“Do or do not. There is no try.” (Master Yoda – “Star Wars”, 1977)

University of Alberta

Evaluation of Mesenchymal Stem Cell-Based Therapies for Inflammatory Lung Diseases

by

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Dedication

To those who have given me all the chances to be who I am. To my grandfather, Ion Leulescu, who has defined me before anything else could have. I miss him to this day and my strongest wish is to be who he believed I will be. To my mother, Carmen Leulescu, who is quietly encouraging everything about me. To my grandmother, Niculina Leulescu, who is not quiet about the value of life. To my younger brother, Leontin Ionescu, for teaching me the joy and trust of sharing by being first to share. To those who smiled with me, to those who smiled for me, to those who were too stubborn to abandon the uncertain path by my side throughout my struggles.

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ABSTRACT

Recent discoveries in stem cell biology have generated enthusiasm about the possibility of harnessing stem cells for organ repair and regeneration. The ability of pluri- and multipotent stem cells to differentiate along various cellular lineages has placed them at the core of research that seeks to protect endogenous stem cell populations or to deliver exogenous stem cells to sites of organ injury. Lung diseases are a major health care concern and the prevalence of chronic lung diseases such as asthma, pulmonary fibrosis and chronic obstructive pulmonary disease is expected to continue to rise over the next decades. Meanwhile, improved perinatal care has allowed the survival of extremely premature infants that constitute a particularly vulnerable subpopulation because of their risk of developing bronchopulmonary dysplasia (BPD) with potential life-long complications.

Research that aims to evaluate the therapeutic potential of exogenous stem cells in lung diseases placed an initial emphasis on the engraftment and differentiation of these cells in the lung. More recent studies demonstrate that multipotent stem cell populations, such as mesenchymal stromal cells (MSCs), exert paracrine activity that can modulate local inflammatory and immune responses in experimental lung disease models including asthma, acute lung injury (ALI) and pulmonary fibrosis. The studies presented hereby demonstrate that factors secreted by adult bone marrow-derived MSCs can prevent the development of inflammatory lung diseases in mouse models of

asthma and ALI and provide mechanistic insight into the anti-inflammatory properties of MSCs.

The perspective of using pluripotent stem cells as therapeutic agents has been revived by the landmark discovery of induced pluripotency, where "embryonic stem cell (ESC)-like" cells can be generated by reprogramming terminally differentiated somatic cells. While the field of induced pluripotent stem cell (iPSC) biology is still in its effervescent infancy, this finding may relieve many ESCs-related ethical concerns and may open the way to large-scale production and evaluation of pluripotent stem cells for organ regeneration and repair. The additional study presented provides proof-of-concept for the utility of iPSC.

Together, the studies presented hereby advocate the potential of stem cells as a novel clinical option for the treatment of severe lung diseases.

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ALI	acute lung injury
AHR	airway hyperresponsiveness
AM	alveolar macrophage
ANOVA	analysis of variance
APC	allophycocyanin
APN	adiponectin
ARDS	acute respiratory distress syndrome
Arg-1	arginase-1
AT2	alveolar epithelial type 2 cells
ASM	airway smooth muscle
AT1	alveolar epithelial type I cell
AT2	alveolar epithelial type II cell
BALF	bronchoalveolar lavage fluid
BASC	bronchoalveolar stem cell
Bmi1	B lymphoma Mo-MLV insertion region 1 homolog
BMC	plastic-adherent bone marrow-derived stromal cell
BMSC	bone marrow-derived stromal cell
BOOP	bronchiolitis obliterans organizing pneumonia
BPD	bronchopulmonary dysplasia
c-kit	proto-oncogene c- (tyrosine-protein kinase) Kit

CCL	chemokine (C-C motif) ligand
CCSP	Clara cell secretory protein (also: CC10, Scg1b1)
CdM	conditioned medium
CD	cluster of differentiation
CFTR	cystic fibrosis transmembrane regulator
CGRP	calcitonin gene-related peptide
CHI3L3	chitinase-3-like-3 (also: ECF-L, Ym1)
COPD	chronic obstructive pulmonary disease
CXC	chemokine (C-X-C motif)
CXCL	chemokine (C-X-C motif) ligand
DALY	disability-adjusted life years
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
ECF-L	T-lymphocyte-derived eosinophil chemotactic factor (also: CHI3L3, Ym1)
ECM	extracellular matrix
ECMO	extracorporeal membrane oxygenation
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
EpCAM	epithelial cell adhesion molecule

ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FcγRIIB	Fc receptor for immunoglobulin G
Fib	fibroblast
FiO_2	oxygen fraction
FITC	fluorescein
FIZZ1	found in inflammatory zone-1 (also: RELM-α)
Foxp3	forkhead-box p3
H & E	hematoxylin and eosin
HELP	Human *Ex Vivo* Lung Project
HSC	hematopoietic stem cell
IBMX	isobutyl methyl xanthine (1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione)
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor 1
IGF-BP-6	insulin-like growth factor binding protein 6
IL	interleukin
IL-1ra	IL-1 receptor antagonist
iNOS	inducible nitric oxide synthase
iPSC	induced pluripotent stem cell
i.n.	intranasal

i.p.	intraperitoneal
i.t.	intratracheal
i.v.	intravenous
IVIG	intravenous immunoglobulin
JAK	Janus-activated kinase
KGF	keratinocyte growth factor
KO	knock-out
LF	lung fibroblast
LFT	lung function testing
LIX	lipopolysaccharide-inducible CXC chemokine
LPS	lipopolysaccharide
M1	classically (canonically) activated macrophages
M2	alternatively activated macrophages
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1 (also: CCL2, small inducible cytokine A2)
M-CSF	macrophage / monocyte colony stimulating factor
MDC	macrophage-derived chemoattractant / chemokine
MDSC	myeloid-derived suppressor cells
MIP-2	macrophage inflammatory protein-2
MIP-1α	macrophage inflammatory protein-1 alpha
MSC	mesenchymal stem cells

NEB	neuroendocrine body
OVA	ovalbumin
PaO_2	partial arterial pressure of oxygen
PBS	phosphate-buffered saline
PE	phycoerythrin
PEEP	positive end-expiratory pressure
PF4	platelet factor 4 (also: CXCL4)
PI3K	phosphoinositide 3-kinase
PLSD	probable least significant difference
PMN	polymorphonuclear cell
PMSF	phenyl-methyl-sulphonyl fluoride
PNEC	pulmonary neuroendocrine cell
PTEN	phosphatase and tensin homolog
PSF	penicillin - streptomycin - fungizone (amphotericin B)
RANTES	regulated on activation, normal T cell expressed and secreted
RIPA	radioimmunoprecipitation assay
RPMI-1640	Roswell Park Memorial Institute medium-1640
qRT-PCR	quantitative reverse transcriptase – polymerase chain reaction
RELM-α	resistin-like molecule alpha (also: FIZZ1)
Sca-1	stem cell antigen-1

SCF	stem cell factor
SDF-1α	stromal-derived factor-1 alpha
SEM	standard error of the mean
SP	side population cells
SP-C	surfactant protein C
STAT6	signal transducer and activator of transcription 6
sTNFRII	soluble TNF-α receptor II
TARC	thymus and activation regulated chemokine
TGF-β	transforming growth factor beta
TLC	total lung capacity
TGF-β	transforming growth factor beta
Th1	T-helper 1
Th2	T-helper 2
Tr1	interleukin-10-induced and –secreting regulatory T cell
Treg	regulatory T cell
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1
VEGFR2	vascular endothelial growth factor receptor 2
WT	wild-type
Ym-1	see: CHI3L3, ECF-L

CHAPTER 1 – GENERAL INTRODUCTION

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1.1. Overview

The respiratory system supports the vital function of breathing. It can be viewed as the interface between the oxygen-rich environment and the carbon dioxide-producing living organism. The failure of the lungs to complete their function is immediately life-threatening.

From a functional and anatomical viewpoint, the respiratory system comprises two compartments: the conducting airways (nasal cavity, pharynx, larynx, trachea, bronchi and bronchioles) and the gas-exchanging airways (respiratory bronchioles and the saccular-alveolar compartment, where alveolar walls come in close contact with capillary walls in order to facilitate the exchange of oxygen and carbon dioxide). Lung injury can occur at any of these levels leading to impairment of breathing function, which can be reversible or irreversible. **Obstructive** respiratory diseases, such as asthma and chronic bronchitis, are caused by damage at the airway level, which limits airflow, whereas **restrictive** pulmonary diseases, such as lung fibrosis, acute respiratory distress syndrome (ARDS) and sarcoidosis are determined by inflammatory processes in the lung interstitium, which lead to reduced lung compliance with limitation of lung expansion. Although the advancements of biomedical research over the past decades have brought novel therapeutic approaches for respiratory disorders, many lung diseases, such as chronic

lung disease of prematurity (or bronchopulmonary dysplasia, BPD), chronic obstructive pulmonary disease (COPD) and cystic fibrosis are still lacking efficient treatments. According to the WHO World Health Report 2000, lung diseases contribute to a total of 17.4% of deaths and 13.3% of disability-adjusted life years (DALY) worldwide [180]. These facts highlight the absolute necessity to study the potential applicability of recent developments in the field of regenerative medicine as therapeutic options for lung diseases.

1.2. Lung Development and Regeneration

According to one of its most recent definitions, regenerative medicine is "an interdisciplinary field of research (…) focused on the repair, replacement or regeneration of cells, tissues, or organs to restore impaired function resulting from any cause (…). It uses a combination of converging technological approaches (…) [which] may include (…) the use of soluble molecules, gene therapy, stem and progenitor cell therapy, tissue engineering, and the reprogramming of cell and tissue types." [32]

This field has evolved dramatically over the past couple of decades and even more so in the recent years. A search for scientific publications using the keyword "regenerative medicine" on the United States Library of Medicine / National Institutes of Health database is returning 12,000

results [158]. As for specialized journals, the 17% increase in the number of articles published in "Cell Transplantation – The Regenerative Medicine Journal" over only one year (2008 compared to 2007) can serve as an eloquent example [109].

The fundamental paradigms in regenerative medicine are:

(i) *in situ* organ regeneration following injury may occur as long as the organ framework has been sufficiently preserved;

(ii) the regeneration principles would normally follow evolutionary principles and would likely recapitulate ontogeny [26, 168]. This brings forth the need to understand organ development, as new developmental concepts may have immediate applicability in regenerative medicine.

The intrauterine development of the lung has traditionally been subdivided in five overlapping stages, on the basis of gross histological features [24]. The respiratory system starts forming as early as the third week of gestation as an outpouching of the primitive forgut bifurcating into the two main stem bronchi (***embryonic stage***). During the following ***pseudo-glandular stage***, rudimentary bronchi divide by dichotomous branching; the resulting tubular structures are lined by columnar epithelium surrounded by mesenchymal tissue. The ***canalicular stage*** is characterized by the bifurcation of the last generations of distal bronchi. In this stage there is also capillary invasion and differentiation of the air

space epithelium into alveolar type 2 cells (AT2, responsible for surfactant production) and type 1 cells (AT1, which form the thin air-blood barriers). Next, during the ***saccular stage,*** the peripheral air spaces expand in length and width, and, around 36 weeks of gestation these spaces form saccules at the expense of the intervening mesenchyme. ***Alveolarization***, the final stage of lung development, begins in the near-term lung prior to birth but primarily occurs postnatally, during the first 2–3 years of life, and may continue beyond childhood, albeit at a slower rate.

The **alveolus** is the definitive gas-exchanging unit- of the lung. Alveoli are, in part, formed by subdivision (*septation*) of the saccules. Septation involves budding of septal crests, which is followed by elongation of the septal walls to form individual alveoli. Septation increases the gas-exchange surface area, without a proportionate increase of lung volume (i.e. alveoli have a larger surface/volume ratio than saccules). ***Microvascular maturation***, the final important step in lung development, follows and partly overlaps the alveolar stage. Initially, capillaries form double layers in the immature gas-exchange region; during the maturation step, these microvessels remodel to form a single capillary layer. The thickness of the alveolar wall decreases by about 20% and the distance between alveolar gas and capillary blood diminishes by about 25%. Morphometric studies show that from birth to adulthood the alveolar and

capillary surface areas expand about 20-fold and the capillary volume 35-fold.

While the histological changes are well described [76, 87, 190], much more needs to be learned about the mechanisms that regulate normal lung development in order to harness these processes for therapeutic purposes [168]. This is particularly relevant for the perinatal care of extremely premature infants who are born at the late canalicular stage, before the completion of the alveolar stage. The immaturity of the lungs, together with the ventilator support required places these infants at risk of developing BPD, which may lead to an irreversible arrest in alveolar development and impaired lung function beyond childhood [179].

The lungs are particularly vulnerable organs due to their role as one of the ports-of-entry for environmental toxins and allergens. The complexity of the lungs and their developmental programme, together with the current lack of efficient therapies that would prevent or repair lung damage, render the lung an especially challenging candidate for the current arsenal of regenerative medicine. Traditionally, respiratory diseases have been assigned to several pathophysiological categories; however, new insights into disease mechanisms may offer new approaches to “old” problems. One example is cystic fibrosis, which is caused by mutations in the gene encoding for the cystic fibrosis

transmembrane regulator (CFTR), an ion channel normally present in epithelial cells. This monogenic disease has been classically regarded as a purely "electrophysiological" disease due to the CFTR dysfunction; however, recent findings suggest that CFTR is also expressed in immune effector cells, such as macrophages [22], which opens new therapeutic perspectives that place more emphasis on the inflammatory aspects of cystic fibrosis.

Aside from and also alike cystic fibrosis, numerous lung diseases are currently lacking efficient therapies: while ALI / ARDS or asthma have an overwhelming inflammatory component, other types of injury may have a more obscure etiology (emphysema, COPD, pulmonary fibrosis). BPD impairs lung development, yet, in recent years, advances in perinatal care have permitted the survival of extremely premature babies whose lungs are in earlier developmental stages; whereas the "old" BPD had a stronger fibrotic component, the "new" BPD is an arrest in alveolarization with minimal inflammation [11]. The prolonged "stalemate" in finding solutions for patients suffering from these incurable diseases has brought stem cell research at the core of RM today. The hallmark abilities of stem cells to self-renew and differentiate along multiple cell lineages ("stem cell plasticity") have rendered both tissue-resident and circulating stem and progenitor cells extremely appealing for tissue regeneration purposes.

Together with advances in creating animal models of lung disease, the promise of stem cells has become a crucial research avenue in lung regenerative medicine [80].

1.3. Stem Cells

The concept of a "plastic" cell type that can respond to the demands of its microenvironment by acquiring traits specific to other cell types was proposed by the German pathologist Julius Cohnheim in 1867 [27]. He theorized that the fibroblasts that participate in wound healing had a bone marrow origin, a hypothesis that to this day has not yet been starkly resolved. It was not until the dawn of the twentieth century that a similar vision of the bone marrow as an origin and reservoir of blood cells was elaborated by the Russian scientist Alexander Maximow. He developed a novel theory of hematopoiesis, in which these elusive cells that he named "stem cells" [92], had an overarching role. It is acknowledged that Maximow's communication during a scientific congress in Berlin in 1908 was the first instance of the term "stem cells". His work described complex cellular interactions where the marrow stroma orchestrated the conditions of hematopoietic stem cell differentiation [46, 93]. The modern milestone of stem cell research was set in 1961 with J.E. Till and E.A. McCulloch's description of colonies consisting of multiple cell types in

the spleens of irradiated mice that had received unirradiated marrow cells [153]. Before the end of that decade, the first allogeneic bone marrow transplantations [54] were to provide the clinical proof for the existence of hematopoietic stem cells (HSCs). These cells are the basis of the complete reconstitution of blood cells following transplantation of bone marrow into irradiated patients. To date, HSCs have been extensively characterized and are often employed as a model of stem cell hierarchy [145, 173], while research methods initially developed to study HSCs, such as lineage tracing methods [45], have been enthusiastically adopted by other fields of research.

The unprecedented emergence of results that suggested a continuous, organism-wide renewal of postnatal tissues also comprised the pioneering reports of Joseph Altman and Gopal Das, indicating postnatal neurogenesis in several animal species: rats (1965) [7], Guinea pigs (1967) [8] and cats (1971) [34]; however, the existence of a neural stem cell had to await more than a few years for the confirmation by Samuel Weiss' group in 1992 [125].

Meanwhile, evidence had started to gather with regards to the presence of a different type of stem cell harbored by the bone marrow, this time in the stromal compartment, which was, at the time, mainly regarded as a "support" for HSCs. These cells were the focus of A. Friedenstein's 1976 report that named them "colony forming units –

fibroblast (CFU-F)" [47]; later, these cells were going to be assigned the term "mesenchymal stem cells (MSCs)" [4, 115].

Stem cells are defined as cells that have clonogenic and self-renewal potential and are able to differentiate along multiple cell lineages [173]. The size, shape and cellular compartments of the adult organs are determined by embryonic and fetal stem/progenitor cell behavior [121].Traditionally, stem cells are categorized based on their origin and differentiation potential into embryonic and adult (postnatal) stem cells.

Embryonic stem cells (ESCs), definitively established in culture in 1981 [41, 90], are isolated from the inner mass of the trophoblast and are pluripotent, i.e. able to differentiate along multiple cell lineages originating in any of the three germ layers: ectoderm, mesoderm or endoderm, whereas the differentiation potential of adult stem cells (multipotent or, for progenitor cells, oligo- or unipotent) has been considered to be restricted to their original germ layer. However, recent studies are challenging this paradigm, as stem cells derived from bone marrow, classically considered to be partially committed to either the hematopoietic or mesenchymal lineages, have been shown to cross lineage boundaries and transdifferentiate along lineages derived from a different germ layer.

The discovery of ESCs determined another golden era of exponential discovery and reconceptualized biomedical research in its entirety by allowing the generation of genetically engineered (specifically "knock-out") mice [25, 40, 138] that are now widely used to model the effects of an abundance of factors. To date, ESCs are better characterized than adult stem cells. Yet, the lineage relationship between embryonic and adult stem/progenitor cells has not been clearly described.

