and mortality, referred to as bronchopulmonary dysplasia (BPD). BPD currently lacks a cure, and mesenchymal stromal cells (MSCs) are being investigated as a potential therapy for preventing and treating many lung diseases, including BPD. Previously, in **Chapters 2 and 3**, we have determined that umbilical cord (UC)-MSCs derived from term and preterm donors with a progenitor-like transcriptome provide optimal therapeutic effects in hyperoxia-induced lung injury in neonatal rats. BPD is a multifactorial disease, and in addition to supplemental oxygen exposure, inflammation plays a major role in the disease's onset and progression. Molecular mechanisms by which postnatal inflammation exposure disrupts lung development are poorly understood. Thus, we have developed a novel 2-hit (2HIT) BPD mouse model with postnatal *Escherichia coli* lipopolysaccharide (*E. coli* LPS) and hyperoxia exposure and characterized its lung pathology and innate immune response. The 2HIT model also represents a robust BPD model for efficient *in vivo* testing of UC-MSCs. Some of the top tasks remaining to obtain optimal clinical outcomes of UC-MSCs as a treatment for BPD are optimization of the dose, the route of cell administration, and the selection of the most suitable timing(s).

In the present study, we used the 2HIT mouse BPD model to investigate the therapeutic efficacy of UC-MSCs with a progenitor-like transcriptome. In addition, we determined the optimal route (intratracheal [*i.t.*] versus intravenous [*i.v.*]), timing, and dose of UC-MSCs injection, and evaluated the therapeutic effects in terms of survival, body weight (BW), lung structure, and function.

5.2 Materials and methods

Please refer to the materials and methods section of the previously mentioned article (1) in **Chapter 4** in addition to the following information.

Preparation of UC-MSCs for *in vivo* administration

Passage number (PN) three UC-MSCs from donor T1, T3, or P5 (which all have a progenitor-like transcriptome) were plated at 5,000 cells/cm² in T75 flask and were placed for 24 h in a cell culture incubator at 37°C, 5% CO₂ and 5% O₂ prior to collection as previously described (2).

Intratracheal administration of UC-MSCs

At postnatal day (PD) four, room air (RA) and 2HIT mice pups were anesthetized by inhalation of isoflurane (anesthesia was induced and maintained with a concentration of 4% and 3% respectively, with an O₂ flow rate of 0.5-1 L/min). Anesthetized pups were injected *i.t.* with 15 µl PBS or UC-MSCs (from donor T3) (in 15 µl PBS) at a dose of 10×10⁶ cells/kg BW (shown to have a therapeutic effect in our hyperoxic neonatal rat BPD model). For each group, n=6. Pups were randomized to receive different treatments.

Intravenous administration of UC-MSCs

At PD1, PD2, or PD3 RA and 2HIT mice pups were anesthetized using isoflurane at a concentration of 3-4% with an O₂ flow rate of 0.5-1 L/min. Anesthetized pups were placed on a “WeeSight Handheld Transilluminator” (WeeSight, Respironics, Murrysville, PA), and 30 µl PBS or UC-MSCs (from donor P5 for the “optimal time of injection study” and from donor T1 for the

“dose-escalation study”) (in 30 μ l PBS) were administered into the temporal (facial) vein at a dose of 10×10^6 , or 20×10^6 or 40×10^6 cells/kg BW. Pups injected at PD3 were either injected three hours before or three hours after *E. coli* LPS administration. Pups were randomized to receive different treatments.

For the “optimal time of injection study”, for RA pups injected with UC-MSCs, n=5 at PD1, n=6 at PD2, and n=11 at PD3. For simplicity, all RA pups injected with UC-MSCs were combined in one group “RA+MSCs”. For 2HIT pups injected with PBS, n=7 at PD1, n=5 at PD2, n=6 at PD3 before LPS injection, and n=12 at PD3 after LPS injection. For simplicity, all PBS-injected 2HIT pups were combined in one group “2HIT+PBS”. For the “dose-escalation study”, for RA pups injected with PBS, n=8 at PD2, and n=6 at PD1+PD2. For simplicity, all RA pups injected with PBS were combined in one group “RA+PBS”. For 2HIT pups injected with PBS, n=8 at PD2 and n=8 at PD1+PD2. For simplicity, all 2HIT pups injected with PBS were combined in one group “2HIT+PBS”.

5.3 Results

5.3.1 Intratracheal injections at PD4 were not tolerated by 2HIT mice pups

We aimed to administer the UC-MSCs during the saccular stage of lung development, as this is the stage at which preterm infants that develop BPD are born. Thus, to determine if *i.t.* injections were possible during that stage in mice (embryonic day (E)17 to PD4), PD4 RA and 2HIT mice were subjected to *i.t.* injection with either 15 μ l PBS or UC-MSCs from donor T3 at a dose of 10×10^6 cells/kg BW in 15 μ l PBS (vehicle control). Following PBS and administration of UC-MSCs, we observed 100% mortality in both 2HIT groups. Thus, *i.t.* injection of cells and PBS

during the saccular phase of lung development at PD4 is not favorable in the 2HIT mice BPD model.

5.3.2 PD1 and PD2 are the optimal times for UC-MSC intravenous injection in 2HIT mice pups

Since we determined that *i.t.* injections were not favorable in the 2HIT mice BPD model, we explored the possibility of using another main route of MSCs' transplantation in animal experiments for BPD: *i.v.* injection. To determine the optimal time of *i.v.* injection during the saccular stage of lung development in 2HIT mice pups, we took advantage of the temporal (facial) vein located just anterior to the earbud, which is clearly visible for the first three days after birth on either side of the head. RA or 2HIT mice were injected with 15 μ l PBS (vehicle control) or UC-MSCs from donor P5 at a dose of 10×10^6 cells/kg BW in 15 μ l PBS either at PD1, PD2, PD3 before LPS injection or PD3 after LPS injection (Fig. 5.1A).

Exposure to hyperoxia and LPS (2HIT) resulted in a significant decrease in survival (33.3%) compared to pups exposed to RA (100%), and UC-MSCs had no adverse effect on survival in RA animals (100%) (Fig. 5.1B). Administration of UC-MSCs at PD1, PD2, and PD3 before LPS administration improved the survival of 2HIT pups (57.1%, 70.0%, and 63.6%, respectively), which reached significance when UC-MSCs were injected at PD2. Contrastingly, administration of UC-MSCs at PD3 three hours following LPS injection results in adverse reactions leading to significant decreased in survival of 2HIT mice (0%).

In addition, 2HIT mice injected with PBS showed severe destruction of the alveolar architecture with widened airspaces and alveolar simplification, which was quantified by measuring the mean linear intercept (MLI). MLI was significantly increased in the 2HIT mice

group injected with PBS compared to RA pups (Fig. 5.1C, D). Administration of UC-MSCs had no adverse effect on the alveolar structure of RA pups (Fig. 5.1C, D). UC-MSCs treatment of 2HIT mice pups at PD1, PD2, and PD3 before LPS partially preserved alveolar growth, and a significant reduction in MLI was observed with UC-MSCs injections at PD1 and PD2 compared to 2HIT PBS injected pups (Fig. 5.1C, D).

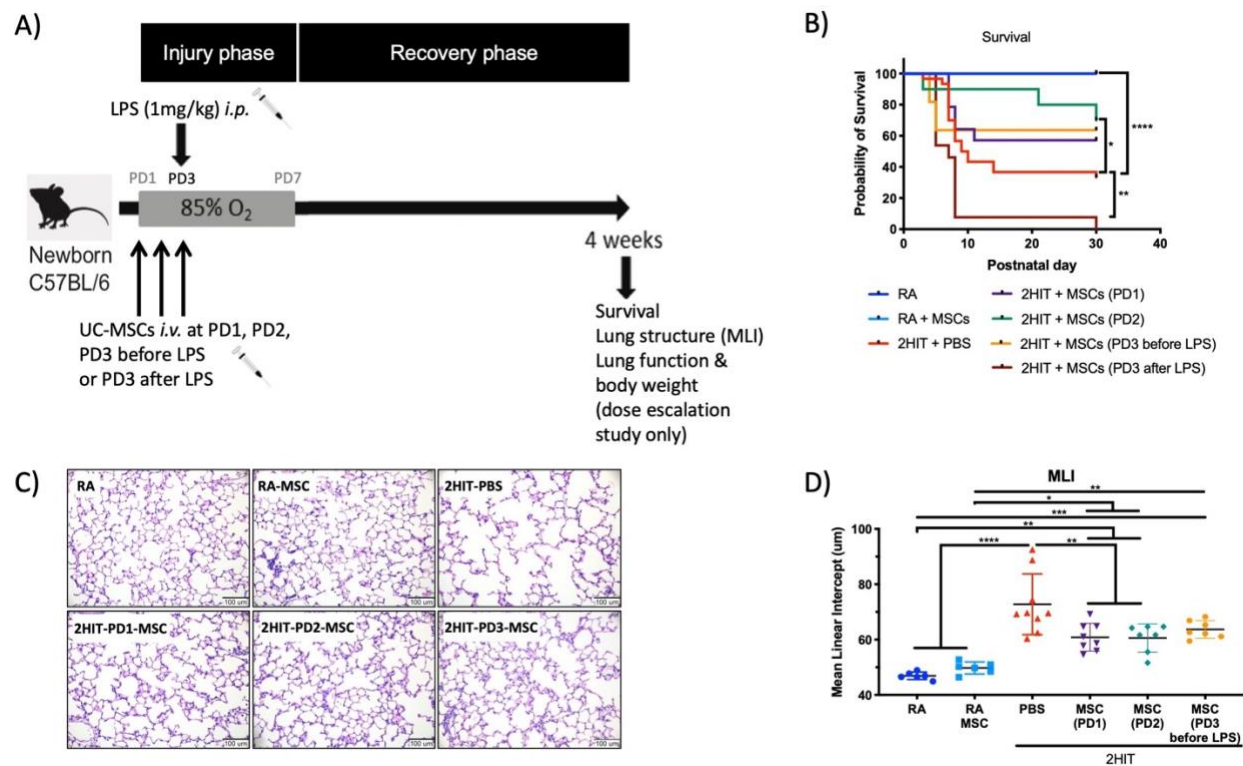


Figure 5.1 UC-MSC intravenous injection at PD2 provides optimal healing effects in terms of survival and lung structure.