One of the defining features of stem cells is their ability to divide either *symmetrically*, generating two identical "daughter" cells or *asymmetrically*, giving rise to an identical "daughter" stem cell and a more specialized, lineage-committed progenitor cell [121, 149] that lacks the self-renewal ability but possesses a higher proliferation rate compared to its parent stem cell.

It becomes obvious that a tight regulatory control of the balance between symmetrical and asymmetrical division, as well as the proliferation rate of these cells, is critical for organ development and homeostasis. For instance, it has been proposed that at each stage of lung development the stem cells divide mostly in an asymmetrical fashion, leaving the specialized progenitor behind as the identical "daughter" cell moves distally with the budding lung tips [121].

Regenerative approaches may therefore follow several therapeutic directions:

1) **Targeting of endogenous local (resident) stem cell populations** – protecting / stimulating these cells (potential regenerative mechanisms effectors) as a means to promote organ regeneration or, conversely, targeting cancer stem cells (e.g. lung cancer stem cells [6]) in a stem cell-oriented therapeutic approach to treat cancer;
2) **Cell replacement by exogenous stem cells**, as stem cells may be able to regenerate damaged organs by differentiating and engrafting *in vivo*. This holds promise for degenerative diseases (e.g. multiple sclerosis, Parkinson's disease) or genetic diseases (cystic fibrosis, alpha-1 antitrypsin deficiency);
3) **Standardized stem-cell based preparations** (e.g. conditioned medium) may eliminate the risks typically associated with heterologous stem cell transplantation (infectious agents carried over to the recipient [130], immune rejection, tumorigenesis [5, 155]) and even allow for autologous therapy.
4) **Regeneration/ reconstruction of damaged organs** using stem cells as a source of terminally differentiated cells for tissue engineering.

1.4. Resident Lung Stem/Progenitor Cells

At birth, the normally developed lung is comprised of more than 40 cell types that originate in both endoderm and mesoderm layers. In healthy adults, lung cellular homeostasis is viewed as a slow process compared to highly proliferating tissues such as the bone marrow, intestine or skin, which makes it more difficult to study lung resident stem/progenitor cells. However, it is widely accepted that stem/progenitor cells contribute to maintenance of lung cell populations and there is evidence that

(i) stem cell proliferation rate in the lung increases dramatically following injury;

(ii) the type and amplitude of injury also determines the intensity, duration and type of cellular response [56].

Current approaches in lung regeneration include therapeutic approaches aimed at the protecting and/or exogenously administering both lung-resident and circulating stem/progenitor cells. Local stem/progenitor cells divide to replace injured or postmitotic cells and require strict control over their proliferation rate. Traditionally, local endoderm-derived adult stem/progenitor cell populations have been considered to reside in well-delineated niches and categorized by lung region. Several cell populations have displayed progenitor-like behavior

following chemical-induced lung injury in rodents, and the common feature generally employed to functionally define these cells has been their ability to incorporate [H3]-thymidine into their DNA [30, 91, 139]. Lung cell populations that have been ascribed stem/progenitor cell functions [reviewed in 18, 84, 105, 119, 120, 122] are summarized in **Table 1-1**.

One particular category of cells, **endothelial progenitor cells (EPCs),** have been traditionally considered to be circulating cells (found in the bloodstream) that contribute to the homeostasis of the endothelium [64, 142, 186, 187], consistent with previous findings demonstrating the beneficial effect of angiogenic growth factors in experimental lung disease models [79, 151]. There is evidence that circulating EPCs may contribute to the maintenance of the lung parenchyma in LPS- [183], hyperoxia- [15] and elastase-induced lung injury [66, 67]. In patients, the number of circulating EPCs correlates with survival and disease severity in acute lung injury [23], severe COPD [106] or restrictive lung diseases [42], idiopathic pulmonary arterial hypertension [36, 71, 146] and pneumonia [184].

Recent findings have identified the presence of resident EPCs within the pulmonary microvascular endothelium with angiogenic capacity [9], highlighting the potential of new tools in stem cell biology to identify resident lung progenitor cells. The significance of these cells in

health and disease as well as their therapeutic potential is currently being explored.

1.5. Therapeutic Potential of Exogenous Stem/Progenitor cells

1.5.1. Cell Replacement by MSCs

Beside local stem/progenitor cell populations, there is evidence that non-resident stem/progenitor cells contribute to lung repair following injury [1, 2, 56, 97, 100, 137, 144, 169-172, 178]. Kotton *et al.* [77] and Krause *et al*. [78] showed that bone marrow-derived stem cells can give rise to "daughter" cells in the airways. This ability of the cells to engraft and differentiate has led to the hypothesis that they may reconstitute injured tissues by replacing the damaged cells. There is now a large body of evidence in support of the hypothesis that bone marrow-derived multipotent MSCs can differentiate into airway [166, 177] or alveolar [159] epithelial cells *in vitro*, engraft and differentiate *in vivo* and prevent lung injury in various disease models including bleomycin-induced lung fibrosis [102, 103, 129, 131, 191], lipopolysaccharide (LPS)-induced ALI/ARDS [57, 181, 183, 184], oxygen-induced BPD [13, 159], radiation [1]- and naphthalene [135]-induced lung injury, haemorrhhagic shock [110]. The ability of adult MSCs to differentiate into lung cells has

rendered them particularly important candidates for lung regeneration approaches; today, MSCs are the most widely used stem cells in regenerative medicine. The establishment of a minimal set of criteria for defining human MSCs [37] created a frame of reference for comparison of reports from different groups; however, an equivalent for rodent MSCs is still lacking. MSCs have proven therapeutic abilities in numerous organ injury models, including myocardial infarction [16], acute renal failure [61, 154], type 1 diabetes [43, 53, 162] and neurodegenerative diseases [70]. As of May, 2012, there are 238 clinical trials listed on clinicaltrials.gov, a website hosted by the United States National Institutes of Health. A so far unique clinical success employed the ability of MSCs to differentiate into chondrocytes that were used to repopulate an acellular tracheal acellular scaffold [86]. The engineered structure was successfully transplanted as main bronchus into a patient whose own airway had been irreversibly damaged. Beyond their classically described mesenchymal lineage differentiation ability, the fact that MSCs can cross lineage boundaries and differentiate into lung epithelial cells could be harnessed for diseases such as cystic fibrosis, in which the symptoms are mainly caused by mutations in the gene encoding for the CFTR, a chloride channel typically expressed in the apical membrane of epithelial cells. The stem cells would be engineered to overexpress functional CFTR and act as a delivery vehicle to the damaged tissues, including the lung [22]. The same

approach would be applicable for other monogenic diseases that severely affect the lung such as alpha-1 antitrypsin deficiency (which leads to irreversible emphysema-like lesions) or surfactant protein B deficiency (which results in fatal respiratory failure in newborns). Moreover, it has been suggested that stem cells could act as drug delivery vehicles [107] based on observed therapeutic effects in myocardial infarction [83] and cancer [73, 85]. The possibility of isolating MSCs from a variety of sources, including the cord blood and the adipose tissue, makes autologous therapy a very promising clinical approach in the close future.

1.5.2. Cell Replacement Versus Paracrine Activity of MSCs

Despite the hope originally placed in cell replacement-driven studies, numerous reports that evaluated the therapeutic potential of stem cell transplantation in animal models of lung disease shared one common feature: the degree of stem cell engraftment in the target organs was generally low and therefore alternate mechanisms needed to be considered in order to account for the observed therapeutic benefit. Moreover, MSCs have been shown effective in inflammatory diseases [136], such as graft-versus-host disease in humans [81] and rodent models of LPS-induced ALI [57, 95], fulminant hepatic failure [108], sepsis [98] and asthma [20, 55, 99], where the local cell engraftment may not be

the primary beneficial component. This has led to the current view that stem cells act through a paracrine mechanism by secreted factors [116]. Indeed, MSCs secrete anti-apoptotic, angiogenic, and immuno-modulatory factors. Since the initial report indicating paracrine-mediated protective effects of MSCs overexpressing the pro-survival gene *Akt* in the ischemic heart [52], this paracrine activity has now extensively been explored *in vitro* [57, 62, 159], showing cell-protective, pro-angiogenic and anti-inflammatory properties. *Ex vivo* [82] and *in vivo* in oxygen- [13], ventilator-[31] and LPS- [57] induced lung injury, MSC-derived conditioned medium conferred therapeutic benefit, even when compared directly with whole-cell therapy [13, 82]. The immunomodulatory, paracrine activity of MSCs may also have therapeutic potential in other inflammatory diseases [68, 116, 136]. Several factors found in the MSCs secretome, among which are interleukin-10 [98], transforming growth factor-beta (TGF-β) [92], stanniocalcin-1 [19] and keratinocyte growth factor (KGF) [82], have been proposed to mediate MSCs cross-talk with various effector cell types, such as macrophages [98]. Recent observations also indicate cell-protective effects of MSCs on endogenous stem cells, such as bronchoalveolar stem cells (BASC) [157]. Identification of these MSC-secreted factors, along with clarification of their mechanisms of action, may allow the development of new treatments.

1.5.3. ESCs and Induced Pluripotent Stem Cells (iPSCs)

ESCs represent the most pluripotent stem cells but the clinical applicability of ESC-based clinical solution is hampered by the ethical controversy surrounding the need to isolate these cells from the early embryo. The recent landmark generation of "ESC-like", induced pluripotent stem cells (iPSC) using viral delivery of pluripotency genes to somatic cells [148] may relieve many ethical concerns related to the use of ESCs for research and has opened the way to large-scale production and evaluation of pluripotent stem cells for lung regeneration and repair.

When maintained in conditions that support the undifferentiated state, pluripotent stem cells show unlimited proliferation potential, which renders them ideal candidates for studies in developmental biology regeneration. ESCs can be directed to differentiate into definitive endoderm from which they may be further differentiated into lung cells using specific factors [128, 161]. Another method employed was the exposure of ESCs to microenvironments mimicking lung conditions (coculture with lung mesenchyme or lung cell extracts) [160]. Although there are isolated reports indicating the attainment of fully differentiated proximal airway-like tissue [28], airway epithelium [133], distal lung progenitors [127] or even pure populations of AT2 cells [165] from ESCs, most of the available literature indicates cellular heterogeneity of the

cultures with a relatively low yield of lung cells. *In vivo* administration of ESCs or progenitor cells derived from ESCs or iPSCs has also generated inconclusive results so far, with limited and transient ESC expression in the lung [128, 174]. Further steps, such as stable differentiation and purification of desired cell populations need to be taken in order to assess the potential of ESCs and iPSCs for lung diseases.

1.5.4. Stem Cells and Carcinogenesis

The term "lung cancer" encompasses several different pathological entities: squamous cell carcinomas, small cell carcinomas and adenocarcinomas which appear with different frequency in different areas of the lung, suggesting that local lung environment may act upon cell fate. The hypothesis of tumor-initiating cells (cancer stem cells) could explain the relapse of certain tumors owing to the fact that these cells might be resistant to many conventional cancer therapies [111, 113, 182]. The identification of putative resident stem cells in lung tumors [75] leads to the question whether the resident cells that survive pollutant-induced injury may in fact be such a cancer stem cell. The existence of cancer stem cells in the lung is supported by work indicating that CD133+ is a marker of self-renewing cells that sustain tumor propagation in mice [39]. These cells are resistant to cisplatin treatment [17]; however, the proportion of

cells expressing this marker lacks prognostic value [132]. Other work suggests that activation of the *k-ras* gene, whose activation was shown to be directly linked to early-onset lung cancer [69], upregulates the SP-C+/CCSP+ (BASC) cells and leads to development of lung adenocarcinomas [75]. Similarly, deletion of phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase (PI3K) or p38a mitogen-activated protein kinase (MAPK) led to proliferation of SP-C+/CCSP + cells simultaneous with the increase in susceptibility to develop lung neoplasms [185], whereas Bmi1 (B lymphoma Mo-MLV insertion region 1 homolog) deletion had opposite effects [38]. Bmi1 has been shown to be crucial for stem cell self-renewal [189]. However, it has not yet been clearly determined whether there is a link between the CD133-expressing and the dual SP-C/CCSP-expressing cell population or whether either of these populations acts as an initiator or propagator of lung malignant tumors. Also, the cells in small cell carcinomas have been shown to express basal cell markers, whereas small cell carcinomas have been found to express markers reminiscent of PNECs [51], but the direct relationship between the putative stem/progenitor cells and the neoplastic cells, as well as the proposed contributions of bone marrow-derived cells to cancer progression [48] has yet to be investigated. Although much work is still needed to identify and characterize cancer

stem cells-initiating cells, the discovery opens therapeutic avenues for designing specific cellular targets for the treatment of cancer [192].

1.6. Biotechnology – Engineering Lung Tissue

Currently, lung transplantation is the only viable solution for incurable lung disease in patients under 65 years of age. These lung diseases include lung fibrosis, COPD, cystic fibrosis, primary pulmonary hypertension, sarcoidosis, lymphangioleiomyomatosis. However, the mortality rate from the moment the potential recipients are placed on the waiting list until they receive the transplant is currently around 30% [118]. Moreover, lung transplantation is not an option for patients with other major accompanying health problems. This highlights the necessity to seek for alternative approaches, such as the development of the artificial lung or bioengineered lung components.

1.6.1. Human Ex-Vivo Lung Project (HELP)

Currently, the supply of donor lungs does not match the demand and one of the facts that contribute to this shortage is that only about 20% of donor organs are considered acceptable for transplantation [118]. Improper oxygenation capacity (reflected by a PaO_2 below 300 mm Hg

after oxygenation with a FiO_2 of 100% for 5 min and PEEP greater than 5 cm H_2O) leads to rejection of donor lungs. HELP involves the concept of reconditioning and transplantation of these otherwise rejected donor lungs. Lungs are reconditioned *ex vivo* by continuous perfusion with a lung evaluation–preservation solution (Steen solution) [175] mixed with erythrocytes for several hours, until the functional parameters reach acceptable values. After reconditioning, these lungs can be transplanted immediately or stored at 8°C in *ex vivo* extracorporeal membrane oxygenation (ECMO) until transplantation can be performed [63]. The first transplant of lungs harvested from a donor and reconditioned *ex vivo* was performed successfully in 2007 [141]. The impact of this promising strategy remains to be evaluated.

1.6.2. Artificial Lung - NovaLung®

The artificial lung is a relatively new method, similar in concept to dialysis and designed to support respiratory function while the potential lung transplant recipient is waiting for the donor lungs [44]. The patient's blood flows into a device that removes carbon dioxide and enriches the blood in oxygen. As compared to conventional ECMO, the artificial lung eliminates the need for an extracorporeal blood pump and can be used for extended periods of time (up to 100 days) [163] in centres where ECMO is

not available. Other advantages of this system over ECMO are reduced anticoagulation and avoidance of long-term mechanical ventilation [150, 164].

1.6.3. Bioengineered Lung Tissue

The structural and functional complexity of the lung has so far restricted the development of bioengineered lung tissue, when compared to the progress made in engineering less complex organs, such as the skin or the urinary bladder [12, 14]. A recent *in silico* model of the alveolar-capillary interface has been developed employing biomaterials and human alveolar epithelial cells at air-liquid interface, along with human pulmonary microvascular endothelial cells [60]. This type of biomimetic microsystems could facilitate drug screening and toxicology studies by allowing high-throughput processing. On a larger scale, so far both ESCs and adult multipotent stem cells, as well as mixed cell populations containing progenitor cells or terminally differentiated cells such as fibroblasts or chondrocytes have been used with promising results to generate lung cell lineages or bioengineered lung components, including recellularization of a human tracheal scaffold with MSCs-derived chondrocytes, followed by surgical implantation as a main bronchus [86]. However, the lack of conclusive information with respect to the

tumorigenic potential of stem cells, especially ESCs, known for their karyotypic instability, together with the unanswered question regarding the local progenitor cells as potential cancer stem cells, demand careful safety evaluation of stem cell-based approaches. Also, the biomaterials used as scaffolds on which the lung tissue would be grown need to be evaluated with regards to their biocompatibility in terms of elasticity, adsorption kinetics, porosity and degradation kinetics [101]. So far, scaffolds composed of natural polymers like collagen, Matrigel (a mixture of basement membrane proteins and / or synthetic polymers, such as polyacrylamide have been used in attempts to engineer lung tissue [12]. Aside from constructed scaffolds, a recent breakthrough in lung bioengineering has been achieved by demonstrating that decellularized lung matrices have the ability to support repopulation with newly-seeded epithelial and endothelial cells and, moreover, to sustain lung function following transplantation into animals [104, 112, 140].

1.7. Clinical Studies: Experience, Outcome, Limitations

Several limitations have hampered clinical trials of stem cell-based therapies for lung diseases. There are certain risks to heterologous cell transplantation. The cells may carry infectious agents, which poses an even enhanced peril in the case of recipients who have developed graft-

versus-host disease [130]. Furthermore, there have been reports of bronchiolitis obliterans organizing pneumonia (BOOP) in patients who had undergone HSC transplantation [58]. Both heterologous and autologous transplantation bear the risk of tumor formation. ESCs and iPSCs develop teratomas *in vivo* and there are also reports indicating that transplantation of neural stem cells led to the development of tumors in the recipient brain [10]. MSCs, generally considered less prone to acquiring karyotypic abnormalities compared to ESCs, may also pose tumorigenic risks [155]. However, these dangers may be overcome: recent findings indicating that stem cell-secreted factors exert therapeutic benefits may abrogate the need to deliver the cells themselves to the damaged tissues.

Another limitation is the insufficient characterization of stem cells in terms of both phenotype and function. For MSCs, minimal criteria for defining human MSCs, established by the International Society for Cellular Therapy [37], have reduced some of the variations with regards to cellular composition of MSC populations isolated according to different protocols. Lung injury prevention obtained with MSCs in various animal models of lung disease, together with their ease of isolation and culture, as well as their immuno-modulatory properties make these cells very promising candidates for clinical trials.

Thus far, MSCs have been transplanted in humans as part of whole bone marrow transplantation for various disorders (including leukemia and genetic diseases of the immune system). Gender-mismatched transplantation (male donor bone marrow to female recipient) has proven to be a useful tool in assessing the impact of stem cell transplantation on other organs than bone marrow. Donor male cells were identified in the lungs of recipients as epithelial and endothelial cells [147] and also in the liver [152], heart [35], brain [29, 96] and kidney [114]. Also, in the reverse case where males were recipients of sex-mismatched organ transplants, the Y chromosome indicating recipient origin was identified in a variable proportion of organ-specific cells. With regards to lungs, the chimerism was present in bronchial epithelial cells, AT2 and seromucous glands [115].

Currently, one phase I clinical trial aimed at evaluating the tolerability and safety of progenitor cells for the treatment of pulmonary arterial hypertension (Pulmonary Hypertension: Assessment of Cell Therapy, PHACeT) is underway. Autologous endothelial progenitor cells are engineered *ex vivo* to express endothelial nitric oxide synthase (eNOS), followed by injection of the cells via a pulmonary artery line. Previous pilot studies have supported the feasibility of this approach in idiopathic pulmonary hypertension [167, 193].

On the basis of initial reports of safety and efficacy following allogeneic administration of MSCs to patients with Crohn's disease or with graft-versus-host disease, several trials studying the effect of MSCs in patients with lung diseases (COPD, BPD, idiopathic pulmonary fibrosis, emphysema) are ongoing and programs such as the Production Assistance for Cellular Therapies (PACT) [124] in the United States have been initiated to facilitate the translation of cell-based therapies to the clinical environment. Information on current clinical trials involving the use of stem cells or stem cell-derived products is regularly updated on the United States National Institute of Health's website www.ClinicalTrials.gov.

Cell type	Location	Phenotype	Lung injury model	Remarks
Basal and parabasal cells	proximal airway epithelium - submucosal glandular ducts and intercarti-laginous zone	cytokeratin 5/14+	polydocanol or SO_2-induced [21]	clonogenic capacity, multilineage differentiation [134] ; repopulate airway epithelium post-injury [59]
Type A (new, variant) Clara cells	bronchioalveolar junction; proximity of neuroepithelial bodies (NEBs) [49]	Clara cell secretory protein (CCSP)+; no secretory granules, no smooth ER	naphthalene- or ozone-induced	retain labeled DNA precursors [121]; repopulate injured airway epithelium with both mature, quiescent Clara cells and ciliated epithelial cells
Bronchio-alveolar stem cells (BASC)		Sca1+/CD34+/CD45−/CD31− [74, 75]		may co-express SP-C
Type II alveolar epithelial cells (AT2)		SP-C		proliferate and generate AT1 cells following injury [4, 123]. AT1 differentiate into AT2 *in vitro* [33]: alternate progenitor cells depending on type of lung injury
Side population (SP) [146]		heterogeneous population efflux the DNA dye Hoechst [50]		derived from bone marrow, identified in lung [88]; differentiate along endoderm- and mesoderm-derived lineages [89, 143]; endothelial potential (newborn mice); decreased in oxygen-induced arrested alveolar growth [65]
Pulmonary neuro-endocrine cells (PNECs)	found with type A Clara cells in NEBs-associated regenerative foci [126].	calcitonin gene-related peptide (CGRP)	naphthalene-induced	oxygen-sensing [188]; if both naphthalene-sensitive and -resistant Clara cells are ablated airway epithelium does not regenerate - PNECs are not airway epithelial progenitors [59]
Lung epithelial progenitors		EpCAM(hi)/CD104+/CD24(low)		give rise to bronchial and alveolar epithelium [94]
Multipotent lung progenitors		c-kit+		self-renewing, clonogenic [72]

Table 1-1. Summary of putative endogenous lung stem cell populations.

1. 8. References

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CHAPTER 2 - AIRWAY DELIVERY OF SOLUBLE FACTORS FROM PLASTIC-ADHERENT BONE MARROW CELLS PREVENTS MURINE ASTHMA

A version of this chapter has been published.

Ionescu LI, Alphonse RS, Arizmendi N, Morgan B, Abel M, Eaton F, Duszyk M, Vliagoftis H, Aprahamian T, Walsh K, Thebaud B. Airway delivery of soluble factors from plastic-adherent bone marrow cells prevents murine asthma. *Am J Respir Cell Mol Biol* 2012 Feb; **46**(2):207-16. Epub 2011 Sep 8.

LII performed the majority of the experiments. RSA performed the BMCs characterization. NA, BM, MA and FE provided technical assistance. MD and HV aided in designing the experimental outlines. TA and KW provided the adiponectin knock-out mice.

The manuscript was written by LII and edited by BT.

2.1. Abstract

Asthma affects an estimated 300 million people worldwide and accounts for 1 in 250 deaths and 15 million disability-adjusted life years lost annually. Plastic-adherent bone marrow-derived cells (BMCs) administration holds therapeutic promise in regenerative medicine. However, given the low cell engraftment in target organs, including the lung, cell replacement cannot solely account for the reported therapeutic benefits. This suggests that BMCs may act by secreting soluble factors. BMCs also possess anti-inflammatory and immunomodulatory properties and may therefore be beneficial for asthma.

Our objective was to investigate the therapeutic potential of BMCs-secreted factors in murine asthma.

In a model of acute and chronic asthma, intranasal instillation of BMCs conditioned medium (CdM) prevented airway hyperresponsiveness (AHR) and inflammation. In the chronic asthma model, CdM prevented airway smooth muscle (ASM) thickening and peribronchial inflammation, while restoring blunted salbutamol-induced bronchodilation. CdM reduced lung levels of the Th2 inflammatory cytokines interleukin (IL)-4 and IL-13 and increased levels of IL-10. CdM upregulated an IL-10-induced and -secreting subset of T regulatory lymphocytes and promoted IL-10 expression by lung macrophages. Adiponectin (APN), an anti-

inflammatory adipokine found in CdM, prevented AHR, ASM thickening and peribronchial inflammation, while the effect of CdM in which APN was neutralized or from APN knock-out mice was attenuated compared to wild-type CdM.

Our study provides evidence that BMCs-derived soluble factors prevent murine asthma and suggests APN as one of the protective factors. Further identification of BMCs-derived factors may hold promise for novel approaches in the treatment of asthma.

2.2. Introduction

Allergic disorders, such as anaphylaxis, hay fever, eczema and asthma, now afflict roughly 20-30% of people in the developed world [12]. Asthma is characterized by airway hyperresponsiveness (AHR), inflammation and remodeling with thickening of the airway smooth muscle (ASM) cell layer refractory to bronchodilator therapy [1, 23]. Despite the progress achieved in the past decades in the management of asthma, refractory asthma still accounts for more than 250,000 deaths worldwide every year [31].

Recent studies have explored the therapeutic potential of stem cells for regenerative medicine in multiple clinical disorders including myocardial infarction, diabetes, sepsis, hepatic and acute renal failure [45]. Bone marrow (BM)-derived cells also prevent various experimental lung diseases including pulmonary fibrosis [43, 47], acute lipopolysaccharide-induced lung injury [14, 29, 37], and chronic oxygen-induced neonatal lung disease [4, 53]. Over the past decade alone, numerous reviews have summarized the increasing amount of evidence in support of the therapeutic benefits exerted by BM-derived cells [22, 33, 47, 60, 61]. However, cell engraftment is consistently disproportionately low for cell replacement to account for the therapeutic benefit. An alternate hypothesis proposed for mesenchymal stem cells (MSCs) is the release of

soluble factors with exertion of their therapeutic benefit through a paracrine-mediated mechanism [45].

In addition, BM-derived cells display immunosuppressive and anti-inflammatory properties [19, 20, 25, 27, 28, 38], suggesting their potential benefit in allergic and inflammatory disease such as asthma. Thus, we hypothesized that airway delivery of plastic adherent BM-derived cells (BMCs) conditioned medium (CdM) prevents AHR, inflammation and remodeling in experimental murine asthma.

2.3. Materials and Methods

2.3.1. BMCs Isolation and Culture

BMCs from adult wild-type (WT) BALB/c mice (8-10 weeks - Charles River Laboratories, Wilmington, MA) and adiponectin (APN) knock-out (KO) mice (Dr. Kenneth Walsh, Boston University) were isolated and differentiated along mesenchymal lineages according to previously described methods [44] based on their adherence to tissue culture plastic. Briefly, mice were anesthetized with sodium pentobarbital (65 mg/kg), the femurs and tibias were dissected, washed twice with phosphate-buffered saline (PBS, Sigma-Aldrich, Oakville, ON) and the bone marrow was flushed with complete cell culture medium - Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin-fungizone (PSF, Invitrogen, Burlington, ON) using a 26-gauge needle attached to a 10 mL syringe. The plastic-adherent cells were cultured at 37 °C in atmosphere containing 20% O_2, 5% CO_2 in humidified incubators.

For adipogenic differentiation, passage 2 BMCs were cultured for 21 days in adipogenic induction cell culture medium (Dulbecco's Modified Eagle Medium, low-glucose, supplemented with 10% fetal bovine serum, 1% PSF, 0.5 mM IBMX, 60 μM indomethacin, 0.5 μM hydrocortisone and 1

μg/mL insulin). After 21 days, the cells were stained with Oil Red O (Sigma-Aldrich) to reveal intracellular lipid droplets indicating the differentiation of BMCs into adipocytes.

For osteogenic differentiation, passage 2 BMCs were cultured in osteogenic induction cell culture medium (Minimal Essential Medium, alpha modification, low-glucose, supplemented with 10% fetal bovine serum, 1% PSF, 10 mM B-glycerophosphate, 0.2 mM ascorbic acid, 0.01 μM dexamethasone). After 21 days in culture, the cells were stained with fresh Alizarin Red solution (Sigma-Aldrich) in order to reveal the extracellular calcium deposits characteristic of osteoblasts.

For chondrogenic differentiation, passage 2 BMCs were cultured as pellets (200,000 cells/ pellet) in 15 mL centrifuge tubes for 21 days. We used a commercially available chondrogenic differentiation kit (R&D Systems, Minneapolis, MN). The chondrogenic induction culture medium was prepared according to manufacturer's instructions. After 21 days, the pellets were embedded in paraffin, cut into sections and stained with Safranin O (Sigma-Aldrich) to reveal the proteoglycan deposits indicative of differentiation into chondroblasts.

For separation of CD45+ and CD45- BMCs subpopulations we used streptavidin-coated magnetic beads (Dynabeads®, Invitrogen) and biotinylated CD45 antibody (BD Biosciences, Missisauga, ON).

2.3.2. Lung Fibroblasts (LFs) Isolation and Culture

Fibroblasts were isolated from adult WT Balb/C mice according to a previously described method [51]. The lungs were washed twice with PBS and finely minced in DMEM supplemented with 15% FBS and 1% PSF. Tissue culture plastic-adherent cells were grown for two passages.

2.3.3. Fluorescence-Activated Cell Sorting (FACS)

For the characterization of BMCs [37, 52, 54], the following antibodies were used: stem cell antigen-1 (Sca-1), CD11b, CD29, CD31, CD34, CD44, CD45, CD73, CD105 (Biolegend, San Diego, CA). The cells were analyzed using a BD FACScan flow cytometer (BD Biosciences, Mississauga, ON).

For FACS analysis of lymphocytes from draining lymph nodes, mediastinal lymph node chains were harvested, minced and passed through a 100 μm cell strainer; for the quantification of regulatory T cells and macrophages in the lung, lymphocytes were isolated using a previously published method [50]. Briefly, lungs of experimental animals were digested using collagenase III (Worthington Biochemical Corporation, Lakewood, NJ) and the lymphocytes stained using IL-10 antibody (Biolegend) and a commercially available kit (Mouse Treg Flow™

Kit, Biolegend) or CD11b (BD Biosciences) and analysed using a BD FACSCanto™ flow cytometer (BD Biosciences).

2.3.4. BMCs and Lung Fibroblasts CdM Preparation

Passage 2 BMCs or lung fibroblasts (~80% confluent) were rinsed twice with PBS and cultured in medium without FBS supplementation for 24 hours. The cell culture supernatants were collected, concentrated (25 times) and desalted by centrifugal filtration (Amicon - Millipore, Billerica, MA). Supernatants were generated from multiple batches of BMCs. Typically, 3 adult mice were used to isolate primary cells from femurs and tibiae seeded in one 75 cm^2 tissue culture flask. At passage 2, 9 identical flasks were generated from the original flask, yielding 600 µL concentrated CdM / flask (used for animal treatment and cytokine quantification) and also cells for lineage differentiation and surface marker characterization (WT BMCs). BMCs (WT or APN KO) did not appear different between batches in terms of cell numbers, rate of growth/proliferation, viability (Trypan Blue counts). In order to further minimize variability:

- BMCs were always isolated and cultured by the same operator, using the same protocol, reagents, mouse strain, age, gender, weight, nutrition status and mouse source;

- CdM was always prepared from the same number of cells; cells would be in the log phase of growth, ~80% confluent to avoid effects of sub- or overconfluency on cells or protein yield;
- CdM was never prepared twice from cells isolated at the same time; after serum starvation and supernatant collection cells were screened for consistent viability between batches using Trypan Blue vital dye and discarded immediately;
- Upon preparation of CdM, CdM prepared on the same day (from cells initially isolated and passaged at the same time) was pooled, frozen at – 80 degrees Celsius and thawed only once, upon use;
- Again, for reproducibility purposes, the same frozen CdM (which would have otherwise been used for treatment) was used for cytokine measurements. Cytokine quantification was always preceded by total protein quantification, to ensure sufficient amounts and consistency.

For neutralization of APN in WT BMCs CdM, we used an APN neutralizing antibody (R&D Systems). Cells were serum-starved for 24 hours in the presence of 100 ng/mL APN antibody [46] and CdM was prepared as described before.

2.3.5. Animal Model

Adult male BALB/c mice (8-10 weeks) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed in specific pathogen-free conditions in the University of Alberta Health Sciences Laboratory Animal Services facilities throughout the entire duration of the experimental protocols. All procedures were approved by the University of Alberta Animal Policy and Welfare Committee. Animals were allowed to adapt to housing conditions for a week before commencing experimental protocol.

Mice were sensitized to ovalbumin (OVA) by intraperitoneal injection of 10 μg OVA (lypopolysaccharide-free chicken egg albumin grade V, Sigma-Aldrich) adsorbed on 2 mg aluminum hydroxide (Sigma-Aldrich) in 0.2 mL saline [9]. Control animals received saline solution alone.

For OVA challenges, mice were lightly anesthetized with a mixture containing ketamine (75 mg/kg) and acepromazine (2.5 mg/kg) and a volume of 25 μL of a solution containing 5% OVA was administered intranasally.

In the acute asthma model (short protocol) [9], mice received two intraperitoneal sensitization injections on days 1 and 6, followed by two intranasal challenges on days 12 and 14 (**Fig. 2-1**A). In the chronic asthma

model (long protocol, adapted from [57]), mice were sensitized on days 1, 8, 15 and 29 and challenged on days 22, 24, 27, 31, 34 and 36 (**Fig. 2-1**B). A volume of 25 µL concentrated, desalted BMCs CdM was administered intranasally after the OVA challenge.

For the APN experiments, recombinant APN (rAPN, R&D Systems, 5 µg/g body weight) was reconstituted in 25 µL of saline and administered upon each OVA challenge, similar to BMCs CdM administration.

2.3.6. Bronchoalveolar Lavage Fluid (BALF) Analysis

Mice not subjected to lung function testing were anesthetized by intraperitoneal injection of sodium pentobarbital. The trachea was cannulated and instilled with 5 successive aliquots of 1 mL sterile, ice-cold phosphate buffered saline (PBS, Sigma-Aldrich) solution. The recovery of the instilled fluid was typically greater than 80%. The total cells in the BALF were counted and cytospin preparations were obtained (Thermo Shandon, Pittsburgh, PA). Differential staining (Hema 3 Manual Staining System, Thermo Fisher Scientific, Nepean, ON) was performed in order to identify the cell types present in the BALF based on their morphological features [9].

2.3.7. Lung Function Testing (LFT)

At 48 hours after the last challenge, mice were anesthetized by intraperitoneal injection of a mixture containing ketamine and acepromazine. After anesthesia was achieved, pancuronium bromide (1 mg/kg, Sigma-Aldrich) was administered intraperitoneally in order to abolish spontaneous breathing by achieving muscular relaxation. An 18-gauge cannula was inserted into the trachea and secured in place. The lung function was assessed using a small animal ventilator (FlexiVent, Scireq, Montreal, QC). All data recordings were obtained while maintaining a positive end-expiratory pressure (PEEP) of 4 cm H_2O. Aerosolized saline (to establish baseline responses), methacholine (2, 8 and 32 mg/ mL) [8] and salbutamol (0.5 mg/mL) solutions were delivered intratracheally. Each dose was nebulized for 10 seconds using an Aeroneb nebulizer. The volume history was standardized to TLC after the administration of each dose and the selected parameters were measured 12 times over 3 minutes. Only data with a coefficient of determination (COD) greater than 0.9 were used and the maximal responses corresponding to each dose were selected for analysis.

We employed the single-compartment model (whole lung mechanics) of lung behavior [55]. The measurement of dynamic lung resistance (R) and dynamic lung elastance (E) was obtained using a preset

volume perturbation during which the regular ventilation was interrupted and a 2.5 Hz (150 breaths/ min) sine wave was applied. The data was automatically fitted to the equation of motion describing the single-compartment linear model of lung behavior:

$$P(t) = RV(t) + EV(t) + P_0$$

where P(t) is the pressure at the airway opening, V(t) is the air flow, V(t) is the volume of gas in the compartment and P_0 is the resting applied pressure (i.e. PEEP).