A) Experimental design and endpoints. **B)** Kaplan-Meier survival curve (RA: n=23, RA + MSCs: n=22, 2HIT + PBS: n= 30, 2HIT + MSCs (PD1): n= 14, 2HIT + MSCs (PD2): n=10, 2HIT + MSCs (PD3 before LPS injection): n=11, 2HIT + MSCs (PD3 after LPS injection): n= 13). **C)** Representative images of H&E-stained lung sections for each group at PD30. **D)** Quantification of alveolar size using the MLI method in lung tissue sections at PD30 (n=6-9/group). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels except panel **B** for which log-rank (Mantel-Cox) test was used. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001.

Thus, we have determined that *i.v.* is the optimal injection route compared to *i.t.* delivery in 2HIT mice pups. Also, *i.v.* administrations of UC-MSCs before LPS injection are preferred, and PD2 is the optimal injection time for the mouse 2HIT BPD model.

5.3.3 Repeated injections of UC-MSCs with either 20×10^6 cells/kg or 40×10^6 cells/kg BW (once at PD1 and once at PD2) partially reverse 2HIT-induced loss in BW, lung growth, and function injury

To identify the optimal dose of UC-MSCs using single and multiple *i.v.* injection strategies in the 2HIT model, we tested four UC-MSCs doses: 10×10^6 cells/kg BW (single *i.v.* injection at PD2), 10×10^6 cells/kg BW injected twice (*i.v.* once at PD1 and once at PD2), 20×10^6 cells/kg BW injected twice (*i.v.* once at PD1 and once at PD2) and 40×10^6 cells/kg BW injected twice (*i.v.* once at PD1 and once at PD2).

No difference in survival was observed between RA pups injected with the vehicle (PBS) (92.3%) and RA pups kept untreated (95.8%) (Fig. 5.2A). Survival of 2HIT mice pups injected with PBS (50.0%) was significantly lower than RA pups (Fig. 5.2A). Survival of 2HIT pups injected with all four doses was higher than 2HIT pups injected with the vehicle and was significantly higher in 2HIT pups injected with a single dose of 10×10^6 cells/kg BW. Survival of 2HIT pups injected twice with 10×10^6 cells/kg BW was significantly lower (62.5%) than RA pups.

No difference in BW was observed between RA pups injected with PBS, and RA pups kept untreated at PD15, PD21, and PD29-30 (Fig. 5.2B-D). The BW of 2HIT mice pups injected with PBS was significantly lower than RA pups at all assessed time points (Fig. 5.2B-D). The BW of 2HIT pups treated with all four doses of UC-MSCs was significantly higher at PD15 compared to the BW of 2HIT pups injected with PBS (Fig. 5.2B). At PD21 and PD29-30, the BW of pups injected with all four doses of UC-MSCs was higher than that of 2HIT pups injected with PBS but

reached significance only in 2HIT pups injected with a single dose of 10×10^6 cells/kg BW (at PD21 only), and in 2HIT pups injected twice with 20×10^6 cells/kg BW (at PD21 and PD29-30) (Fig. 5.2C, D).

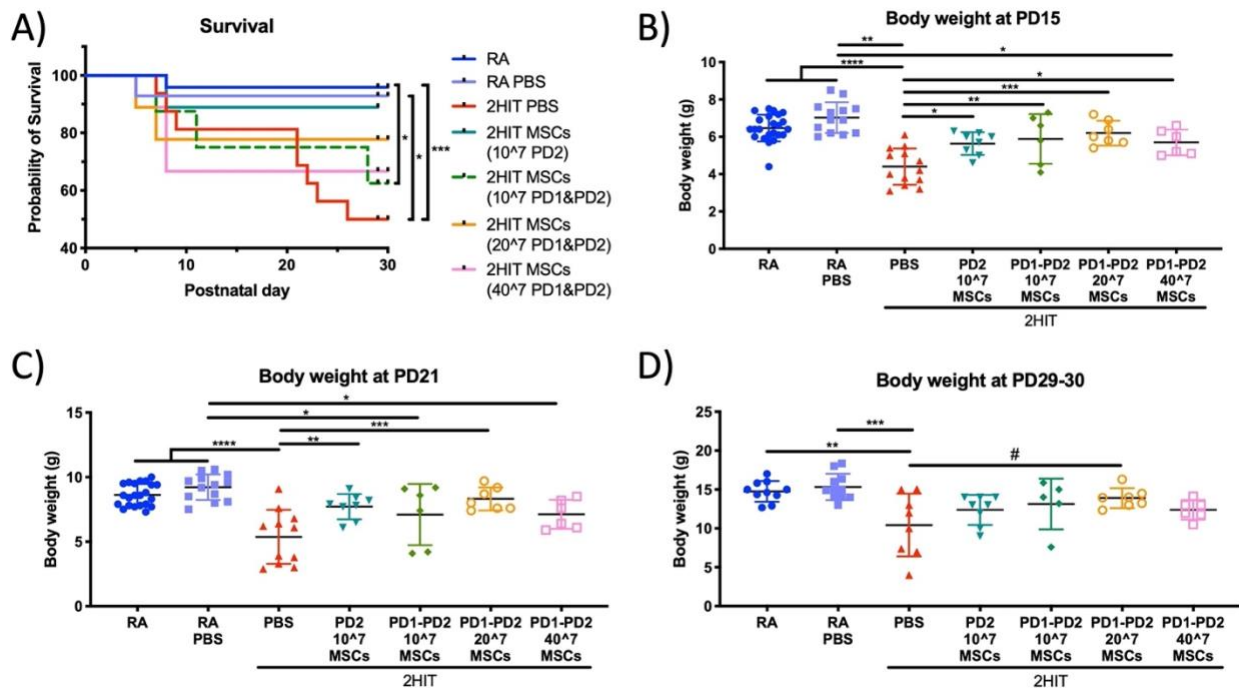


Figure 5.2 Treatment with UC-MSCs improved survival and body weight of 2HIT mice.

A) Kaplan-Meier survival curve (RA: n=24, RA PBS: n=14, 2HIT PBS: n= 16, 2HIT PD2 MSCs 10^7 : n= 9, 2HIT PD1+ PD2 MSCs 10^7 : n=8, 2HIT PD1+ PD2 MSCs 20^7 : n=9, 2HIT PD1+ PD2 MSCs 40^7 : n=9). **B)** BW of animals at PD15 in each group (RA: n=23, RA PBS: n=13, 2HIT PBS: n= 13, 2HIT PD2 MSCs 10^7 : n= 8, 2HIT PD1+ PD2 MSCs 10^7 : n=6, 2HIT PD1+ PD2 MSCs 20^7 : n=7, 2HIT PD1+ PD2 MSCs 40^7 : n=6). **C)** BW of animals at PD21 in each group (RA: n=23, RA PBS: n=13, 2HIT PBS: n= 11, 2HIT PD2 MSCs 10^7 : n= 8, 2HIT PD1+ PD2 MSCs 10^7 : n=6, 2HIT PD1+ PD2 MSCs 20^7 : n=7, 2HIT PD1+ PD2 MSCs 40^7 : n=6). **D)** BW of animals at PD29 or PD30 in each group (RA: n=10, RA PBS: n=11, 2HIT PBS: n= 8, 2HIT PD2 MSCs 10^7 : n= 8, 2HIT PD1+ PD2 MSCs 10^7 : n=5, 2HIT PD1+ PD2 MSCs 20^7 : n=7, 2HIT PD1+ PD2 MSCs 40^7 : n=6). Values are represented as the mean $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels except panel **A** for which log-rank (Mantel-Cox) test was used. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001. For panel **D**, *t* test (unpaired, two-tailed) was used to compare results between two groups only, and significant results are shown with # (*P* $\leq$ 0.05 was considered significant).