2.3.8. Cytokine Quantification

Lungs from animals that had not been subjected to LFT or BALF collection were snap-frozen in liquid nitrogen and homogenized by sonication on ice in modified RIPA buffer (Millipore) supplemented with aprotinin, leupeptin, sodium orthovanadate and phenyl-methyl-sulphonyl fluoride (PMSF, all from Sigma-Aldrich).

Cytokine concentrations were determined using commercially available ELISA kits for detection of IL-4, IL-10, IL-13 (eBioscience, San Diego, CA) according to manufacturer's instructions. APN was quantified in the wild-type and APN KO BMCs CdM and LFs CdM using a commercially available ELISA kit (Millipore).

2.3.9. Lung Histological Analysis

The trachea was cannulated and the lungs were inflated with 10% zinc-formalin while maintaining a constant pressure of 20 cm H_2O. The lungs were excised and placed in formalin for 24 hours, embedded in paraffin and cut into 5-μm sections according to a previously described method [54] in order to maximize the number of intact airways per section. Hematoxylin & eosin (H & E) - stained lung sections were analyzed using a computer-controlled light microscope (Leica Microsystems Canada, Richmond Hill, ON). The airway smooth muscle layer thickness and peribronchial inflammatory infiltrate area were measured using the Openlab imaging software (Improvision, Coventry, UK). All non-obliquely cut (maximum diameter / minimum diameter < 2), intact medium-sized airways (100-300 μm diameter) were selected for airway analysis, according to a previously published set of criteria [17]. The airway smooth muscle (ASM) layer thickness was measured using the formula:

ASM layer thickness = (outer airway diameter – inner airway diameter)/ 2

where the outer airway diameter was measured to include the smooth muscle layer and the inner airway diameter was measured as lumen diameter + epithelial layer.

Peribronchial inflammatory area was delineated and quantified using the OpenLab selection and measurement tool. All quantifications were performed by an investigator blinded to the experimental groups.

2.3.10. Statistical Analysis

Statistical analysis was performed using the StatView 5.0 software (Abacus Concepts, UK). The group means were compared using the analysis of variance (ANOVA) - one-way or repeated measurements (FLT), followed by Fisher's probable least significant difference (PLSD) post-hoc test. All values are expressed as mean (+/- standard error) and a value of $p<0.05$ was considered significant.

2.4. Results

2.4.1. BMCs Differentiation Along Mesenchymal Lineages And Surface Marker Profile

BMCs differentiated into adipocytes (**Fig. 2-2A**), osteocytes (**Fig. 2-2B**) and chondrocytes (**Fig. 2-2C**) and displayed a surface marker profile indicative of a heterogeneous cell population (**Fig. 2-3**).

2.4.2. BMCs CdM Prevents Airway Inflammation in Experimental Asthma

BMCs CdM significantly reduced the total number of cells in the BALF in OVA-CdM animals compared to untreated OVA mice in both the acute (n=8, $p<0.0001$, **Fig. 2-4**A) and the chronic (n=6, $p=0.0001$, **Fig. 2-4**B) asthma model. Polymorphonuclear cells (PMN), predominantly eosinophils (~50% of total BALF cells) were significantly increased in the BALF of OVA mice compared to control animals, whereas BMCs CdM significantly attenuated lung PMN influx (acute model: $p<0.0001$, **Fig. 2-4**C; chronic model: $p=0.0006$, **Fig. 2-4**D). BMCs CdM had no effect on control saline animals in the acute model (**Fig. 2-4**A). Control CdM from mouse lung fibroblasts (Fib CdM) had no effect when compared to

untreated OVA animals in the acute model (**Fig. 2-4**B). Fib CdM significantly lowered the total number of cells and PMN in the BALF in the chronic model (**Fig. 2-4**D) suggesting some anti-inflammatory properties of Fib CdM.

2.4.3. BMCs CdM Prevents AHR in Experimental Asthma

In the acute model, the methacholine dose-dependent increases in dynamic whole-lung resistance (**Fig. 2-5**A) and elastance (**Fig.2-5**B) were exacerbated in untreated OVA mice (n=11) compared to control saline (n=9). BMCs CdM treatment attenuated the dose-dependent increase in both parameters - dynamic whole-lung resistance (**Fig. 2-5**A) and elastance (**Fig. 2-5**B) - by 50%. In the chronic model, there were no differences between groups in the dynamic lung resistance (**Fig. 2-5**C). BMC CdM attenuated the increase in dynamic lung elastance in the chronic model by over 50% (**Fig. 2-5**D) Conversely, control Fib CdM had no beneficial effect compared to untreated OVA animals (**Fig. 2-5**A-D) suggesting that the benefit was specific to the CdM of BMCs. BMCs CdM had no deleterious effect in control saline animals (**Fig. 2-5**A-D).

2.4.4. BMCs CdM Improves Bronchial Responsiveness to Salbutamol and Prevents Chronic ASM Thickening and Peribronchial Inflammation

The bronchodilator response to salbutamol was significantly blunted in the chronic OVA model as documented by a persistent increase in dynamic lung resistance (**Fig. 2-6**A) and elastance (**Fig. 2-6**B). The response to salbutamol remained blunted in OVA-Fib CdM-treated animals as quantified by the persistent increase in dynamic lung resistance (**Fig. 2-6**A) and elastance (**Fig. 2-6**B). Conversely, BMCs CdM restored the bronchodilator response to salbutamol similar to the control groups (**Fig. 2-6**A and B).

Medium-sized bronchi had a significant thickening of the ASM layer and an increase in peribronchial inflammatory infiltrate in OVA-sensitized animals compared to controls as quantified by ASM thickening (**Fig. 2-7**A) and increased peribronchial inflammatory infiltrate (**Fig. 2-7**B) and shown in representative H&E sections for each group (**Fig. 2-7**C-I). Increased ASM thickening (**Fig. 2-7**A) and peribronchial inflammatory infiltrate (**Fig. 2-7B**) were significantly reduced by 20% and 40% respectively in BMCs CdM-treated OVA-mice compared to untreated OVA-mice. Fib CdM had no beneficial effect on these parameters.

2.4.5. BMCs CdM Attenuates T helper-2 (Th2) Lymphocyte Cytokine Response

The Th2 cytokines IL-4 and IL-13 were significantly lower in the lungs of OVA-CdM mice compared to untreated OVA mice and OVA-Fib CdM mice in both the acute (**Table 2-1**) and the chronic (**Table 2-2**) model. The levels of the anti-inflammatory cytokine interleukin-10 (IL-10), were significantly increased in the lungs of OVA BMCs CdM mice compared to untreated OVA mice. IL-10 was present in BMCs CdM (29.4 ± 3.26 ng/mL) but not detectable in Fib CdM by ELISA.

2.4.6. APN Mediates Protective Effects of BMCs CdM on AHR

We also found APN, an anti-inflammatory adipokine, to be expressed in BMCs CdM but not in Fib CdM (**Fig. 2-8**A) suggesting that some of the beneficial effects of BMCs CdM could be mediated by APN. APN was produced predominantly by BM-MSCs (5-fold compared to $CD45^+$ BMCs) (**Fig. 2-8**A).

To test for the contribution of APN to the beneficial effects of CdM, we tested the effects of APN absence in BMCs CdM using a neutralizing antibody and BMCs from APN knock-out (KO) mice. APN inhibition with a neutralizing antibody efficiently decreased APN in BMC CdM (NeutCdM,

Fig. 2-8A). APN inhibition significantly attenuated the beneficial effect of BMC CdM on dynamic lung elastance in the acute asthma model (**Fig. 2-8**B).

Likewise, APN was undetectable in BMCs of APN KO mice (**Fig. 2-8**A). In addition, APN KO BMCs CdM was unable to prevent AHR (**Fig. 2-8**C) and bronchodilator responsiveness (**Fig. 2-8**D). Conversely, recombinant APN (rAPN) treatment alone significantly improved AHR (**Fig. 2-8**C) and bronchodilator responsiveness to salbutamol (**Fig. 2-8**D).

2.4.7. APN Mediates Protective Effects of BMCs CdM on Airway Remodeling

Representative H&E stained lung sections showed that compared to control saline mice (**Fig. 2-9**A), the chronic OVA model had significant airway remodeling (**Fig. 2-9**B). Airway remodeling was prevented in OVA-CdM (**Fig. 2-9**C), but not by APN KO BMCs CdM (**Fig. 2-9**D). Treatment with rAPN in turn prevented airway remodeling (**Fig. 2-9**E). These data were confirmed by quantitative airway morphometry showing attenuated ASM thickness (**Fig. 2-9**F) and peribronchial inflammation (**Fig. 2-9**G) in OVA-CdM and rAPN-treated mice but not in APN KO BMCs CdM-treated mice, further suggesting that APN contributes to the beneficial effects of BMCs CdM.

2.4.8. BMCs CdM Induces Subsets of IL-10-Producing T Regulatory Cells (Tregs) and Macrophages

FACS analysis indicated that, while CdM did not impact the proportion of CD4+CD25+Foxp3+ cells in the lung (**Fig. 2-10**A) or the draining lymph node (**Fig. 2-10**B) lymphocytes, it led to the upregulation of a subpopulation of lung lymphocytes co-expressing the surface markers CD25 (**Fig. 2-11**A) and CD4 (**Fig. 2-11**B) and intracellular IL-10, but lacking expression of the transcription factor Foxp3 (**Fig. 2-11**C-F). This population was significantly upregulated in OVA-CdM mice compared to OVA and control mice (**Fig. 2-11**G). FACS also revealed a subpopulation of IL-10-expressing $CD11b^{+}$ macrophages (**Fig. 2-11**H-K). This population was significantly upregulated in BMCs CdM- and rAPN-treated mice (**Fig. 2-11**L).

2.5. Discussion

We show that airway delivery of CdM obtained from BMCs prevents the development of AHR, airway inflammation and airway remodeling while restoring airway responsiveness to salbutamol in a murine model of acute and chronic asthma. BMCs CdM also attenuated Th2-associated cytokine release, induced a subset of anti-inflammatory IL-10-secreting Tregs and promoted IL-10 production by macrophages. Our data support the potential for APN as a target for asthma therapy by showing that APN contributed significantly to prevent AHR, inflammation and histological features of airway remodeling. We propose APN as a candidate for asthma therapy. BMCs-derived soluble factors may be a viable alternative to whole-cell delivery and could therefore serve as a novel approach for the treatment of asthma.

Recent evidence suggests that stromal stem cells prevent organ damage through a paracrine effect rather than cell replacement [45]. In addition, these cells display anti-inflammatory and immunomodulatory properties [19, 20, 25, 27, 28, 38]. These properties render stromal stem cells particularly appealing and amenable to the treatment of inflammatory diseases. *In vitro*, these cells have been shown to inhibit the proliferation of immune effector cells such as B lymphocytes and T

lymphocytes [19]. In humans, a phase II clinical trial suggested that stromal stem cells attenuate steroid-resistant graft-versus-host disease after haemopoietic stem cell transplantation [27]. Inhaled and systemic steroids have been successfully employed in asthma since the recognition of the contribution of airway inflammation to asthma pathogenesis, but refractory asthma is still responsible for 250,000 deaths worldwide every year [31]. Refractory asthma is a pathological entity characterized by reduced or lack of responsiveness to conventional bronchodilator therapy [1, 23]. Here we show that BMCs-derived CdM exerts potent anti-inflammatory effects in acute and chronic murine asthma, preventing airway inflammation (**Fig. 2-4**) and AHR in both models (**Fig. 2-5**). In chronic asthma, BMCs CdM restored the response to salbutamol, a commonly prescribed bronchodilator (**Fig. 2-6**), and prevented ASM layer thickening and peribronchial inflammation (**Fig. 2-7**). Meanwhile, Fib CdM failed to significantly impact AHR, bronchodilator responsiveness and airway remodeling. This suggests that the benefits were specific to BMCs CdM.

Asthma is an inflammatory disease caused by a complex set of interactions between multiple effector cell types. The presence of the antigen triggers Th2 lymphocyte activation, leading to an acute cascade of events, which include recruitment of PMN to the lung parenchyma

(typically eosinophils) [18] and secretion of Th2 cytokines such as IL-4 and IL-13 [13, 32, 62]. In our study, PMN recruitment was inhibited by BMCs CdM treatment, as indicated by BALF analysis in both acute and chronic asthma models (**Fig. 2-4**). Fib CdM also decreased total BALF cellularity and PMN numbers. This effect was limited to the chronic model and reduced compared to BMCs CdM. A similar partial reduction in BALF cellularity and eosinophilia following skin fibroblast administration has been reported [39]. These observations highlight the importance of using appropriate cell controls in order to further our understanding about the mechanism of action of MSCs and CdM.

In humans, chronic airway remodeling is characterized by airway wall thickening, mainly due to smooth muscle hypertrophy and hyperplasia, accompanied by the narrowing of the airway lumen with goblet cell hyperplasia, peribronchial and perivascular fibrosis [17]. These structural changes in chronic asthmatic airways may be a consequence of prolonged inflammation [53]. These changes may contribute to fatal asthma refractory to bronchodilator therapy. We evaluated the impact of BMCs CdM administration on airway remodeling by quantifying the degree of ASM layer thickening and peribronchial inflammation in the chronic asthma model, where remodeling was expected to occur. Both parameters were reduced in OVA-CdM animals compared to the OVA and

OVA-Fib CdM groups (**Fig. 2-7**C and D), suggesting that BMCs CdM prevents the development of airway remodeling. This histological improvement was accompanied by restoration of the bronchial responsiveness to salbutamol, a conventional bronchodilator (**Fig. 2-6**). Administration of a single dose of salbutamol on maximally constricted airways led to a significant decrease in dynamic resistance and elastance in the OVA-CdM similar to controls, while this response was blunted in untreated OVA mice and OVA-Fib CdM mice, a major feature in the occurrence of refractory asthma.

We sought to determine the mechanisms through which CdM acts to prevent the development of asthma features. We found that the levels of IL-4 and IL-13, two Th2 cytokines generally elevated in asthma [62], were attenuated in the lungs of OVA-CdM animals compared to untreated OVA mice (**Tables 2-1** and **2-2**). Our findings are consistent with recent reports showing that BMCs administration decreases lung inflammation and BALF levels of Th2 cytokines in an acute, ragweed-induced model of allergic asthma [39] and OVA-induced mouse models of AHR [5, 11, 21]. Moreover, in our study the levels of the anti-inflammatory IL-10 were significantly increased in the OVA-CdM lungs suggesting that BMCs CdM attenuates allergic Th2 inflammation through an IL-10-dependent mechanism. IL-10 has been shown to contribute to the reduction of airway

inflammation [41]. BMCs CdM itself acted as an exogenous source of IL-10, however, we did not find a significant difference between the levels of IL-10 in the lungs of BMCs CdM-treated control animals and untreated controls, suggesting that endogenous production of IL-10 in the allergic inflammatory environment may be an important component of elevated IL-10 levels found only in OVA-CdM lungs. We therefore sought to identify cellular effectors that would mediate the actions of BMCs-secreted factors and also potential local IL-10 sources. Regulatory T (Treg) cells have recently been shown to diminish inflammation in asthma [35] and BMCs are able to induce IL-10 secretion by Treg cells [10] and macrophages [24, 40]. Naturally occurring Treg cells are most commonly defined as cells that express the surface markers CD4, CD25 and the intracellular marker Foxp3 upon activation [34]. We did not find significant changes in the proportion of naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory Treg cells in the draining lymph nodes or lung lymphocytes (**Fig. 2-10**), suggesting that local BMCs CdM administration does not act on a systemic cellular immunity level in the acute model. This is consistent with reports indicating that short-term exposure to ovalbumin may lead to decreased lung $CD4^+CD25^+Foxp3^+$ cells [6] or spleen $CD4^+Foxp3^+$ cells [59]. A recent study suggests upregulation of $CD4^+CD25^+Foxp3^+$ in the lungs of BALB/c mice following allogeneic BMCs treatment in an OVA-induced model of AHR [21]. However, in this report the allogeneic BMCs were also

administered at the time of sensitization to OVA, not only upon antigen challenges. We did not attempt to prevent antigen sensitization, as we sought to mimic the clinical conditions in which the patient would have already been hypersensitive to specific triggers. Instead, we found that BMCs CdM prevented the OVA-induced downregulation of Treg cells expressing CD4, CD25 and IL-10 and lacking expression of Foxp3 (**Fig. 2-11**A-G), a subset of inducible Treg cells that have been described to be IL-10-induced and –secreting Treg cells (Tr1) [15, 16, 56]. This correlates with our finding that IL-10 is present in BMCs CdM and consistent with other reports indicating the presence of IL-10 in the human stromal stem cell secretome [58]. Simultaneously, using CD11b as a macrophage marker, we found a significant upregulation of $CD11b^{+}$ coexpressing IL-10 in OVA-CdM lungs (**Fig. 2-11**H-L). It has been proposed that BMCs may contribute to reduction of allergic airway inflammation by promoting a switch from Th2 to Th1 phenotype in an OVA-induced murine model of AHR [11]. Recently, a population of CD11b+Gr1+F4/80+ immunoregulatory myeloid-derived suppressor cells (MDSCs) have been shown to contribute to blunted Th2 responses following pre-allergen LPS exposure in a house dust-mite murine model of AHR. MDSCs were also able to prevent airway eosinophilia and IL-13 production when transplanted in vivo post-OVA exposure. The hypothesis that IL-10 has a

direct role in reducing Th2 inflammation is supported by the finding that Th2 cytokine levels were restored with neutralization of IL-10 [3].

BMCs have also been shown to act via TGF-ß production to alleviate airway inflammation in a ragweed-induced model of murine asthma [39].

In our quest to identify candidate factors mediating the beneficial effects of BMCs, we found APN, an anti-inflammatory adipokine to be present in BMCs CdM. APN is a white adipose tissue-derived anti-inflammatory cytokine that is found in lower levels in obese patients. Obesity is associated with increased incidence and worse outcomes of asthma, suggesting that APN levels may serve as a biomarker in asthma [49]. It has been shown that APN KO mice develop more severe features of chronic allergic asthma, including airway inflammation and remodeling, compared to wild-type animals [36]. In addition, rAPN decreased AHR and neutrophil and eosinophil counts, as well as BALF IL-13 and IL-5 levels in acute murine OVA-induced asthma [48]. Consistent with these observations, we found that administration of CdM from WT BMCs in which APN was neutralized (acute asthma model) or CdM from BMCs of APN KO mice (chronic model) was less effective than WT CdM in attenuating AHR (**Fig. 2-8**B and D) and remodeling (**Fig. 2-9**) in chronic OVA-induced asthma. Conversely, administration of rAPN alone restored airway responsiveness to normal levels (**Fig. 2-8**C and D) and prevented

ASM thickening (**Fig. 2-9**F) and peribronchial inflammation (**Fig. 2-9**G) in the chronic model.

Given the relative scarcity of reports regarding the phenotype of BMCs from the BALB/c mouse strain [7, 44] and the heterogeneity of the BMCs used in this study (**Fig. 2-3**), we sought to determine the cellular source of APN. We separated the BM-MSCs by negative CD45-based selection. BM-MSCs displayed a $CD45^{-}Sca1^{+}CD29^{+}$ phenotype, whereas the positively selected population ($CD45^{+}$ BMCs) were $CD45^{+}Sca1^{lo}CD29^{+}$ (**Fig. 2-12**). The BM-MSCs were found to be the major contributor to APN production compared to $CD45^{+}$ BMCs (**Fig. 2-8**A).

We sought to clarify the role of APN in our study and we tested whether APN alone could contribute to the upregulation of IL-10-expressing cellular subtypes. rAPN administration did not affect Tr1 levels when compared to untreated OVA animals (**Fig. 2-11**A-G). APN has been shown to induce IL-10 secretion by macrophages [26, 42], another cellular source potentially responsible for the increased IL-10 found in the OVA-CdM lungs compared to the untreated OVA animals. In the chronic asthma model, rAPN induced the upregulation of IL-10-expressing macrophages (**Fig. 2-11**H-L), similar to BMCs CdM administration. It is therefore possible that administration of BMCs CdM would induce the Tr1 through IL-10 found in the CdM, with a similar, simultaneous upregulation of IL-10 production by lung macrophages through APN. This would lead to a

sustained production of endogenous IL-10 that may contribute to attenuation of inflammatory events [41].

We aimed to determine the effects of CdM alone in order to come as close as possible to the clinical setting: a relatively quick delivery of a cell-free preparation that can be administered via the airways. The former paradigm for the benefit of BMCs administration focused on cell replacement; however, this concept encompassed the concerns related to cell tumorigenic potential and allogeneic transplantation [2, 30]. We believed that the delivery of a cell-free preparation would somewhat alleviate concerns related to tumorigenesis. A recent report [29] has compared the effects of BM-MSCs and BM-MSCs CdM in endotoxin-induced lung injury (an inflammatory model) and did not find significant differences between the effects of the CdM and the administration of cells themselves.

In summary, this is the first study to investigate the therapeutic potential of MSCs CdM and to propose APN as a MSCs-derived protective factor in experimental asthma.

Overall, our data not only highlights the therapeutic benefit of BMCs in asthma, but also suggest BMCs-derived soluble factors as a novel, practical and clinically relevant approach for the treatment of asthma and

other inflammatory disorders, thereby alleviating the potential risks of whole cell therapy. Further identification of BMCs-derived factors may hold promise for novel approaches in the treatment of asthma.

2.6. References

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Table 2-1. Cytokine profile in the acute asthma model (mean ± SEM)

Group	Interleukin-4 (pg/mg protein)	Interleukin-13 (pg/mg protein)	Interleukin-10 (pg/mg protein)
Saline (n=11)	39.39 ± 5.26	152.37 ± 8.48	652.75 ± 88.64
Saline-CdM (n=9)	27.87 ± 1.96	147.19 ± 6.32	654.13 ± 88.02
OVA (n=17)	66.37 ± 4.81*,**	207.04 ± 12.99**	753.63 ± 55.65
OVA-CdM (n=18)	51.62 ± 4.16**	126.44 ± 5.08	1156.50 ± 95.78**
OVA-Fib CdM (n=7)	63.21 ± 3.27**	190.44 ± 18.18*,**	992.91 ± 60.40*

IL-4: **-p<0.01 OVA and OVA-Fib CdM vs. saline, saline-CdM; OVA-CdM vs. saline-CdM; *-p<0.05 OVA vs. OVA-CdM.

IL-13: **-p<0.01 OVA vs. saline, saline-CdM, OVA-CdM; OVA-Fib CdM vs. OVA-CdM; *-p<0.05 OVA-Fib CdM vs. saline, saline-CdM.

IL-10: **-p<0.01 OVA-CdM vs. saline, saline-CdM, OVA; *-p<0.05 OVA-Fib CdM vs. saline, saline-CdM.

Table 2-2. Cytokine profile in the chronic asthma model (mean ± SEM)

Group	Interleukin-4 (pg/mg protein)	Interleukin-13 (pg/mg protein)	Interleukin-10 (pg/mg protein)
Saline (n=5)	19.18 ± 3.12	99.42 ± 4.90	662.09 ± 22.87
OVA (n=7)	131.93 ± 12.67**	165.60 ± 11.39**	840.54 ± 60.94
OVA-CdM (n=7)	87.78 ± 9.71*,**	118.56 ± 5.56**	1365.95 ± 100.24**
OVA-Fib CdM (n=7)	121.57 ± 10.97**	140.07 ± 9.16*,**	862.35 ± 35.13

IL-4: **-p<0.01 OVA, OVA-CdM, OVA-Fib CdM vs. saline; OVA-CdM vs. OVA; *-p<0.05 OVA-CdM vs. OVA-Fib CdM.

IL-13: **-p<0.01 OVA and OVA-Fib CdM vs. saline; OVA-CdM vs. OVA; *-p<0.01 OVA-Fib CdM vs. OVA.

IL-10: **-p<0.01 OVA-CdM vs. saline, OVA, OVA-Fib CdM.

2.7. Figures

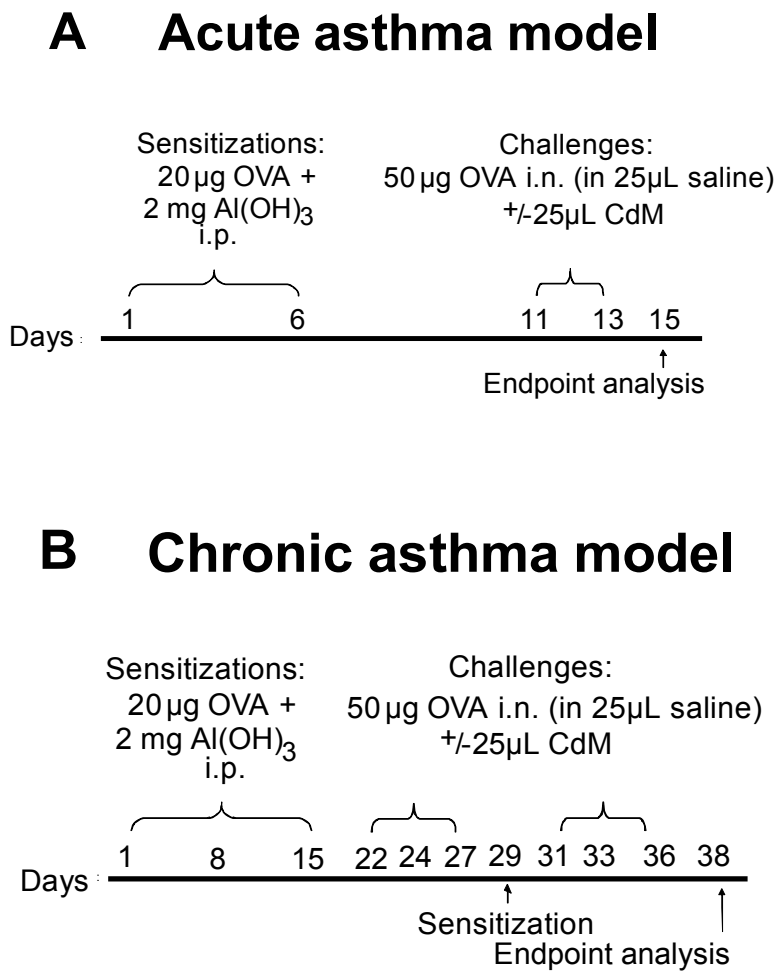

Figure 2-1. Experimental protocols for the acute (A) and chronic (B) OVA-induced allergic asthma models.

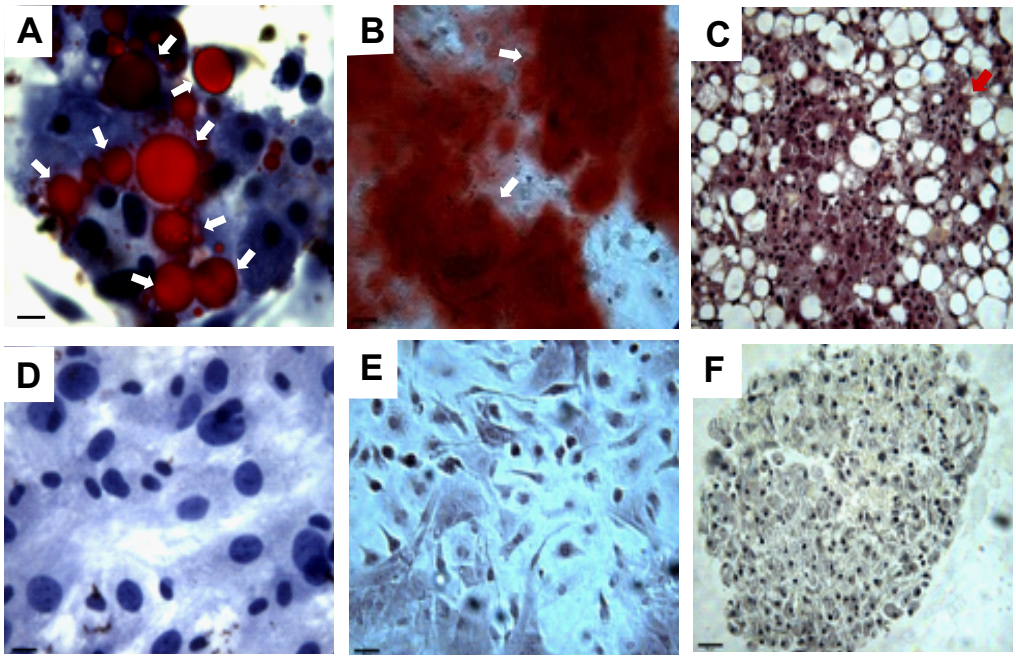

Figure 2-2. Mesenchymal lineage differentiation of BALB/c BMCs. BMCs differentiate into adipogenic (**A**, arrows indicate Oil Red O-stained lipid droplets), osteogenic (**B**, arrows indicate Alizarin Red-stained calcium deposits) and chondrogenic (**C**, arrows indicate Safranin O-stained proteoglycans) lineages following three weeks in culture with specific induction factors. (**D-F**) Undifferentiated BMCs cultured in DMEM. Images representative from a series of n=3 experiments for each differentiation assay.

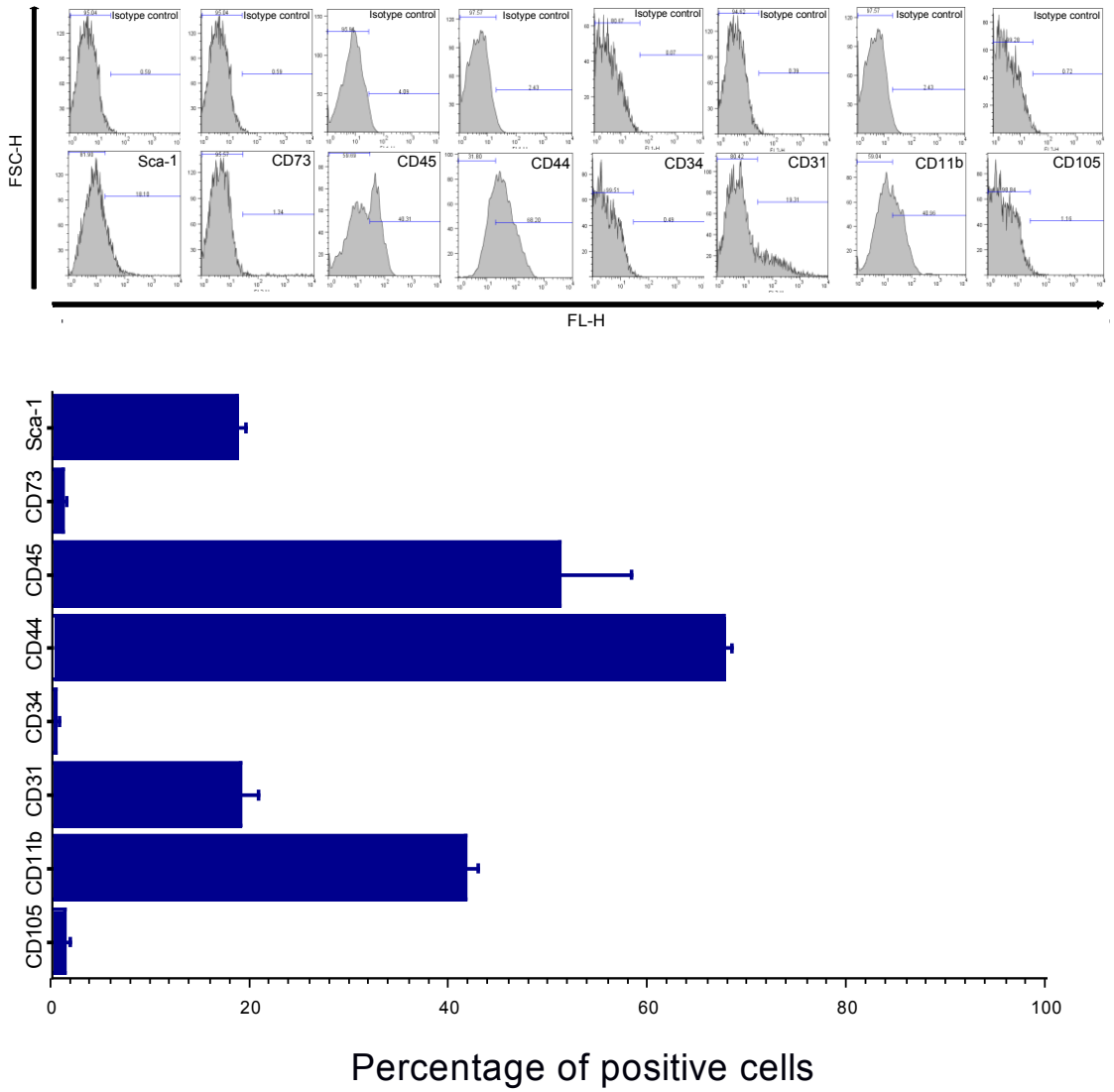

Figure 2-3. Flow-cytometric characterization of BALB/c BMCs surface marker profile.

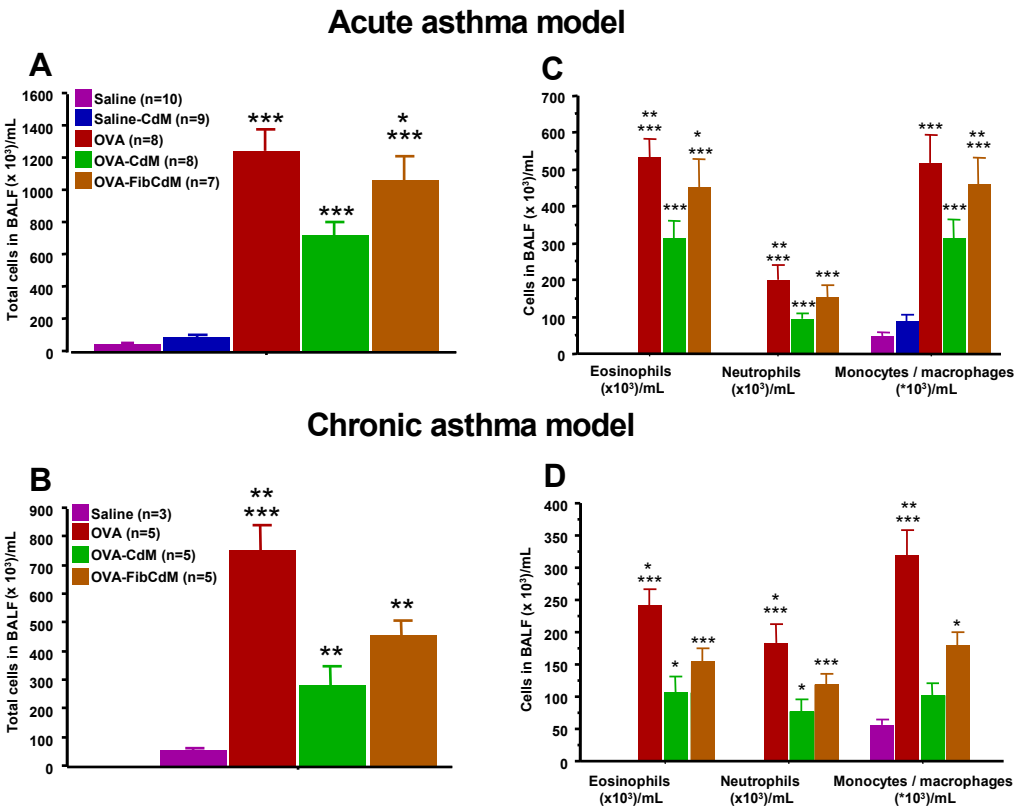

Figure 2-4. BALF analysis. BMCs CdM significantly decreased the total cell number (**A** – acute model, **B** – chronic) and lung PMN influx **(C –** acute, **D -** chronic**)** in BALF. **(A) Total cells in BALF (acute)**: ***-p<0.001 OVA, OVA-FibCdM and OVA-CdM vs. saline and saline-CdM, OVA vs. OVA-CdM; *-p<0.05 OVA-FibCdM vs. OVA-CdM. **(B) Total cells in BALF (chronic)**: ***-p<0.001 OVA vs. saline, OVA-CdM; **-p<0.01 OVA vs. OVA-FibCdM and OVA-FibCdM vs. saline; *-p<0.05 OVA-CdM vs. saline. **(C) Differential cell counts in BALF (acute)**: ***-p<0.001 OVA, OVA-FibCdM and OVA-CdM vs. saline and saline-CdM; **-p<0.01 OVA vs. OVA-CdM; *-p<0.05 OVA-FibCdM vs. OVA-CdM (eosinophils only). **(D) Differential cell counts in BALF**

(chronic). ***-p<0.001 OVA vs. saline, OVA-CdM; (eosinophils and neutrophils) ***-p<0.001 OVA-FibCdM vs. saline; *-p<0.05 OVA vs. OVA-FibCdM and OVA-CdM vs. saline; (monocytes/macrophages) **-p<0.01 OVA-CdM vs. OVA-FibCdM and *-p<0.05 OVA-FibCdM vs. saline. The values are expressed as means ± standard error of the mean (SEM). Data representative from a series of n=5 separate experiments.