The 2HIT mice injected with PBS showed severe alveolar destruction and significantly higher MLI compared to RA pups (Fig. 5.3A, B). Injection of PBS (vehicle control) in RA pups did not alter the alveolar structure and MLI (Fig. 5.3A, B). 2HIT mice treated with all UC-MSCs doses showed improvement in alveolar structure and significantly lower MLI than the 2HIT mice injected with PBS (Fig. 5.3A, B).

No difference in lung function was observed between RA pups injected with the vehicle compared to RA pups left untreated (Fig. 5.3C-G). An upward shift of the pressure-volume (PV)-loop and a significant increase in volume at 30 cmH₂O in the 2HIT mice pups injected with the vehicle compared to RA pups were observed (Fig. 5.3C, D). In addition, static compliance (Cst), inspiratory capacity (A), and hysteresis (area under the PV-loop) were significantly increased in 2HIT pups injected with the vehicle compared to RA pups (Fig. 5.3E-G). Treatment with all four doses of UC-MSCs induced a downward shift of the PV-loop compared to 2HIT pups injected with PBS (Fig. 5.3C). Only treatment with a repeated dose of 20×10^6 cells/kg BW significantly reduced the lung volume at 30 cmH₂O in 2HIT pups compared to 2HIT pups injected with PBS (Fig. 5.3D). In addition, treatment with a repeated dose of 20×10^6 cells/kg BW significantly improved Cst, A, and hysteresis compared to 2HIT pups injected with PBS (Fig. 5.3E-G). Treatment with a repeated dose of 40×10^6 cells/kg BW also significantly improved lung function in terms of hysteresis (Fig. 5.3G).

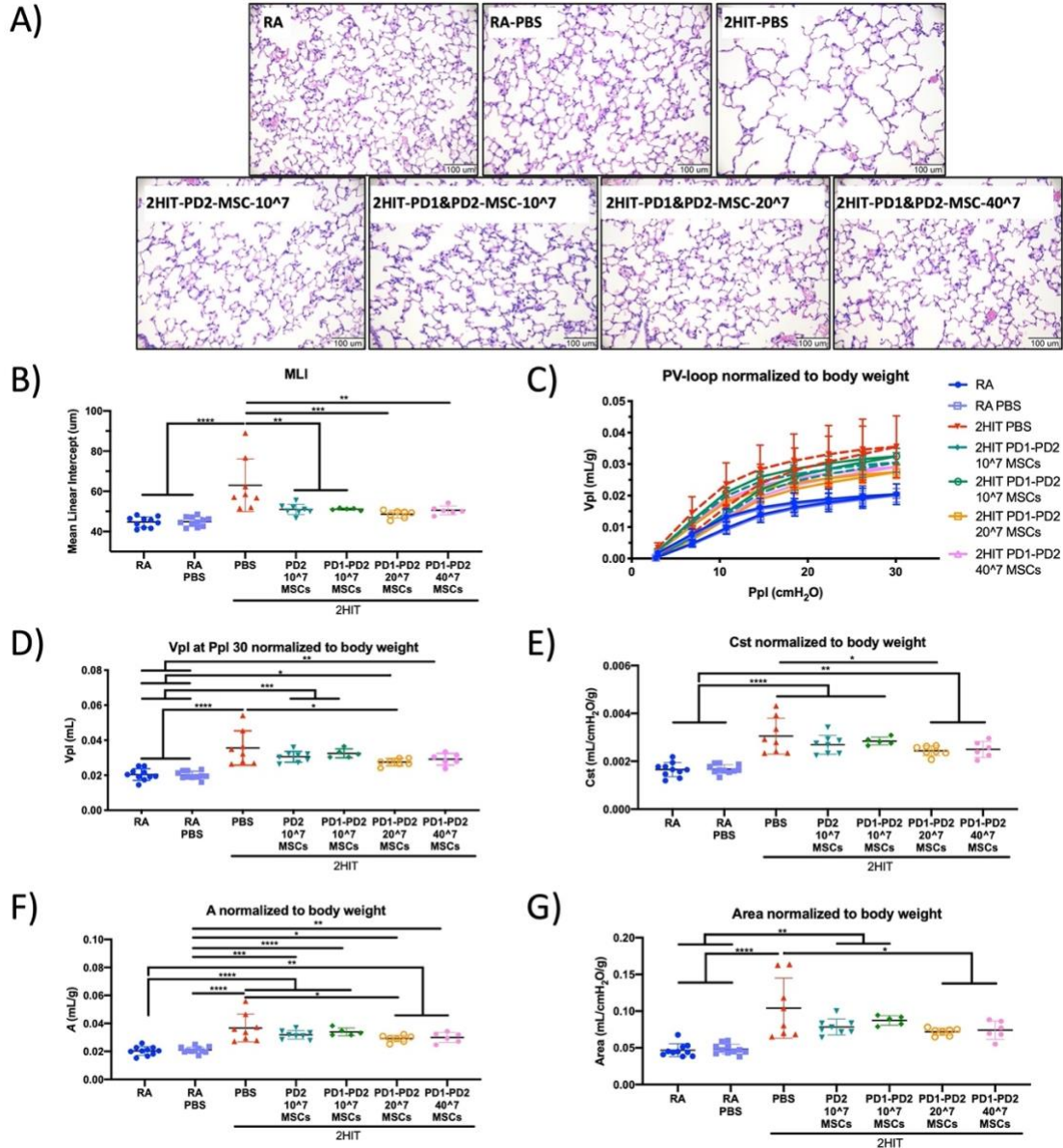


Figure 5.3 Repeated injections of UC-MSCs with either 20×10^6 cells/kg or 40×10^6 cells/kg BW partially reverse 2HIT-induced lung growth and function injury.

A) Representative images of H&E-stained lung sections for each group at PD29-30. **B)** Quantification of alveolar size using the MLI method in lung tissue sections at PD29-30. **C)** BW-adjusted PV-loop for each group at PD29 or PD30 (Vpl: volume plethysmography, Ppl: pressure plethysmography) and **D)** BW-adjusted lung volume at peak pressure 30cmH₂O. **E)** BW-adjusted static compliance (Cst), **F)** BW-adjusted inspiratory capacity (A) and **G)** BW-adjusted hysteresis (area under PV-loop) for each group at PD29-30. PV-loops are shown as the mean volume of all animals within one group at each pressure point. Values are represented as the mean $\pm$ STD (n=5-11/group). *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels. Significant results are shown as **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001.

5.4 Conclusion

In this study, we identified UC-MSCs' optimal route of administration, dose, and administration time in a novel 2HIT mouse BPD model with postnatal LPS and hyperoxia exposure. A dose of 20×10^6 cells/kg BW injected twice *i.v* (once at PD1 and once at PD2) showed significant therapeutic effects in terms of BW, lung structure, and function. A lower dose of 10×10^6 cells/kg BW injected *i.v* either once at PD2 or twice (PD1 and PD2) also showed therapeutic effects, but not to a significant degree in terms of BW and lung function. Moreover, a higher dose of 40×10^6 cells/kg BW injected twice *i.v* (PD1 and PD2) also showed therapeutic effects, but to a lower extent in terms of lung function compared to the dose of 20×10^6 cells/kg BW injected twice. Therefore, there is an optimal therapeutic dose of UC-MSCs for experimental BPD and these results may provide basic data to decide on an optimal treatment regime of UC-MSCs for BPD patients.

Our previous experiments in **Chapters 2 and 3** have shown that *i.t.* administration of UC-MSCs with a progenitor-like transcriptome has the ability to reduce lung damage in hyperoxia-exposed neonatal rats. However, *i.t* injection in 2HIT mice at PD4 was not well tolerated, and mortality of 100% was observed. The small size of 2HIT mice at PD4 due to the exposure to two different stressors (hyperoxia and LPS) might have contributed to the high percentage of mortality following *i.t.* injection.

To explore the therapeutic efficacy of UC-MSCs via the *i.v.* route, we performed facial vein injections of cells in PD1 to PD3 2HIT mice. UC-MSCs improved the survival and lung structure of all 2HIT mice pups, except when the injection was performed three hours after *i.p.* LPS injections. Thus, injection of UC-MSCs following the high increase of inflammation triggered by LPS administration results in adverse reactions leading to 100% mortality of 2HIT mice pups.

Therefore, the best timings for UC-MSCs *i.v.* delivery via the facial vein are PD1, PD2, and PD3 before LPS administration.

As far as we know, this is the first dose-finding study of MSCs in experimental BPD using the *i.v.* route. Dose-finding studies using *i.t.*, intranasal (*i.n.*), and *i.p.* delivery routes were previously accomplished in rodent hyperoxia BPD models in which 5×10^3 , 5×10^4 , 1×10^5 , 5×10^5 , and 1×10^6 cells were administered (3,4). Low and high doses of *i.n.* MSCs had no significant effect on lung structure and function, whereas the highest *i.t.* dose used (5×10^5) and *i.p.* dose used (1×10^6) were the most efficacious at mitigating hyperoxia-induced lung injury. Considering the approximate BW of the animals in each study, these doses were in the order of 10×10^6 to 10×10^7 cells/kg BW. Previous studies using the *i.v.* route in rodent models of BPD have shown improvement in lung structure with the administration of 5×10^4 and 1×10^5 cells, which correspond to approximately 2×10^6 to 20×10^6 cells/kg BW (5–8). Thus, for our study, we considered a similar starting dose of 10×10^6 cells/kg BW, which also demonstrated therapeutic benefits in our hyperoxia-induced lung injury model in neonatal rats, and wanted to determine if we could see a dose-dependent response up to 40×10^6 cells/kg BW injected twice, once at PD1 and once at PD2. In addition, early phase clinical trials to assess the safety and feasibility of MSCs' administration have also tested similar doses ranging from two doses of 1×10^6 cells/kg BW up to one dose of 20×10^6 cells/kg BW (9–11).

Administration of different UC-MSCs' doses before LPS injection all provided therapeutic efficacy, with the dose of 20×10^6 cells/kg BW injected twice (once at PD1 and once at PD2) showing the most favorable results.

These data may provide important basic data for determining the route of injection, timing, and dosage in the clinical application of UC-MSCs in BPD patients.

5.5 References

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CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

6.1 Many challenges remain to translate MSC as a treatment for BPD

Based on their beneficial immunomodulatory and other pleiotropic effects, MSCs have emerged as a promising therapeutic agent for many diseases, including BPD (1,2), the chronic lung disease of prematurity. In this context, MSCs derived from the umbilical cord (UC-MSCs) represent an interesting candidate. Already, early-phase clinical trials have proven that the administration of MSCs in infants at high risk of developing BPD is safe and feasible (3–7). However, many challenges remain for the clinical application of MSCs to fully harness their potential for promoting tissue repair and modulating inflammation (8). Within the research chapters included in this thesis, we provide new information on 1) the heterogeneity of different UC-MSC populations, 2) the relation between UC-MSC transcriptome and their therapeutic potential in hyperoxia-induced lung injury in neonatal rats, 3) the immune response and lung injury in a novel 2HIT mice BPD model, and 4) the optimal route, timing, and dose of UC-MSCs in the 2HIT model.

6.2 Characterization of term and preterm UC-MSCs using scRNA-seq

MSCs exhibit *in vitro* functional heterogeneity, and their exact molecular characteristics remain unclear, limiting clinical applications (9,10). Indeed, different functional attributes are specific to distinct MSC subpopulations, where certain subsets are prone to differentiate into specific lineages (11), while some are better at self-renewal (12,13), and some show senescence earlier than others (14). Also, the proportion of cells with high regenerative properties varies across MSC donors (10,15,16). Therefore, a better understanding of the composition of MSC subpopulations is critical for the reproducible generation of functionally equivalent MSCs and for enhancing their efficacy in clinical trial settings.

Among MSCs of various sources, UC-MSCs are regarded as an attractive candidate for cell therapy due to their easy availability, higher proliferation, and lower immunogenicity (17,18). Additionally, in the prospect of using MSCs as a treatment for BPD, UC-MSCs represent an opportunity for autologous cell therapy. In **Chapter 2**, we took advantage of scRNA-seq technology to determine the transcriptome of cultured UC-MSCs isolated from five term donors and HNDFs, a non-progenitor control cell line (generally known to have no therapeutic effect (19)). Limited heterogeneity within UC-MSCs was observed with only two identified clusters composed of UC-MSCs from mixed-donors (clusters 0 and 2), and a unique transcriptome was identified for one UC-MSC donor T2 (cluster 1) and HNDFs (cluster 3). Also, GO enrichment analysis revealed two distinct UC-MSC subpopulations: 1) UC-MSCs from donor T1, T3, T4, and T5 showed enriched GO terms related to cell division, cell proliferation, cell cycle, gene expression, cell signaling, and protein folding functions and were termed “progenitor-like” UC-MSCs, and 2) UC-MSCs from donor T2 were enriched in terms related to ECM organization and collagen processes, reminiscent of HNDFs’ phenotype, and were termed “fibroblast-like” cells.

As mentioned earlier, UC-MSCs could provide the opportunity for an autologous treatment for BPD, but so far, only term UC-MSCs have been considered for cell therapy. Furthermore, to identify if autologous treatment is safe and feasible, it is important to determine if UC-MSCs derived from preterm donors with or without pregnancy-related complications often associated with prematurity, such as chorioamnionitis and preeclampsia, are affected by these conditions. In **Chapter 3**, alongside the term UC-MSCs and HNDFs, we investigated the transcriptomic profile of UC-MSCs isolated from 11 preterm donors with or without pregnancy-related complications (chorioamnionitis and preeclampsia). scRNA-seq analysis revealed that regardless of pregnancy-

related conditions, following cell culture, most preterm UC-MSCs clustered together with term UC-MSCs in clusters 0, 1, and 5 (referred to as “main UC-MSC clusters”). Only donor T2, two preterm UC-MSC donors with preeclampsia (donors LPP3 and PP1), and HNDFs formed distinct clusters (clusters 3, 2, 4, and 6, respectively). Moreover, the main UC-MSC clusters were significantly enriched in genes involved in cell division, transcriptional and translational processes, similar to our previous results for progenitor-like UC-MSCs in **Chapter 2**. Conversely, UC-MSC donors that formed their own cluster were enriched in genes related to ECM organization and collagen processes, similar to our previous results for fibroblast-like cells in **Chapter 2**.

In addition, UC-MSCs and HNDFs met the MSCs’ criteria (20): all adhere to plastic, all were positive for *CD73*, *CD90*, and *CD105*, and lack the expression of *CD14*, *CD11B*, *CD34*, *CD45*, *CD79A*, *CD19* and *HLA-DR*, and all could differentiate into osteoblasts, chondrocytes, and adipocytes *in vitro*. This is in line with previous studies, which found that fibroblasts exhibit MSC phenotype and multilineage differentiating potential (21–23). Thus, UC-MSCs are indistinguishable from HNDFs using the common MSCs’ minimal criteria (20), and better identification methods for UC-MSCs are needed.

To our knowledge, within the timeframe of this thesis, eight other scRNA-seq studies of cultured UC-MSCs have been published, each including UC-MSCs derived from four or fewer term donors only (24–31). Six of those studies focused on characterizing the differences in mRNA expression profiles between UC-MSCs and other sources of MSCs such as the BM, AT, dermis, foreskin, and synovial fluid (25–27,29–31). The remaining two studies compared the mRNA expression profiles between UC-MSCs and the UC tissue (28) or between different UC-MSCs’ stimulation states (with or without inflammatory cytokines) and PNs (24). Differences in gene expression involving numerous biological processes, including apoptosis, angiogenesis, ECM, and

wound-healing via immune regulation, were found (25,29). Similar to our results, DEGs within UC-MSC subpopulations were mainly enriched in cell proliferation, cell cycle activity, DNA metabolic process, ECM organization, protein folding, post-transcriptional regulation of gene expression, regulation of apoptotic process, RNA splicing, translation, transcription factor activity, senescence and antigen processing/presentation (26–29). Moreover, cell cycle activity was noted as one of the primary sources of UC-MSCs' heterogeneity (24,25,27), which was also the case in a scRNA-seq study using thawed UC-MSCs (cryopreserved UC-MSCs which were thawed, and right away processed for scRNA-seq, without reviving the cells in culture) (32). Taken together, these results uncover the molecular heterogeneity of cultured UC-MSCs and pave the way for exploring the distinct functional properties of each subpopulation.

6.3 Identification of a UC-MSC subpopulation that provides a therapeutic effect in a neonatal rat model of hyperoxia-induced lung injury

All other UC-MSC scRNA-seq studies were limited to *in vitro* work, and the exact relationship between *in vitro* characteristics of UC-MSCs and *in vivo* therapeutic potential remains unknown. In **Chapters 2 and 3**, we explored the association between the transcriptome of cultured UC-MSCs and their therapeutic effects in hyperoxia-induced lung injury in neonatal rats. We showed that progenitor-like UC-MSCs, including term, preterm and preterm UC-MSCs with pregnancy related-complications (donors T3, T4, P5, PP2, and PC1) could mitigate hyperoxia-induced lung injury in newborn rats, but not fibroblast-like UC-MSCs (donors T2, LPP3, and PP1), nor HNFs.

As far as we know, we are the first to demonstrate that UC-MSCs isolated from different donors have different therapeutic potentials in experimental BPD. On the other hand, recent studies

have demonstrated differences in the healing effect of various MSC sources in hyperoxia-induced models of BPD. Benny *et al.* have shown that treatment with UC-MSCs provided superior improvement in some morphometric measurements of alveolarization with improved lung epithelial cell healing and showed fewer macrophage infiltration as compared to treatment with BM-MSCs (33). In another study, BM-MSCs, UC-MSCs, and AF-MSCs had therapeutic effects in a hyperoxia-induced BPD model, with the important remark that UC-MSCs were shown to have the best immunosuppressive effect compared to BM-MSCs and AF-MSCs, while the latter had better therapeutic effects in regards to pulmonary alveoli histological changes and neovascularization (34). These results demonstrate that MSCs derived from various biological sources exhibit healing effects by different modes of action. Moreover, in our data, although fibroblast-like UC-MSCs do not exert the same repair effect as progenitor-like UC-MSCs in experimental BPD, there is a possibility that these cells may be helpful in different medical conditions in which ECM-related gene expression could be beneficial (e.g., repairing degenerated cartilage (35)). Taken together, these results support the idea that it may be advantageous to carefully select the MSC subpopulation (source and donor) that exhibits a phenotype corresponding to the function most appropriate for treating the pathology of interest.