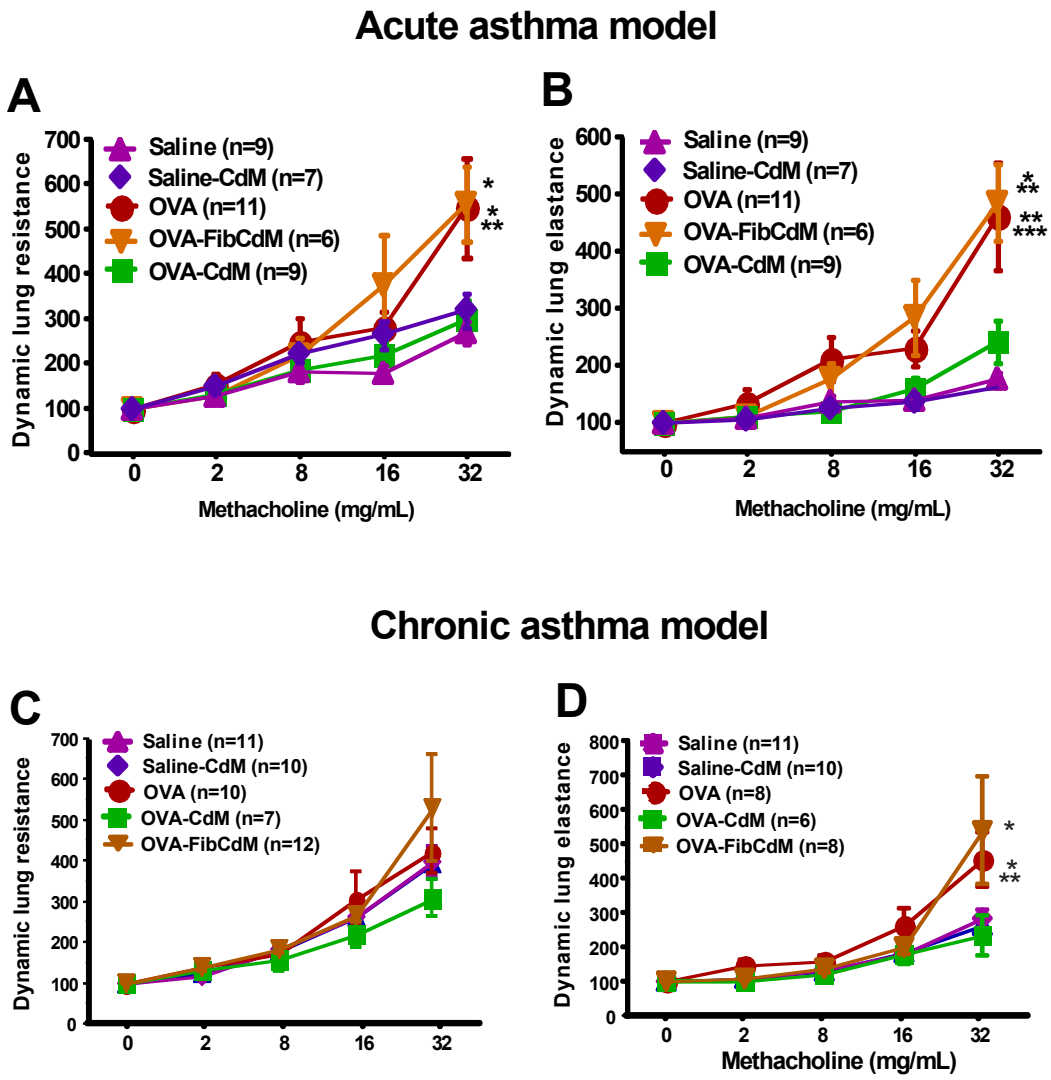

Figure 2-5. Invasive LFT. BMCs CdM significantly reduced the methacholine-induced increase in dynamic lung resistance (acute) and elastance (acute and chronic models). **(A) Dynamic lung resistance (acute):** *-p<0.05 OVA vs. OVA-CdM, OVA-Fib vs. saline and OVA-CdM, **-p<0.01 OVA vs. saline. **(B) Dynamic lung elastance (acute):** *-p<0.05 OVA-Fib vs. OVA-CdM, **-p<0.01 OVA vs. OVA-CdM, OVA-Fib vs. saline and saline-CdM, ***-OVA vs. saline and saline-CdM. Data for each methacholine

dose expressed as means ± SEM of the peak percent increase compared to baseline-response to aerosolized saline (n=4 separate experiments). “n” indicates the number of animals in combined experiments**. (C) Dynamic lung resistance (chronic). (D) Dynamic lung elastance (chronic)**. *-$p<0.05$ OVA and OVA-Fib CdM vs. OVA-CdM, saline-CdM; OVA-Fib CdM vs. saline; **-$p<0.01$ OVA vs. saline. Data for each methacholine dose expressed as means ± SEM of the peak percent increase compared to baseline-response to aerosolized saline (n=5 separate experiments). “n” indicates the number of animals in combined experiments**.**

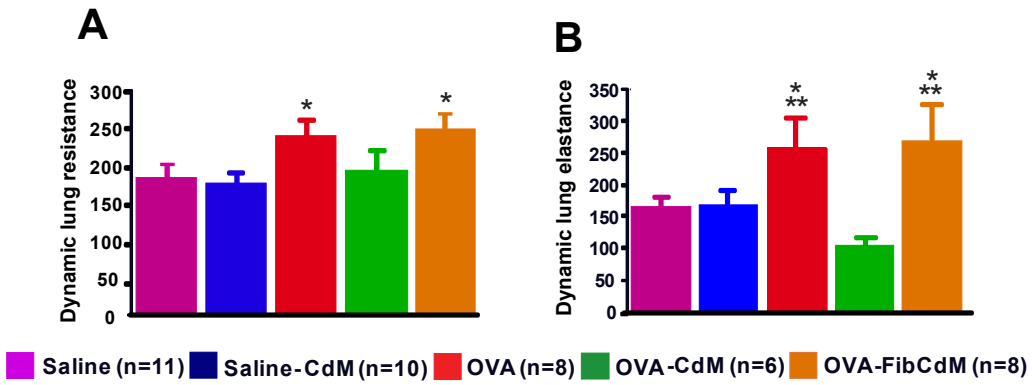

Figure 2-6. Bronchodilator response to salbutamol. Bronchodilation in response to salbutamol was blunted in the chronic asthma model. BMCs CdM restored the bronchodilator response to salbutamol. **(A) Dynamic lung resistance**: *-p<0.05 OVA and OVA-Fib CdM vs. saline and saline-CdM. **(B) Dynamic lung elastance**: *-p<0.05 OVA vs. saline, OVA-Fib CdM vs. saline and saline-CdM; **-p<0.01 OVA and OVA-Fib CdM vs. OVA-CdM. The values are expressed as means ± SEM. (n=5 separate experiments). "n" indicates the number of animals in combined experiments**.**

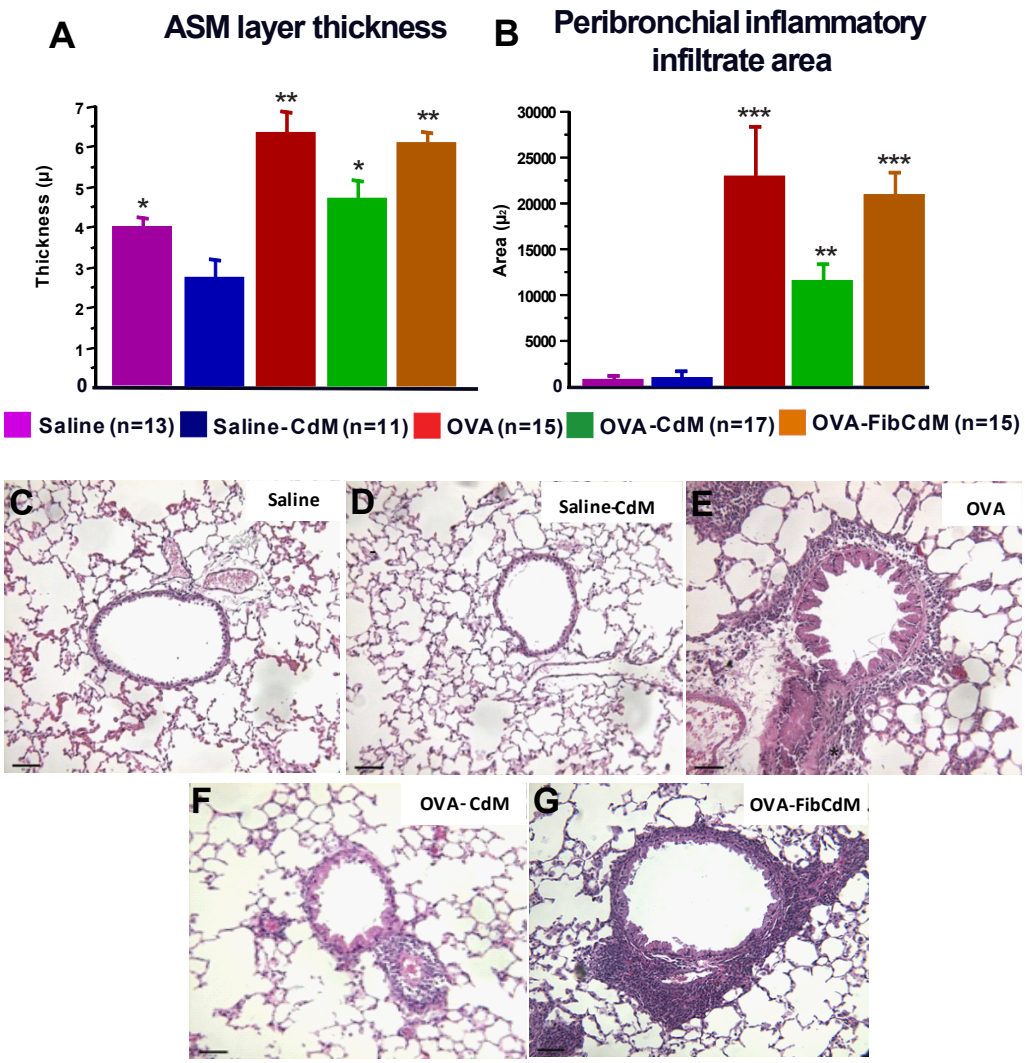

Figure 2-7. Airway remodeling in chronic asthma. (A) Bronchial smooth muscle layer thickness: *-p<0.05 saline vs. saline-CdM, OVA-CdM vs. OVA-Fib CdM; **-p<0.01 OVA and OVA-Fib CdM vs. saline and saline-CdM, OVA-CdM vs. OVA and saline-CdM. **(B) Peribronchial inflammatory infiltrate area:** **-p<0.01 OVA-CdM vs. saline, saline—CdM, OVA, OVA-Fib CdM. ***-p<0.001 OVA and OVA-Fib CdM vs. saline, saline-CdM. (**C-G**) Hematoxylin and eosin (H & E) staining showing the

characteristic features of airway remodeling of chronic asthma including increased ASM layer thickening and peribronchial inflammatory infiltrate in chronic OVA-exposed mice. BMCs CdM significantly reduced both. Scale bar: 65 μm. Data representative from a series of n=5 separate experiments. Data expressed as means ± SEM. “n” indicates the number of airways analyzed in combined experiments.

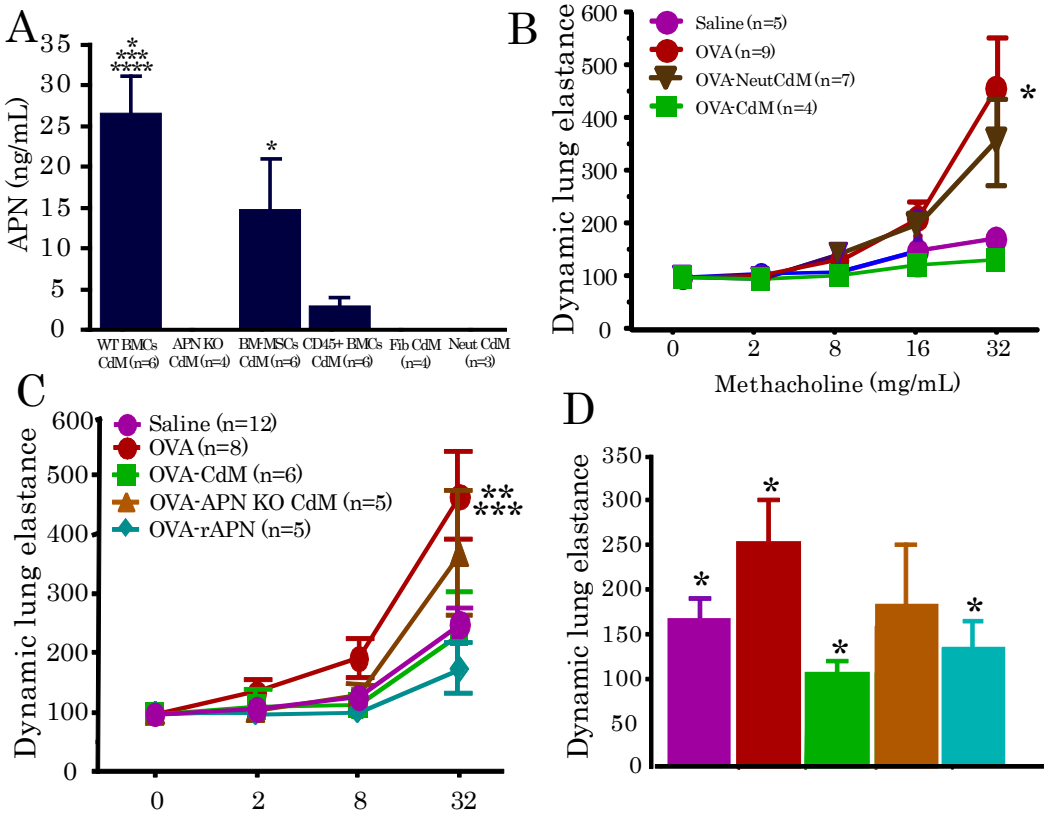

Figure 2-8. Effects of APN on AHR. (A) APN concentration in CdM preparations. *-p<0.05 WT CdM vs. CD45- CdM; CD45- vs. APN KO CdM, CD45+ CdM, Fib CdM, NeutCdM; ***-p<0.001 WT CdM vs. NeutCdM; ****-p<0.0001 WT CdM vs. APN KO CdM, CD45- CdM, Fib CdM. Results from two separate experiments.Data expressed as means ± SEM. "n" indicates the number of independent CdM batches. **(B) Invasive LFT in the acute asthma model.** NeutCdM failed to prevent acute OVA-induced AHR. *-p<0.05 OVA vs. saline, OVA-CdM. **(C) Invasive LFT in the chronic asthma model.** BMCs CdM and rAPN significantly reduced the increase in dynamic lung elastance. **-p<0.01 OVA vs. OVA-CdM, saline, ***-p<0.001 OVA vs. OVA-rAPN. Data for each methacholine dose expressed as means ± SEM of

the peak percent increase compared to baseline-response to aerosolized saline (n=5 separate experiments). “n” indicates the number of animals in combined experiments**.** (**D) Bronchodilator response to salbutamol.** Bronchodilation in response to salbutamol was blunted in the chronic asthma model. BMCs CdM and rAPN restored the bronchodilator response to salbutamol *-$p<0.05$ OVA vs. OVA-CdM, OVA-rAPN and saline. The values are expressed as means ± SEM. (n=5 separate experiments). “n” indicates the number of animals in combined experiments**.**