6.4 Identification of therapeutic UC-MSC molecular signature

The absence of specific molecular markers to identify therapeutic UC-MSCs hinders progress towards harnessing the putative repair potential of these cells and contributes to significant controversy (36). In **Chapters 2 and 3**, using scRNA-seq analysis, we identified marker genes for the therapeutic UC-MSC and non-therapeutic cell populations (fibroblast-like UC-MSCs and HNDFs). Among them, the high expression of HLA-A, -B, and -C (HLA-ABC), key

determinants of allograft rejection, was found within the non-therapeutic cell populations. While MSCs were initially believed to be immune-privileged, several studies have suggested otherwise, where immune responses against donor antigens following MSCs' administration were reported (37–39). MHC I mismatch might contribute to the rejection of MSCs from the host and could harm their therapeutic potential. In this regard, we observed that two to three days following the administration of therapeutic (low HLA-ABC expression) and non-therapeutic (high HLA-ABC expression) UC-MSCs, more therapeutic UC-MSCs were retained in the lungs of hyperoxia-exposed rat pups compared to non-therapeutic UC-MSCs. Moreover, while therapeutic UC-MSCs expressing high levels of HLA-ABC had reduced healing potential in experimental BPD, non-therapeutic UC-MSCs expressing either high or low levels of HLA-ABC did not provide therapeutic potential. Hence, HLA-ABC low expression marked the therapeutic UC-MSC population, but other factors contribute to the lack of therapeutic effect by non-therapeutic cells.

Within the DEGs characteristic of therapeutic UC-MSCs, we identified *MKI67*, a marker of proliferation, as well as the transcription factor *HMGB2*, previously shown to be highly expressed in proliferating MSCs and contributing to suppressing MSC differentiation (40). *IGFBP5* was identified as a marker of the non-therapeutic UC-MSCs and has been shown to enhance osteogenic differentiation of UC-MSCs (41). Thus, these results suggest that the therapeutic subpopulation of UC-MSCs maintains proliferative capacity, while the non-therapeutic one might be more committed, further restricted to a particular differentiated fate. Correspondingly, gene-set enrichment analysis showed that biological processes related to cell cycle activity and cell proliferation were enriched in therapeutic cells. In contrast, non-therapeutic cells were enriched in ECM synthesis and collagen-related processes and displayed a more senescent phenotype. In addition, our observations are consistent with the other scRNA-seq studies

that discovered similar UC-MSC subpopulation marker genes. Indeed, *MKI67* was identified as a DEG in UC-MSC subpopulations in many other scRNA-seq studies (27,28,30,31). Furthermore, Wang *et al.* identified *MKI67*, *TPX2*, *TOP2A*, *HMGA1*, and *HMGB2* as markers of a first UC-MSC subpopulation, which is in line with the DEGs characteristic of our therapeutic UC-MSCs, and *IGFBP5*, *IGFBP3*, and *COL3A1* as markers of a second UC-MSC subpopulation, which correspond to DEGs characteristic of our non-therapeutic UC-MSCs (28). In line with another scRNA-seq study of uncultured UC-MSCs (UC-MSCs freshly isolated from the UC), four out of the top ten marker genes of UC-MSCs expressing high ECM production genes *TIMPI*, *COL3A1*, *COL1A2*, and *NDUFA4L2* were found in our molecular signature of non-therapeutic cells (42). Moreover, Wang *et al.* suggested that *TNFRSF12A* might be a promising cell surface marker to isolate UC-MSC with high proliferative capacity (28). However, in our data, this marker is not differentially expressed between progenitor-like and fibroblast-like cell subpopulations. Other than HLA-A, -B, and -C, few cell surface markers were identified within the list of DEGs. Nevertheless, using flow cytometry, we could confirm protein expression of intracellular therapeutic cell markers PTX3, MARCKS, and IDH2 in therapeutic UC-MSCs, which could serve as markers for predicting the therapeutic effect of UC-MSCs in the context of BPD. Indeed, in the future, cut-off values of PTX3, MARCKS, and IDH2 levels may be useful in screening UC-MSCs with optimal repair potential for BPD by testing an aliquot of cells for these markers before clinical use. However, it may be possible that more markers will be necessary to discriminate between therapeutic and non-therapeutic cells completely, and this will require further experimental investigations.

Furthermore, DEGs characteristic of therapeutic UC-MSCs might also help us better understand UC-MSC biology and mechanisms of action which provide their therapeutic effect. It

is well-accepted that MSC repair capacity lies mainly in the production of paracrine factors and cell-to-cell contact-mediated mechanisms. Previous findings suggest that MSC-secreted PTX3 reduces inflammation by inducing macrophage polarization towards the M2 state, which contributes to MSCs' therapeutic potential in experimental BPD (43). PTX3 might not only mark the therapeutic UC-MSC cell population but might also be involved in the cells' mode of action.

Also, in **Chapter 3**, to better understand intercellular communications and unveil the possible interactions between UC-MSCs and neonatal lung cell populations (NLCPs), we used CellChat, a platform able to quantitatively infer and analyze communication probabilities of ligand-receptors interactions associated with each signaling pathway using scRNA-seq data. The results of CellChat analysis further confirmed the high activity related to collagen processes within the non-therapeutic cells since more interaction possibilities via collagen-related pathways were found in those cells compared to therapeutic UC-MSCs. In addition, we were particularly interested in the signaling pathways known to be dysregulated during the alveolarization process in BPD, including TGF β , VEGF, and FGF (44–47). However, although ligand-receptor interactions were possible between UC-MSCs and NLCPs via those pathways, none of those were specific to therapeutic cells only. On the other hand, signaling via NEGR and NXRN pathways was exclusively present between therapeutic cells and NLCPs. Nonetheless, the role of NEGR and NXRN pathways in the lungs has not yet been reported in the literature. Thus, we still need to improve our understanding of the mechanisms underlying UC-MSC therapeutic action in BPD.

6.5 Develop and characterize a novel murine 2HIT BPD model

The importance of accurate modeling of BPD is highlighted by the challenges emanating from the multifactorial aspect of the disease, poor understanding of the disease pathogenesis, and

restricted access to patient lung samples. The most common animal models of BPD involve the exposure of term rodents to hyperoxia to induce oxygen toxicity (48,49). However, hyperoxia-induced lung injury models mimic only some of the pathological features of BPD. The use of a second hit to induce postnatal inflammation was suggested to better recapitulate the inflammatory aspect of the disease and to better mimic the clinical conditions of BPD for preclinical testing of novel therapies (50–52).

In **Chapter 4**, we developed a novel 2HIT murine BPD model with postnatal LPS and hyperoxia exposure and described the lung injury and evolution of the innate immune response. We found that the 2HIT model caused an increase in mortality, a decrease in body weight, altered lung structure and function, and RVH in experimental animals.

The influx of pulmonary macrophages, as well as neutrophils, is an important marker of chronic inflammatory states. Exposure to inflammation induces a proinflammatory M1 phenotype in macrophages with increased expression of CD86, IL-6, and IL-1 β (53–55). Several studies have detected an association between the increase in inflammatory markers in both tracheal aspirates (56,57) and serum (58–60) of preterm infants and the occurrence of BPD. Additionally, a longitudinal study has shown that systemic inflammation occurs early in the neonatal period in infants with BPD (61). Consistent with these previous findings, perturbations observed in the 2HIT animals included an early local pulmonary and systemic type 1 cytokine/chemokine response, including high levels of IL6 and IL1 β . This was accompanied by the recruitment of innate immune cells, including macrophages, neutrophils, and monocyte with alveolar macrophages polarized towards a proinflammatory M1 phenotype.

Moreover, recent evidence demonstrated that a type 2 immune response is important for normal lung development (62,63). In this respect, a decrease in type 2 anti-inflammatory cytokine

expression and a decrease in anti-inflammatory M2-type alveolar macrophages were observed in 2HIT compared to control animals. Although macrophages are known to produce more than the simplified M1/M2 functional phenotypes, a simplistic view can allow for a more practical approach for mechanistic studies of novel therapy, including UC-MSCs (53,55). In brief, the 2HIT model represents a robust BPD mouse model recapitulating key disease phenotype, enabling a better understanding of the disease pathogenesis and preclinical studies of potential novel therapies, including UC-MSCs with a focus on inflammation.

6.6 Determine the optimal treatment regimen for progenitor-like UC-MSCs in 2HIT mice

Perinatal inflammation is an important risk factor for the development of BPD in preterm infants (64), and MSCs are promising therapeutic modalities for the treatment of BPD. Most studies that have assessed MSC therapeutic potential have used single-hit hyperoxia-induced lung injury models in small neonatal rodents (reviewed in (65)), with only one study using a combination of hyperoxia exposure and prenatal LPS (66). Thus, until now, the effect of MSCs in a neonatal hyperoxia-induced lung injury model with an additional postnatal inflammatory aspect has been unexplored.

In **Chapter 5**, we tested the therapeutic potential of progenitor-like UC-MSCs (donors T1, T3, and P5) in the 2HIT model of BPD. Injection via the *i.t.* route during the saccular stage of lung development at PD4 was not tolerated in 2HIT mice pups. We hypothesized that the procedure's invasiveness at an early age, combined with the exposure to two different stressors (hyperoxia and LPS) and the small size of the animals, contributed to the high mortality following airway delivery of the vehicle and cell treatment. Consequently, we opted for the *i.v.* route via the facial vein for administering UC-MSCs in the 2HIT animals. We determined that PD1 and PD2 were the optimal

times for *i.v.* administration of UC-MSCs in 2HIT mice pups. Moreover, treatment with 10×10^6 cells/kg BW UC-MSCs improved survival and lung growth of 2HIT animals.

The most effective cell dosages for clinical application of UC-MSCs are still unclear. Thus, we tested different UC-MSC doses in the 2HIT model. We determined that 20×10^6 cells/kg BW injected at PD1 and PD2 was the optimal UC-MSC dose in that model and improved the BW, lung structure, and function. So far, early phase clinical trials have shown the safety and feasibility of MSCs' administration in preterm infants of similar doses ranging from two doses of 1×10^6 cells/kg BW up to one dose of 20×10^6 cells/kg BW (3,5,7).

Mice and rats do share many similarities, but they can exhibit different phenotypes when exposed to the same injury. For example, opposite findings in lung function assessment have been reported in mouse and rat hyperoxia models of BPD. Indeed, while neonatal rats exposed to hyperoxia showed a decrease in lung compliance typical of restrictive lung diseases such as fibrosis (19), an increase in lung compliance was shown in mouse hyperoxia models (55). Similarly, although both the 2HIT model in neonatal mice and the hyperoxia model in neonatal rats resulted in simplifying the alveolar structure, opposite findings were observed regarding lung function. 2HIT animals showed emphysematous functional changes, including increased compliance and an upward shift in the PV-loop, reflecting decreased elastic recoil, and no signs of long-term lung fibrosis. In contrast, hyperoxic rat pups showed a decrease in lung compliance. Nevertheless, progenitor-like UC-MSCs were able to provide therapeutic effects in terms of improved lung function in both animal models. Therefore, we believe that assessing treatment efficacy in more than one BPD model is advantageous to discern which type of patients will respond to the therapy.

Even though the investigation of UC-MSCs' mechanism of action was not the main aim of our study, we speculate that macrophage polarization induced by the injected cells might contribute to the repair efficacy of UC-MSCs. This is based on our previous findings in **Chapter 4**, suggesting an impaired type 2 cytokine environment with a reduced number of M2 homeostatic alveolar macrophages in the 2HIT neonatal lung, together with previously published studies showing that MSCs can modulate the macrophage polarization state from M1 proinflammatory to M2 anti-inflammatory in part through the release of PTX3, a marker of our therapeutic UC-MSCs (43,55,67).

6.7 Limitations of our study

This study has some limitations. First, the scale of the scRNA-seq study is limited to the number of donors for each perinatal condition: n=5 for term, preterm and preterm donors with preeclampsia, n=4 for late preterm donors with preeclampsia, and n=1 for preterm donor with chorioamnionitis. Contamination issues, regardless of the disinfection and antibiotic-supplementation procedure, contributed partly to the low number of donors with chorioamnionitis included in this research. Second, therapeutic efficacy *in vivo* was only tested for nine out of 21 donors. However, from a transcriptomic point of view, the remaining donors were indistinguishable from the other donors tested *in vivo*. Third, we cannot fully explain the cause behind the differences in transcriptome between donors. Finally, side-by-side delivery method (*i.t.* versus *i.v.*) comparison could not be achieved in the 2HIT model due to the smaller size of 2HIT mice pups and would require further assessment in a larger animal model.

6.8 Recommendations for future research

In this study, we combined molecular transcriptomic data with functional *in vivo* readouts to better characterize UC-MSCs and identify subsets of cells with superior repair potential in experimental BPD. In the future, experimental studies should include: 1) the screening of UC-MSCs using FACS for high expression of UC-MSCs' therapeutic markers (including PTX3, MARCKS, and IDH2) and low expression of non-therapeutic markers (including HLA-ABC) before administration in BPD models to determine if those markers do in fact select for therapeutic cells, 2) the use of agonists or inhibitors of certain genes to promote cell proliferation of UC-MSCs in culture to enhance their therapeutic potential for the treatment of BPD, and 3) selective deletion of HLAs, which might benefit the cells by protecting them from immune rejection.

Moreover, translational studies in larger animals which can be conducted in settings closer to the clinical situation of BPD patients compared to rodent models, such as the piglet model, remain essential to test the efficacy of UC-MSCs (68). In this respect, we are currently working in our lab to develop a stable, reliable, and reproducible piglet acute injury lung model. Side-by-side comparison of different UC-MSCs' delivery routes could also be performed in that model.

Finally, knowledge of a therapeutic agent's mechanism of action is key to the success of a clinical trial (9). While our recent findings of progenitor-like UC-MSCs-mediated lung repair are encouraging, our understanding of UC-MSC biology and mode of action still is incomplete. More in-depth research on MSCs' paracrine properties and the mechanism underlying MSC-based therapy remain to be achieved. For example, the 2HIT model could be used to determine how UC-MSCs modulate inflammation by assessing inflammatory markers that we have previously characterized in that model. Moreover, gene knockdown studies could be performed to establish whether the expression of genes found within the molecular signature of therapeutic and

non-therapeutic cells is only a marker or a true mediator of their phenotype. Once these fundamental aspects of UC-MSc biology will be better understood, we will be able to apply this cell therapy to the clinic more confidently.

6.9 Conclusion

BPD is the most severe complication of extreme prematurity for which no effective treatment exists. MSCs have emerged as a new promising therapy to treat the disease, with UC-MSCs being of particular clinical relevance for treatment of neonatal disorders. Experiments in this study concentrated on UC-MSc transcriptome characterization at the single-cell level, assessment of UC-MSCs' therapeutic effect in experimental BPD, and the development and characterization of a novel 2HIT murine BPD model.

In this series of experiments, it was shown that:

1. UC-MSCs isolated from term, preterm, and preterm donors with underlying pregnancy-related complications have limited variability in their transcriptome, independent of their perinatal state.
2. Treatment (*i.t.*) with UC-MSCs from term, preterm and preterm donors with pregnancy-related complications (preeclampsia and chorioamnionitis), which displayed a progenitor-like transcriptome with high cell cycle and cell proliferation activity, mitigated hyperoxia-induced lung injury in neonatal rats.
3. Treatment (*i.t.*) with UC-MSCs from term and preterm donors with preeclampsia, which displayed a fibroblast-like transcriptome enriched in ECM and collagen-related processes, did not provide optimal repair potential in a neonatal rat hyperoxia-induced lung injury model.

4. Therapeutic and non-therapeutic UC-MSCs can be distinguished based on distinct molecular signatures.
5. Hyperoxia and postnatal LPS exposure in the novel 2HIT murine BPD model impaired lung compliance and growth and caused a transient type 1 inflammatory cytokine/chemokine response and a decrease in type 2 anti-inflammatory cytokine expression and the number of anti-inflammatory M2 type alveolar macrophages.
6. Treatment with UC-MSCs at a dose of 20×10^6 cells/kg BW administered twice *i.v* (once at PD1 and once at PD2) improved BW, lung compliance and growth of 2HIT animals.

This study broadens our understanding of UC-MSCs' molecular transcriptome in relation to their *in vivo* therapeutic effect in experimental BPD. It has also created a novel BPD model with an additional inflammatory aspect and provides a long-term analysis of the innate immune changes associated with the 2HIT model. Moreover, we show that progenitor-like UC-MSCs over fibroblast-like UC-MSCs are favorable for treating BPD. These results will enable further screening and testing of UC-MSCs with superior repair potential, with the ultimate goal being the clinical translation for the treatment of BPD.

6.10 References

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7. SCIENTIFIC COMMUNICATIONS

7.1 List of publications

7.1.1 Original research (authored as other than first author)

Single-Cell RNA Sequencing-Based Characterization of Resident Lung Mesenchymal Stromal Cells in Bronchopulmonary Dysplasia.

Mižíková I, Lesage F, **Cyr-Depauw C**, Cook DP, Hurskainen M, Hänninen SM, Vadivel A, Bardin P, Zhong S, Carpén O, Vanderhyden BC, Thébaud B. *Stem Cells*. 2022;40(5):479-492

Pulmonary and Neurologic Effects of Mesenchymal Stromal Cell Extracellular Vesicles in a Multifactorial Lung Injury Model.