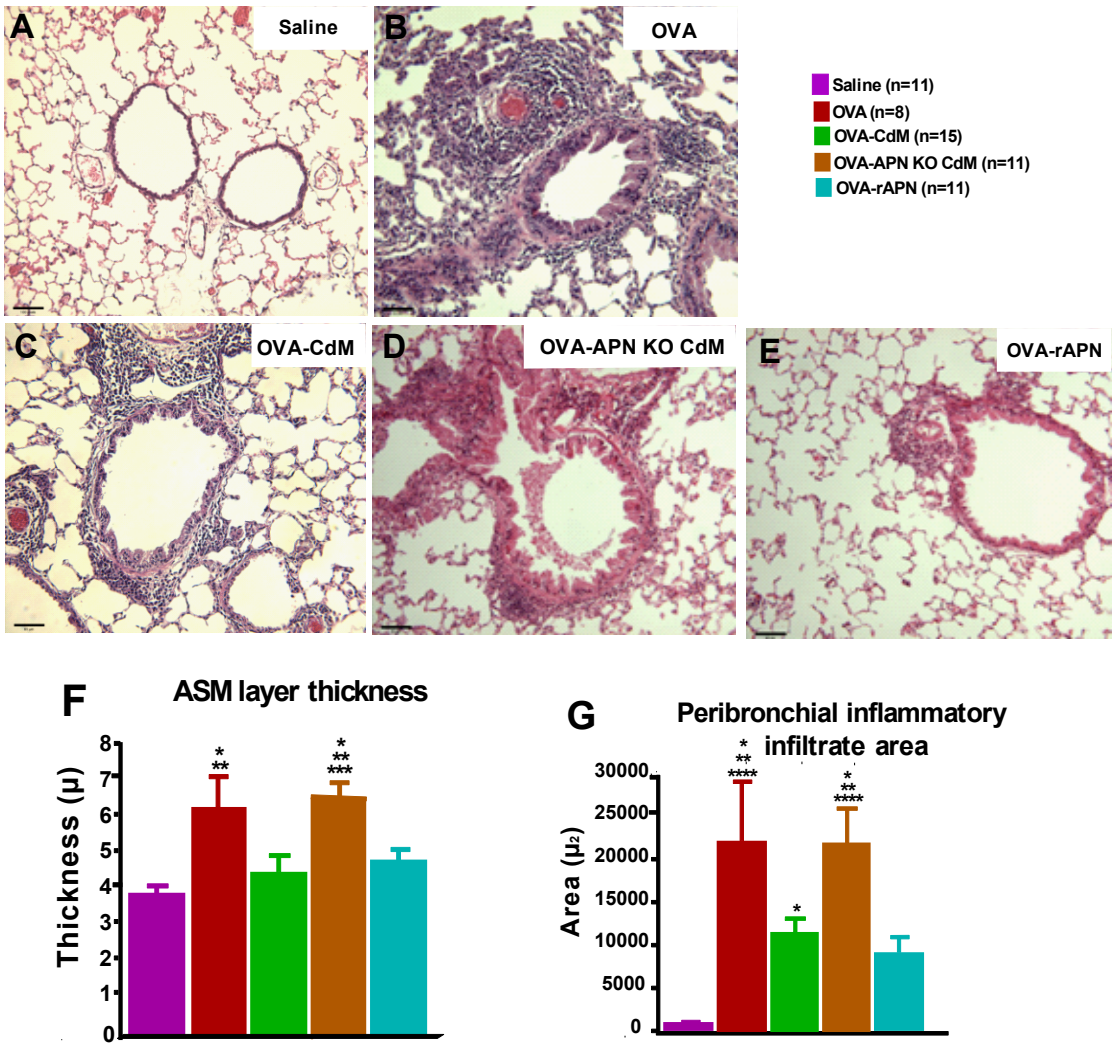

Figure 2-9. Effects of APN on chronic airway remodeling parameters. **(A-E) Airway remodeling parameters in chronic asthma.** Representative H & E-stained lung sections showing the characteristic feature of airway remodeling in the chronic asthma model including increased ASM layer thickening and peribronchial inflammatory infiltrate in chronic OVA-exposed mice. BMCs CdM and rAPN significantly reduced both. **(F) Bronchial smooth muscle layer thickness:** *-p<0.05 OVA vs. OVA-CdM, OVA-rAPN, **-p<0.01 OVA vs. saline, OVA-KO CdM vs. OVA-

CdM, OVA-rAPN, ***-p<0.001 OVA-KO CdM vs. saline. **(G) Peribronchial inflammatory infiltrate area:** *-p<0.05 OVA and OVA-KO CdM vs. OVA-CdM, OVA-CdM vs. saline, **-p<0.01 OVA and OVA-KO CdM vs. OVA-rAPN, ****-p<0.0001 OVA and OVA-KO CdM vs. saline. Scale bar: 65 μm. Data representative from a series of n=5 separate experiments. The values are expressed as means ± SEM. “n” indicates the number of airways analyzed in combined experiments.

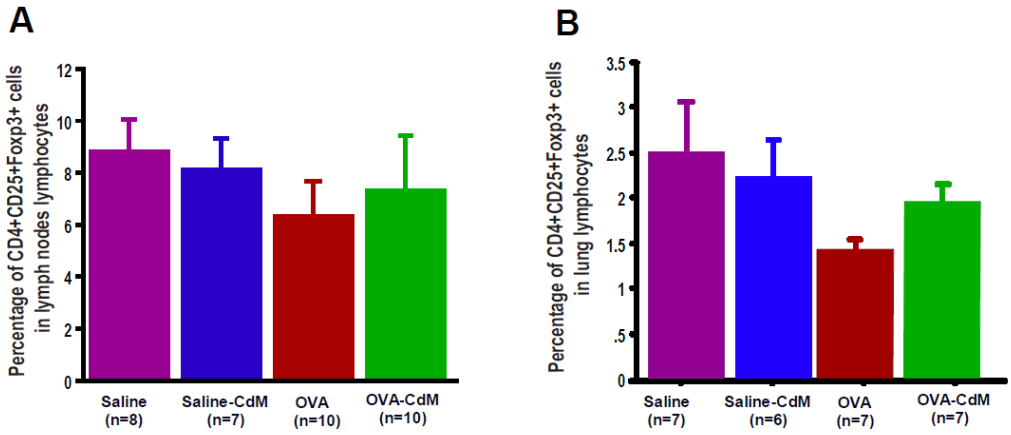

Figure 2-10. FACS of lung and draining lymph nodes lymphocytes. (**A**) OVA or BMCs CdM administration did not significantly impact the proportion of $CD4^{+}CD25^{+}Foxp3^{+}$ cells in draining lymph nodes or (**B**) lungs. Data expressed as means ± SEM. "n" indicates the number of animals in combined experiments.

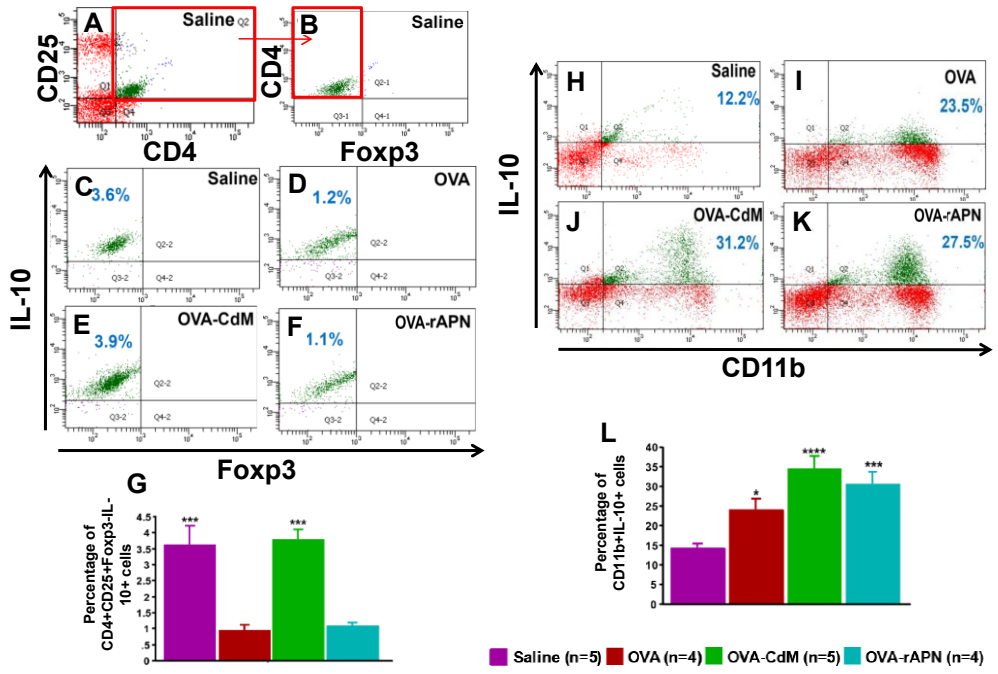

Figure 2-11. FACS of lung lymphocytes. (**A**) Gating for activated CD4+ lymphocytes (CD4+CD25+) followed by (**B**) gating for Foxp3- within this cell population. (**C-F**) Representative scatterplots for CD4+CD25+Foxp3-IL-10+ cells from each experimental group. (**G**) BMCs CdM increased the proportion of lung CD4+CD25+Foxp3-IL-10 cells.***-p<0.001 saline and OVA-CdM vs. OVA and OVA-rAPN. (**H-K**) Representative scatterplots for CD11b+IL-10+ cells from each experimental group. **(L)** BMCs CdM increased the proportion of lung CD11b+IL-10+ cells. *-p<0.05 OVA vs. OVA-CdM and saline, ***-p<0.001 OVA-rAPN vs. saline, ****-p<0.0001 OVA-CdM vs. saline. Data representative from n=2 experiments. The values are expressed as means ± SEM.

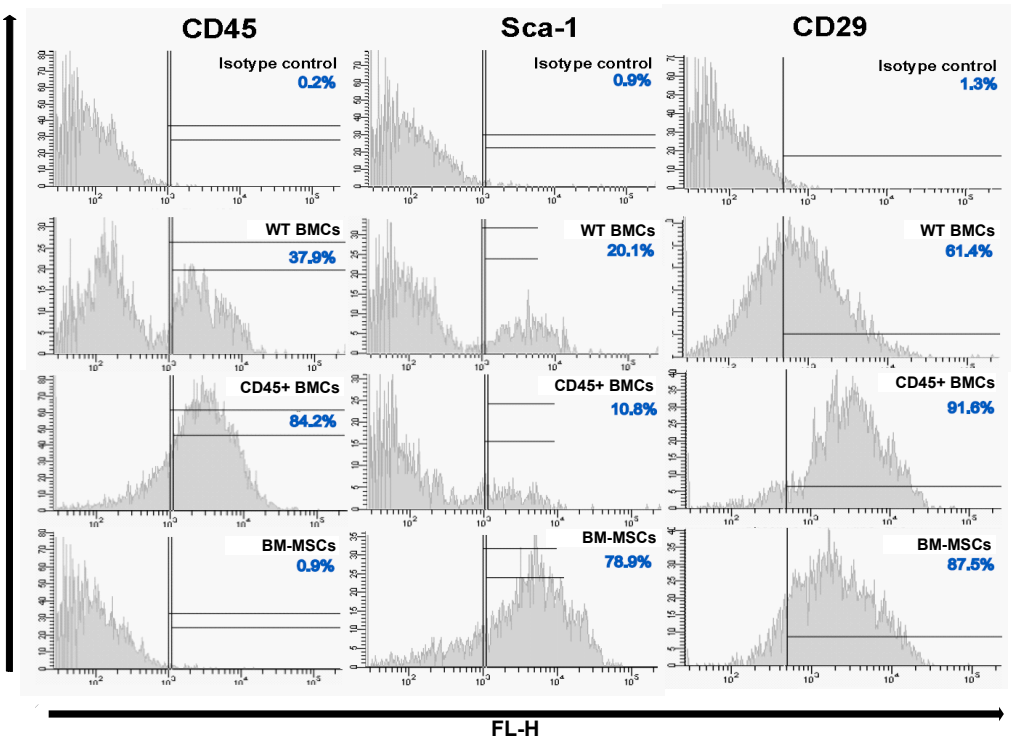

Figure 2-12. Flow-cytometric evaluation of CD45, Sca-1, CD29 expression by WT BMCs, CD45⁺ BMCs and BM-MSCs.

CHAPTER 3 - STEM CELL CONDITIONED MEDIUM PREVENTS ACUTE LUNG INJURY IN MICE: IN VIVO EVIDENCE FOR STEM CELL PARACRINE ACTION

A version of this chapter is being reviewed for publication.

Ionescu LI, Byrne RN, van Haaften TJ, Vadivel A, Alphonse RS, Rey-Parra GJ, Weissmann G, Eaton F, Thébaud B. Stem Cell Conditioned Medium Prevents Acute Lung Injury in Mice: In Vivo Evidence for Stem Cell Paracrine Action. *Am J Physiol Lung Cell Mol Biol*

LII performed the majority of the experiments. RNB performed preliminary experiments. TvH, AV, RSA, GJRP, GW and FE provided technical assistance. The manuscript was written by LII and edited by BT.

3.1. Abstract

Mortality and morbidity of acute lung injury and acute respiratory distress syndrome (ALI/ARDS) remain high in part because of the lack of pharmacological therapies to prevent injury or promote repair. Stem cells have been proposed to regenerate damaged organs. Mesenchymal stem cells (MSCs) prevent lung injury in various experimental models, despite a low proportion of donor-derived cell engraftment. Increasing evidence suggest that MSCs exert their beneficial effects via a paracrine mechanism. We hypothesized that soluble factors secreted by MSCs prevent lung injury in part by modulating macrophage function. We tested the protective effect of MSC-derived conditioned medium (CdM) compared to whole MSCs, lung fibroblasts and fibroblast (Fib)-CdM *in vivo* and *in vitro*. Intratracheal MSCs and MSC CdM significantly attenuated lipopolysaccharide (LPS)-induced lung neutrophil influx, lung edema and lung injury as assessed by an established lung injury score. MSC CdM increased arginase-1 activity in LPS-exposed alveolar macrophages (AMs) and enhanced expression of Ym1 while decreasing iNOS in AMs from LPS-MSC and LPS-MSC CdM mice as compared to control mice, which suggests the occurrence of MSC CdM-driven alternative macrophage activation to an M2 “healer” phenotype. Comparative multiplex analysis between MSC CdM and lung fibroblast-CdM demonstrated that MSC CdM contained a

range of proteins that may confer therapeutic benefit in lung injury. In summary, MSCs exert their beneficial effects through a paracrine activity. MSC CdM prevents LPS-induced lung injury by attenuating lung inflammation and promoting a wound healing/anti-inflammatory M2 macrophage phenotype.

3.2. Introduction

Despite improvements in management, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) remain major causes of morbidity and mortality in critically ill patients of all ages [55]. The incidence of ALI/ARDS in the US is 138:100,000 persons per year and is anticipated to double in the next 25 years [55]. Treatment of ALI/ARDS remains primarily supportive [34].

Bone-marrow derived mesenchymal stem cells (MSCs) differentiate into cartilage, fat and bone, but also into muscle, liver, kidney, heart and brain cells [50]. These properties have been harnessed for organ regeneration [58]. In the lung, bone marrow derived cells, including MSCs, engraft and adopt a lung epithelial cell phenotype [2, 17, 27, 28, 47, 54, 62]. MSCs can also suppress local immune responses and have the capacity to avoid immune rejection in allotransplantation [40, 24, 29, 30, 51]. These immunomodulatory properties might be of therapeutic benefit in ALI/ARDS and other lung diseases characterized by inflammation. Both intratracheal and systemic MSC administrations improve lipopolysaccharide (LPS)-induced ALI in mice [17, 37]. These findings are congruent with the therapeutic benefits of MSCs described in other lung injury models [2, 21, 31, 46, 54, 62]. In all these studies, the engraftment rates of MSCs were low [26-28, 54, 62]. Together with the rapid

therapeutic benefits seen within 48 to 72 hours of MSC delivery in the ALI models [17, 37], these findings suggest that beyond cell replacement, MSCs may be releasing factors responsible for the beneficial effects of cell therapy. In addition, since the use of MSCs as whole-cell therapy may hold some risks to the patient [1, 32], we investigated the effects of MSC-derived conditioned medium (CdM) in LPS-induced ALI in mice.

3.3. Materials and Methods

All procedures were approved by the Animal Welfare Committee of the University of Alberta. Additional detail on all the methods is provided in the online data supplement.

3.3.1. MSCs and Lung Fibroblasts Isolation, Culture and Characterization

Bone marrow was harvested from adult (8-10 weeks) C57BL/6 mice (Charles River, ON, Canada), and MSCs were cultured and characterized as previously described [21]. The femur and tibia were excised and the marrow was flushed with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Burlington, ON) containing 10% fetal bovine serum and 1% PSF. The extracted marrow was dissociated and plated in a tissue culture flask. After 24 hours, the media was aspirated, adherent cells were rinsed three times with PBS and replenished with fresh media. Cells were grown to ~80% confluency, trypsinized, and re-seeded at a density of 10^5 cells/cm^2. Differentiation of MSCs was performed over 21 days on passage 2-3 cells [21]. MSCs were evaluated for expression of a panel of surface markers according to established criteria [12, 15, 37, 49, 60]. Antibodies against the following markers were obtained from Becton

Dickinson (BD, Mississauga, ON): stem cell antigen-1 (Sca-1, fluorescein isothiocyanate [FITC]), CD31 (phycoerythrin [PE]), CD11b (FITC), CD45 (FITC), CD44 (FITC), CD73 (PE), CD14 (FITC), CD34 (FITC), c-kit (FITC), Flk-1 (PE), CD106 (vascular cell adhesion molecule-1 [VCAM-1]), CD29 (PE) and from BioLegend (San Diego, CA): CD105 (PE). MSCs between passages 7-11 were detached from culture surfaces, counted, and divided into aliquots of ~ 0.5-1x10^6 cells/sample in 12x75 mm polystyrene round-bottom tubes (BD Falcon). Cells were washed twice with flow buffer (0.05% sodium azide, 0.1% bovine serum albumin in PBS), incubated with the respective antibodies at 4 °C with gentle shaking for 30 min, washed twice, re-suspended in flow buffer and analyzed by flow cytometry (FACSCalibur, BD). Cellquest (BD) and FlowJo (version 5.7.2) software were used for analyses.

Lung fibroblasts were isolated from adult (8-10 weeks) C57BL/6 mice [59]. Fibroblast identity was confirmed by immunofluorescence staining for the intermediate filament protein vimentin.