Lithopoulos MA, Strueby L, O'Reilly M, Zhong S, Möbius MA, Eaton F, Fung M, Hurskainen M, **Cyr-Depauw C**, Suen C, Xu L, Collins JJP, Vadivel A, Stewart DJ, Burger D, Thébaud B. *Am J Respir Crit Care Med*. 2022;205(10):1186-1201.

Single cell transcriptomic analysis of murine lung development on hyperoxia-induced damage.

Hurskainen M, Mižíková I, Cook DP, Andersson N, **Cyr-Depauw C**, Lesage F, Helle E, Renesme L, Jankov RP, Heikinheimo M, Vanderhyden BC, Thébaud B. *Nat Commun*. 2021;12(1):1565.

A lung tropic AAV vector improves survival in a mouse model of surfactant B deficiency.

Kang MH, van Lieshout LP, Xu L, Domm JM, Vadivel A, Renesme L, Mühlfeld C, Hurskainen M, Mižíková I, Pei Y, van Vloten JP, Thomas SP, Milazzo C, **Cyr-Depauw C**, Whitsett JA, Noguee LM, Wootton SK, Thébaud B. *Nat Commun*. 2020;11(1):3929.

7.1.2 Review article

Insights into the mechanisms of alveolarization - Implications for lung regeneration and cell therapies.

Hurskainen M, **Cyr-Depauw C**, Thébaud B. *Semin Fetal Neonatal Med*. 2022;27(1):101243

7.1.3 Book chapter

Chapter – 74, Developmental Biology of Lung Stem Cells.

Mižíková I, **Cyr-Depauw C**, Thébaud B. In: Fetal and Neonatal Physiology, 2-Volume Set. 6th edition, 2021, Polin RA, Abman SH, Rowitch D., Benitz WE. Publisher: Elsevier, pp. 768-775

7.2 List of communications in scientific meetings

Single-cell RNA sequencing analysis of term and preterm human umbilical cord mesenchymal stromal cells identifies repair cell characteristics for experimental neonatal lung injury, *Till & McCulloch meetings*, Vancouver, BC, Canada, 2022

scRNAseq analysis identifies unique transcriptome of therapeutic umbilical cord MSCs in a hyperoxic rodent model of bronchopulmonary dysplasia, *Till & McCulloch meetings*, Vancouver (virtual), BC, Canada, 2021

scRNAseq analysis identifies unique molecular signature of therapeutic umbilical cord MSCs to attenuate lung injury in a hyperoxic rodent model of bronchopulmonary dysplasia, *International Society for Cell and Gene Therapy (ISCT) Annual Meeting*, New Orleans (virtual), USA, 2021

Captain Repair to the rescue of preemies! WINNER of the Elevator Pitch Competition, *OHRI Research Day*, Ottawa (virtual), ON, Canada, 2020

Identifying the impact of donor-origin on the therapeutic potential of umbilical cord MSCs to attenuate lung injury in a hyperoxic rodent model of bronchopulmonary dysplasia, *European Respiratory Society (ERS) International Congress*, Vienna (virtual), Austria, 2020

Identifying the impact of donor-origin on the therapeutic potential of umbilical cord MSCs to attenuate lung injury in a hyperoxic rodent model of bronchopulmonary dysplasia, *Till & McCulloch meetings*, Vancouver (virtual), BC, Canada, 2020

scRNAseq analysis identifies unique molecular signature of therapeutic umbilical cord MSCs to attenuate lung injury in a hyperoxic rodent model of bronchopulmonary dysplasia, *International Society for Cell and Gene Therapy (ISCT) Annual Meeting*, Paris (virtual), France, 2020

Development and application of a novel murine model mimicking bronchopulmonary dysplasia (BPD) for evaluating cell-based therapies, *Till & McCulloch meetings*, Montreal, QC, Canada, 2019

Therapeutic potential of MSCs isolated from preterm umbilical cord in neonatal lung injury, *Joint Queen's/OHRI research day*, Kingston, ON, Canada, 2019

Therapeutic potential of MSCs derived from preterm umbilical cord in neonatal lung injury, WINNER of the Elevator pitch competition, *Stem Cells, Cell Therapies, and Bioengineering in Lung and Biology Conference*, Burlington, VT, USA, 2019

Therapeutic potential of preterm umbilical cord-derived MSCs in neonatal lung injury, *Cardio-pulmonary Organ Repair and Regeneration (SCORR) meeting*, Ottawa, ON, Canada, 2019

Lung protective effects of hUC-MSCs isolated from preterm delivery in neonatal lung injury, *Till & McCulloch meetings*, Ottawa, ON, Canada, 2018

7.3 Travel grants

Cellular and Molecular Medicine (CMM) Program Director Travel Grant, 2022

Stem Cell Network (SCN) complimentary registration fee TMM2021, 2021

Stem Cell Network (SCN) complimentary registration fee TMM2020, 2020

Stem Cell Network (SCN) travel award TMM2019, 2019

Graduate and Postdoctoral Studies Office Research Travel Grant, 2018

7.4 Other grants

Ontario Graduate Scholarship (OGS) and University of Ottawa Excellence Scholarship, 2021-2022

European Respiratory Society – Short-Term Research Fellowship, 2020

Canadian Lung Association Breathing as One award, 2019-2021

Ontario Graduate Scholarship (OGS) and University of Ottawa Excellence Scholarship, 2019-2020

Queen Elizabeth II Graduate Scholarship in Science and Technology (QEII-GSST), declined, 2019-2020

University of Ottawa Admission Scholarship, 2017-2021

8. APPENDIX

8.1 European Respiratory Society – Short-Term Research Fellowship

Head of research: Dr. Daniel Freund

Address of the host unit: Center for Regenerative Therapies Dresden (CRTD), Dresden University of Technology, Fetscherstrasse 105, 01307, Dresden, Germany

Date of the fellowship: September 20th 2021- December 3rd 2021

Principal objectives of the fellowship: Gain experience working with UC-MSCs under Good Manufacturing Practice (GMP) at the GMP facility of the CRTD and performing isolation, expansion, and characterization experiments to optimize the cells to eventually use them to treat neonatal lung injury.

Summary report: MSCs have been identified as potent repair cells with pleiotropic effects. Pioneering work in our lab (Dr. Bernard Thébaud's lab) showed that BM-MSCs and, subsequently, UC-MSCs, prevent and rescue injury to the developing lung in rat models mimicking bronchopulmonary dysplasia, a neonatal chronic lung disease (1,2).

Since then, early-phase clinical trials have shown the safety and tolerability of MSCs in preterm infants at high risk of developing the chronic lung disease (3–7). However, the therapeutic functions of UC-MSCs administered to humans are not as robust as demonstrated in preclinical studies, and the translation of UC-MSCs is impaired, in part, by the lack of molecular and functional characterization of UC-MSCs (8–10). The ultimate goal of my Ph.D. research project is to characterize and fine-tune a high-quality UC-MSC product for the treatment of neonatal lung disease.

During my Short-Term Research Fellowship, I learned and performed several experiments using UC-MSCs in order to optimize UC-MSC cell products.

First, I learned the protocol to isolate the MSCs from the UC under GMP conditions, followed by the expansion period of the cells and cryopreservation protocol. I expanded UC-MSCs up to passage number 10 (PN10).

Next, following regulatory authorities' demand to determine if we could distinguish between immunosuppressive characteristics of early versus late UC-MSC PNs and directly thawed (D.T.) UC-MSCs versus fresh UC-MSCs (cells revived in culture), we assessed some of the *in vitro* characteristics of UC-MSCs from four healthy term donors. Cells at early versus late passages (PN2 to PN10) were examined, as well as D.T. PN2 UC-MSCs and D.T. PN2 UC-MSCs incubated at room temperature for six hours (to mimic what could happen in the clinic) versus fresh PN2 UC-MSCs (cells were in culture until they reached 90% confluency). All UC-MSCs conditions were challenged *in vitro* with IFN γ for 24 hours (Fig. 8.1). Exposure to IFN γ is known to cause the release of indoleamine 2,3 deoxygenase (IDO) by MSCs and has been proposed as a marker of MSCs' immunomodulatory potency as MSCs can suppress T cell activation in an IDO-dependent manner (11). Following IFN γ exposure, the cells were harvested, and RNA was extracted from each sample. Then, RT-qPCR experiments were performed to determine the expression of *IDO* (Fig. 8.1).

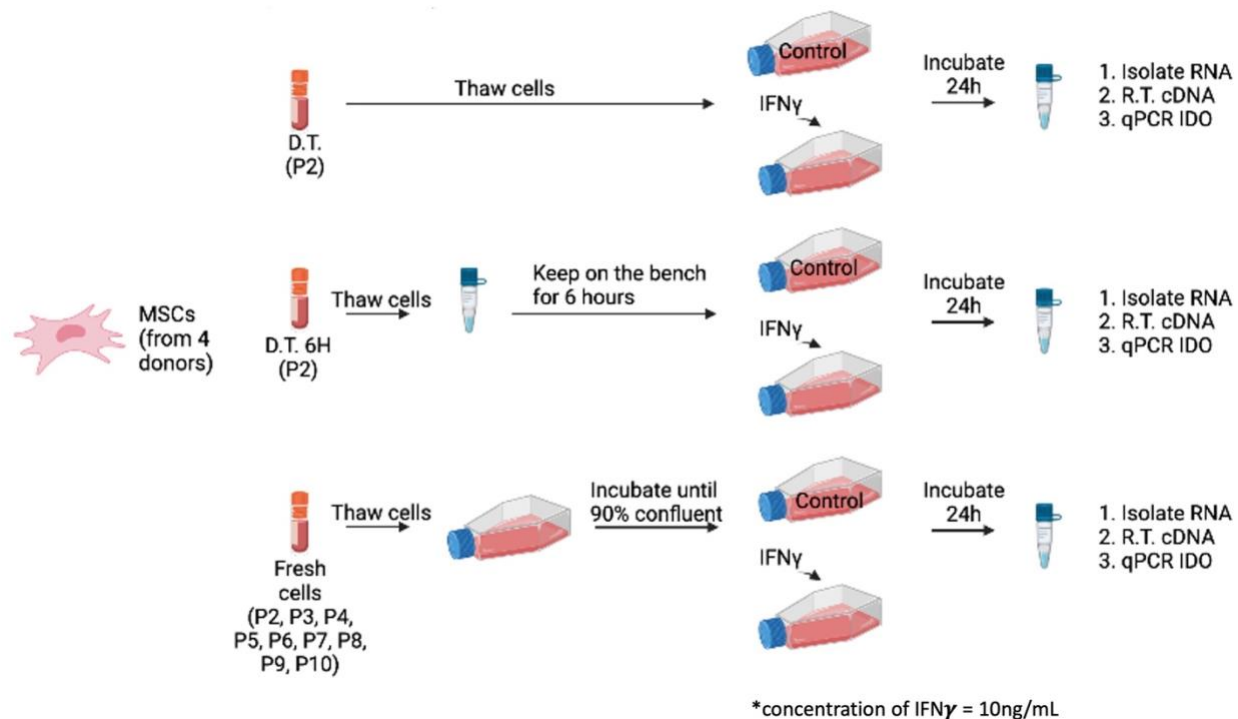


Figure 8.1 Experimental design to compare *IDO* mRNA expression between PN2 D.T. UC-MSCs to PN2 D.T. UC-MSCs and incubated at room temperature for six hours, as well as to fresh PN2 to PN10 UC-MSCs.

UC-MSCs were isolated from four healthy term donors. All three conditions of UC-MSCs were incubated with IFN γ (10 ng/mL) for 24 hours and harvested for RNA isolation, followed by RT-qPCR experiments to determine *IDO* expression. Cells cultured for 24 hours without IFN γ stimulation were used as control.

No significant difference in mRNA relative expression of *IDO* between fresh cells challenged with IFN γ at early PNs versus late PNs was observed (Fig. 8.2A). Previous work has shown that in late MSC PNs, although *IDO* protein expression was reduced compared to MSCs of earlier PNs, *IDO* was transcriptionally induced at a similar level. In fact, the reduction of *IDO* protein expression was due to proteasomal degradation (12). Thus, it is possible that at the protein level, *IDO* expression between early and late MSC PNs differs, and mRNA expression of *IDO* is not enabling us to differentiate between early and late UC-MSC PNs. In addition, we observed that mRNA relative expression of *IDO* of D.T. UC-MSCs compared to D.T. UC-MSCs and incubated at room

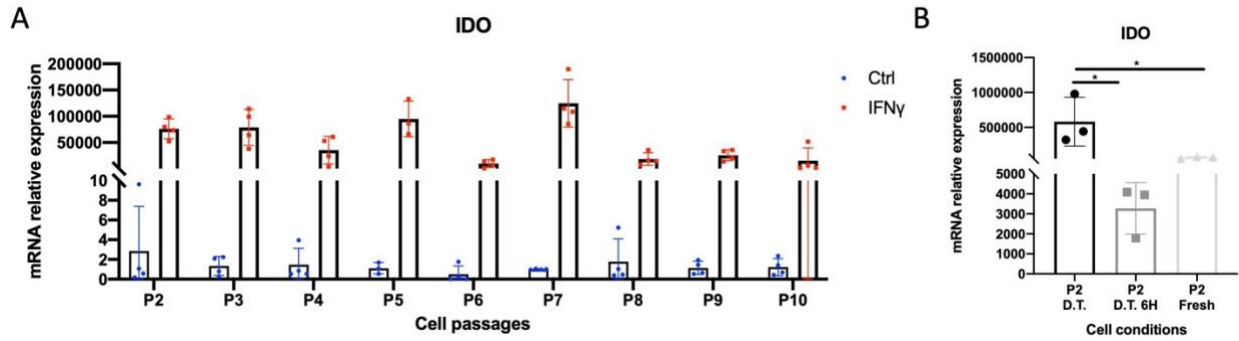


Figure 8.2 Assess the immunosuppressive potential of UC-MSCs by determining mRNA relative expression of *IDO* after stimulation with IFN γ for 24h.

A) mRNA expression levels of *IDO* were measured by RT-qPCR from fresh UC-MSC at PN3 up to PN10 from four donors. Unstimulated UC-MSCs at the corresponding passage were used as control. **B)** Comparison of mRNA expression levels of *IDO* measured by RT-qPCR in PN2 UC-MSCs D.T. versus D.T. and incubated at room temperature for six hours versus freshly harvested UC-MSCs from three donors. GAPDH RNA was used as an internal control. Figures show the results for each donor as indicated by the dots and the means $\pm$ STD. *P* values were calculated with one-way ANOVA with Tukey's multiple comparison post hoc test for all panels. Multiple comparisons was used for panel A. Significant results are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

temperature for six hours, as well as compared to fresh UC-MSCs, was significantly higher. The difference in expression between D.T. and D.T. incubated at room temperature for six hours was expected as cells would lose viability while at room temperature. However, the results obtained for D.T. versus fresh were unexpected as they contradict a previously published study showing that D.T. thawed BM-MSCs are resistant to IFN γ -induced upregulation of *IDO* (13). The reason for this somewhat contradictory result is still not entirely apparent and further investigation will be required to better understand the cause of the increased mRNA expression of *IDO* in D.T. cells compared to fresh cells.

Moreover, we compared the mRNA expression of differentiation, senescence, and stemness markers between UC-MSCs at early PN3 and late PN10. To do this, UC-MSCs isolated from four

donors were expanded in culture, and RNA from PN3 and PN10 cells was isolated. Then, RT-qPCR experiments were performed to determine the expression of differentiation and senescence markers (markers of chondrogenesis ACAN and SOX9; markers of osteogenesis RUNX2, ALP, COL1A1, SPP1, and BGLAP; a marker of adipogenesis PPAR γ ; a marker of differentiation into fibroblasts CTGF/CCN2; and senescence marker CDKN2A (14–18)) and stemness markers (FGF2, GREM1, ISLR/Meflin, MCAM/CD146, and SERPINF1, (19–21)). We observed that three out of five stemness markers were more highly expressed at PN3 compared to PN10 (*ISLR/Meflin*, *MCAM/CD146*, and *SERPINF1*). No difference in expression was observed for stemness marker *FGF2* between PN3 and PN10. Two differentiation markers and the senescence marker were downregulated at PN3 compared to PN10 (*BGLAP*, *CTGF/CCN2*, and *CDKN2A*), whereas the expression of differentiation markers *ALP*, *PPAR γ* , and *SPP1* were unchanged. Expression of differentiation markers *ACAN*, *SOX9*, *RUNX2*, and *COL1A1* were higher at PN3 compared to PN10.

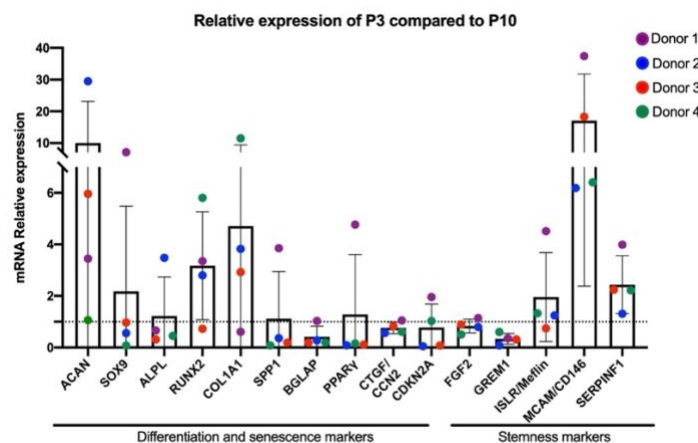


Figure 8.3 mRNA relative expression of differentiation, senescence, and stemness markers in PN3 compared to PN10 fresh UC-MSCs.

mRNA expression levels were measured by RT-qPCR in four UC-MSC donors cultured to PN3 and PN10. Relative expression of PN3 compared to PN10 is shown in the figure. GAPDH RNA was used as an internal control. The figure shows the results for each donor as indicated in the legend and the means $\pm$ STD.

Among all the markers, *MCAM/CD146*, was the most highly expressed gene by PN3 UC-MSCs compared to PN10 and might be a good marker to use in the future to distinguish between early and late passage UC-MSCs.

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