3.3.2. Conditioned Medium (CdM) Preparation

Passage 2- 8 MSCs and fibroblasts were grown to >80% confluency. Medium (DMEM) was aspirated and cells were rinsed three times with PBS. Cells were cultured with serum-free DMEM (+ PSF) for 24 hours. CdM

was collected and filtered through a 0.2 μm filter to remove cellular debris. Adherent cells were trypsinized, stained with trypan blue, and counted. The medium from 5 x 10^6 cells yielded 15 mL of primary CdM that was further de-salted and concentrated ~25-fold, yielding 600 μL CdM, using ultrafiltration units with a 3 kDa molecular weight cut-off (Amicon Ultra-PL 3, Millipore, Billerica, MA). Similar to work by others [20], serum-free DMEM + PSF (de-salted and concentrated 25-fold) was the vehicle control.

3.3.3. Murine LPS-Induced ALI

Eight to ten week-old male C57BL/6 mice were anaesthetized with 5% isoflurane and injected intratracheally (i.t.) with 4 mg/kg LPS (E. *coli.* 055:B5, Sigma-Aldrich, Oakville, ON). Four hours post-LPS, mice were re-anesthetized and received a 30 μL i.t. instillation of MSCs, LFs, MSC CdM, Fib CdM or DMEM. We ensured equivalence between cell-based and CdM-based treatment by administering the same number of cells (250,000 cells/ 30 μL DMEM) that produced 30 μL concentrated CdM.

Mice (n>=5 per group per endpoint) were sacrificed via an intraperitoneal injection of pentobarbital at 48 hours post-LPS for either bronchoalveolar lavage fluid (BALF) or lung histological analysis.

3.3.4. BALF Analysis and Alveolar Macrophages (AM) Isolation

Lungs were lavaged with 2.5mL ice-cold phosphate buffered saline (PBS) injected at 0.5mL increments via a 20 gauge catheter inserted in the trachea. BALF was centrifuged for 10 minutes at 400 g and BALF cells were enumerated using the Scepter automated cell counter (Millipore, Billerica, MA). Differential cell counts were performed on cytospin preparations (Thermo Shandon, Pittsburgh, PA) stained with Hema 3 Manual Staining System (Fisher Scientific, Nepean, ON) by counting 300 cells per cell-smear and multiplying by total cell number per mL.

For AMs isolation, an established protocol was followed [65]. Briefly, BALF was centrifuged at 300 g for 10 minutes and the cellular pellet was washed with PBS, re-suspended in red blood cell lysis buffer [8.3 g NH_4Cl, 1 g $KHCO_3$, 1.8 mL of 5% EDTA in 1 L of distilled water) for 5 minutes at room temperature and centrifuged again at 300 g for 10 minutes. The pellet was re-suspended in RPMI-1640 medium and plated at a density of 600,000 cells/mL in a 24-well tissue culture plate. After 2 hours, medium was removed and adherent cells were washed three times with PBS. AMs were stained with Hema 3 Manual Staining System for morphological assessment. AMs were evaluated for the expression of characteristic surface markers [18] using antibodies against CD11b (FITC)

and CD11c (allophycocyanin [APC]) (BD Biosciences) by flow cytometry according to the same protocol described for MSC characterization.

3.3.5. Assessment of Lung Permeability

Lung edema due to LPS-induced increase in lung permeability was measured using the wet / dry weight ratio of lung lobes as previously described [17]. Briefly, lungs were weighed upon excision (wet weights), homogenized in 1 mL of water and placed in a drying oven at 55 °C for 24 hours, dry weights recorded and wet / dry ratio calculated.

3.3.6. Lung Histological Analysis

Lungs were inflated and fixed with 4% formaldehyde solution through a tracheal catheter at a constant pressure of 20 cm H_2O [21]. Lungs were processed, paraffin-embedded and 4 μm thick serial sections were stained with hematoxylin and eosin (H&E). Images were captured (Openlab, Improvision, version 5.0.2.) with Leica CTRMIC microscope and 40 high-powered fields per lung were examined by a blinded investigator to quantify the histopathology score. Parameters assessed were: alveolar septal congestion, alveolar hemorrhage, intra-alveolar fibrin and intra-alveolar infiltrates. A score from 0 to 3 was given for each criterion and a

total score was established, all according to a previously published protocol [36, 37]. Briefly, lung injury score = [(alveolar hemorrhage points/no. of fields) + 2 x (alveolar infiltrate points/no. of fields) + 3 x (fibrin points/no. of fields) + (alveolar septal congestion/no. of fields)]/total number of alveoli counted.

3.3.7. AM LPS Exposure and AM Phenotype Characterization

Freshly isolated AMs were cultured with RPMI-1640 media containing 10% FBS in a 12-well plate. After overnight adherence, RPMI-1640 media was replaced with either DMEM alone, DMEM+LPS (1 μg/ml), or MSC CdM+LPS (1 μg/ml). After 48 hours in culture, media was aspirated and cells were rinsed 3X with PBS. The monolayer of cells was scraped, cells were spun down and pellets were frozen. Arginase activity in AM was measured by determining the amount of urea generated by the enzyme [9, 57]. Briefly, the frozen AM pellets were thawed and homogenized in 100 μl per sample of lysis buffer (0.1 % Triton X-100 with protease inhibitors: phenyl-methyl-sulphonyl fluoride [PMSF] (1 mM), leupeptin (0.5 μg/ml), aprotinin (5 μg/ml), EDTA (2 mM)). The samples were sonicated twice for 3-5 seconds and stored at -80 °C. Samples were thawed and 50 μL of the homogenate was added to 50 μl Tris-HCl (25 mM, pH 7.5) containing $MnCl_2$ (5 mM) and incubated at 56 ºC for 10 min to

activate the enzyme. 50 µL of this heat-activated supernatant mixture was incubated with 50 µL L-arginine (0.5 mol/L) at 37ºC for 60 min. The reaction was stopped by adding 400 µL of an acid mixture (1 H_2SO_4: 3 H_3PO_4: 7 H_2O). 25 µL of α-isonitrosopropiophenone (9 % dissolved in 100 % ethanol) was added to the above mixture and incubated at 100 ºC for 45 min for color development. The mixture was cooled at room temperature in the dark for 10 min. A standard curve for urea (0-30 µg) was prepared. Urea concentration in the homogenate was measured using a colorimeter at 550/540 nm with 200 µL of the aliquot. Arginase activity was expressed as units / mg protein / hour.

For further macrophage phenotype characterization by multicolor flow cytometry, AMs were isolated from experimental animals and allowed to adhere to plastic as described above. The following antibodies were used: CD11b (Pacific Blue), CD11c (PE, both from Biolegend), rabbit anti-mouse inducible nitric oxide synthase (iNOS, Abcam) and donkey anti-rabbit secondary (FITC, Biomeda Corporation, Foster City, CA), goat anti-mouse Ym1 (R&D Systems) and donkey anti-goat secondary (Cy5, Biomeda). The staining was performed as described for MSC characterization, with the addition of a 30 minute incubation of the cells at 4 ºC in Cytofix / Cytoperm Buffer (BD), followed by resuspension in Permeabilization Buffer (BD) before incubation with primary antibodies. Analysis was performed using FACSCanto with FACS Diva software (BD).

3.3.8. Antibody array of MSC CdM

MSC CdM and Fib CdM were prepared, concentrated and de-salted as described above. Samples were analyzed for a panel of 96 factors using an antibody array (RayBio Mouse Antibody Array G Series 1000, RayBiotech Inc., Norcross, GA). Relative intensity of each factor in MSC CdM and Fib CdM is reported compared to a positive and negative control, as well as fold-change of specific factors in MSC CdM versus Fib CdM.

3.3.9. Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Group comparisons were analyzed by one-way ANOVA with a Fisher's PLSD post hoc test. An unpaired t-test was used when appropriate. Data was assessed with statistical software Statview (version 5.0.1, SAS Institute, Cary, NC). Values were considered significant with $p<0.05$.

3.4. Results

3.4.1. MSC Display Functional Characteristics and Surface Marker Phenotype of Murine MSCs

Bone marrow-derived MSCs differentiated into adipogenic, osteogenic and chondrogenic mesenchymal lineages (**Fig. 3-1**). Cell surface antigen phenotype was assessed by flow cytometry. MSCs expressed high levels of Sca-1 (92.93% of cells) and CD29 (89.99%), moderate levels of CD105 (33.92%), CD106 (14.36%), CD11b (12.10%), and CD45 (11.62%) and were considered negative for CD14 (0.30%), c-kit (0.53%), CD34 (1.13%), CD73 (1.49%), Flk-1 (3.01%) and CD31 (4.08% of cells) (**Fig. 3-2**).

3.4.2. MSC CdM Decreased Lung Inflammation and Lung Vascular Permeability in LPS-Induced Lung Injury

LPS significantly increased the total cell and neutrophil count in the BAL after 12 hours compared to uninjured controls (**Fig. 3-3**A). This inflammatory influx was attenuated by MSCs and MSC CdM treatment, but not by treatment with DMEM, the vehicle control, Fib or Fib CdM (**Fig. 3-3**A). MSCs and MSC CdM, but not DMEM, Fib or Fib CdM prevented the

LPS-induced increase in lung vascular permeability, as assessed by lung wet/dry ratio (**Fig. 3-3**B).

3.4.3. MSC CdM Failed to Prevent LPS-Induced Body Weight Loss

All mice given LPS were lethargic and had reduced activity and decreased body weight over 48 hours compared to control mice (**Fig. 3-4**A).

3.4.4. MSC CdM Improved LPS-Induced Lung Injury

Histological assessment of lung injury using a semi-quantitative histopathology score revealed that mice treated with LPS had increased septal thickening, alveolar hemorrhage, alveolar infiltrates, and fibrin strands compared to controls, whereas treatment with MSCs or MSC CdM significantly attenuated these features (**Fig. 3-4**B and C).

3.4.5. MSC CdM Determined Alternative Activation of AMs Following LPS Exposure *In Vitro* and *In Vivo*

Isolated AMs were macroscopically (**insert Fig. 3-5**A) and phenotypically ($CD11c^{+}CD11b^{-}$) (**Fig. 3-5**A) consistent with AMs. *In vitro*,

AMs were exposed to LPS in order to test the role of MSC CdM on the induction and maintenance of the M2 phenotype in AMs. LPS increased arginase activity in AMs as compared with non-LPS-exposed cells, and MSC CdM further elevated these levels compared to LPS-exposed cells cultured with DMEM (**Fig. 3-5**B).

In order to further investigate the effects of MSC CdM *in vivo*, AMs were isolated from LPS-exposed animals that had received cell or CdM treatment. MSC CdM induced an iNOS-Ym1+ phenotype in AMs from both control (11.6% vs. 0.8%) and LPS-treated animals (69% vs. 6.8%). This effect was enhanced in LPS-MSC recipients (79.9%). Fib and Fib CdM markedly induced an iNOS+Ym1- phenotype (52% and 41.9%, respectively) compared to control AMs (1.3%), similar to the proportion found in LPS-DMEM AMs (41.8%) (**Fig. 3-6**).

3.4.6. MSC CdM Contains Soluble Factors That May Convey Therapeutic Benefit

The levels of 96 different factors were compared between MSC CdM and Fib CdM (**Fig. 3-7**). Cluster analysis of both CdMs revealed the following distribution: chemokines (31%), binding proteins (27%), cytokines (21%), growth factors (14%), cytokine antagonists (4%) and molecules involved in extracellular matrix repair (3%) **(Fig. 3-7).**

Significant fold-variations of certain factors were found (**Fig. 3-8**). MSC CdM contained higher concentrations of stem cell factor (SCF), IL-12p40/p70, stromal-derived factor-1 alpha (SDF-1α) and osteoprotegerin and lower concentrations of IL-6, macrophage inflammatory protein-3 alpha (MIP-3α) and thymus and activation regulated chemokine (TARC).

3.5. Discussion

We provide evidence that the therapeutic benefits of MSCs are attributable to a paracrine mechanism. *In vivo*, administration of MSC CdM alone attenuated lung neutrophil influx and improved lung histology. This effect was comparable with administration of an equivalent number of MSC and absent in mice treated with control cells (lung fibroblasts) or control cell CdM. MSC CdM induced an M2 phenotype in AMs exposed to LPS *in vitro* and *in vivo* in LPS-exposed animals. We also suggest that MSC CdM contains soluble factors capable of attenuating lung injury. Identification of these soluble factors secreted by MSCs may yield new therapeutic options for ALI/ARDS.

Recent studies have shown that MSCs modulate immune cell function [23, 40, 51] and have cell-protective effects through the release of cytokines and growth factors [14, 20, 31]. MSC CdM decreased hypoxia-induced cell death and improved tube formation of human aortic endothelial cells [20]. CdM derived from MSCs engineered to over-express the pro-survival gene *Akt* protected cardiomyocytes from hypoxia-induced cell death and limited myocardial infarct size in vivo [13]. MSC CdM improved healing in an excisional wound splinting model in mice [7], oxygen-induced AT2 and pulmonary microvascular endothelial cell injury *in vitro* [62], neonatal oxygen-induced lung injury *in vivo* [2] and reversed

hepatocyte death and increased survival in Gal-N induced fulminant hepatic failure [41]. Moreover, human MSC CdM attenuated endotoxin-induced lung injury in an *ex vivo* perfused human lung model [31] and mouse MSC CdM prevented the development of murine asthma [21].

In the current study, the therapeutic benefit seen with CdM mirrored the protective effects described with whole cell therapy. Similar to previous findings with whole cell therapy after LPS injury [17, 37], we observed that a single intratracheal injection of MSC CdM or MSCs 4 hours after LPS administration decreased lung neutrophil influx, lung permeability and improved the lung histopathology score as compared to control media, lung fibroblasts or their CdM. These findings open new therapeutic options by identifying potential healing molecules contained in MSC-derived CdM and understanding their mechanism of action.

Our data suggest that MSC CdM promote a M2 "healer" macrophage phenotype. In the development of ALI/ARDS, neutrophils and macrophages are activated in order to eliminate pathogens, but also contribute to tissue injury through the release of antimicrobial compounds [63]. Macrophages are phenotypically heterogeneous because they respond to stimuli in their microenvironment and they also differ genetically [16]. Classically activated macrophages (M1) kill invading microorganisms and tumors and promote type I immunity by secreting high levels of pro-inflammatory cytokines and low levels of anti-

inflammatory cytokines [33]. In contrast, alternatively activated macrophages (M2) secrete lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines. M2 macrophages promote type II immunity and are thought to dampen the immune response and to promote wound healing, angiogenesis, and debris scavenging [10]. M1 and M2 macrophages can be characterized by receptor expression, effector function, cytokine and chemokine production [33] or by a set of marker genes: M1 macrophages generate nitric oxide (NO) by upregulation of inducible nitric oxide synthase (iNOS), whereas M2 macrophages express Ym1 (also known as T-lymphocyte-derived eosinophil chemotactic factor [ECF-L] and chitinase 3-like 3 [CHI3L3]), FIZZ1 (found in inflammatory zone-1, also known as resistin-like molecule alpha [RELM-alpha]), and arginase-1 (Arg-1) [16, 43]. The upregulation of arginase-1 expression and activity is crucial in the metabolic switch from M1 to M2 phenotype in mice [52]. In our LPS-induced inflammation model, we screened for the effect of MSC CdM on AM phenotype *in vitro*. AMs isolated from healthy mice and subsequently exposed to LPS followed by MSC CdM had higher Arg-1 activity characteristic of M2 macrophages. In order to more thoroughly test for the occurrence of alternative macrophage activation *in vivo*, we isolated AMs from experimental animals and found that LPS, as well as Fib and FibCdM, increased the numbers of macrophages expressing iNOS in the absence of

Ym1 (a pattern corresponding with the M1 phenotype), while MSCs and MSC CdM upregulated Ym1+ macrophages lacking iNOS expression (M2).

These data support the immunomodulatory capacity of MSC CdM shifting the immune environment from pro- to anti-inflammatory via the induction of a "healer" M2 AM phenotype from a "killer" M1 macrophage. Our results are in line with several recent reports indicating that MSCs exert anti-inflammatory properties via macrophage reprogramming [25, 42, 44]. However, the murine MSC populations differ amongst these reports, mainly due to the current lack of consensus in establishing the universal murine MSC phenotype [6, 49]. Comparison is also limited by differences in experimental protocols and variations in CdM preparation methods.

The paracrine mechanism of action of MSCs opens new therapeutic perspectives. Indeed, several paracrine mediators that can mediate restorative effects of MSCs have been identified, including interleukin-10, IL-1 receptor antagonist (IL-1ra), and keratinocyte growth factor (KGF). IL-1ra was identified as an MSC-derived paracrine factor that reduced the severity of bleomycin-induced lung injury [46]. Allogeneic human MSCs or their CdM attenuated endotoxin-induced lung injury in an *ex vivo* perfused human lung model partly through KGF, a well-known growth factor to reduce lung injury [31]. In our study, we found that MSC CdM contained higher levels of KGF and HGF than Fib CdM. IL-10 was shown to be

responsible for the therapeutic benefits of exogenous MSCs in murine sepsis [44]. Our group has recently reported that BMSC-derived adiponectin contributes to the anti-asthmatic effect of BMSC CdM in a murine ovalbumin-induced asthma model [21]. Human cord blood-derived MSCs, but not fibroblasts, produce high levels of angiotensin converting enzyme 2, an exopeptidase recently shown to be lung protective [53]. Further identification of soluble factors may lead to the development of critically missing pharmacological therapies for ALI/ARDS and other inflammatory diseases [5].

In an attempt to broaden the search for candidate soluble factors, we performed a multiplex analysis screening for 96 factors present in MSC CdM and showed that their secretory profile differed from that of lung fibroblasts. In a similar manner, Schinköthe et al. screened human MSCs to attain the first large-scale description of factors they secrete and categorized these into functional groups: anti-apoptotic, immunosuppressive, pro-proliferative and angiogenic modulating [58]. Amongst factors that may account for the alternative macrophage activation, we found that MSC CdM contained several known M2 activators. Interleukins (IL)-4, -10 and -13 were identified in our multiplex screen of MSC CdM (**Fig. 3-7**). These cytokines can activate the JAK-STAT6 signaling pathway and promote STAT6 binding to the promoter region of target genes, which ultimately drives the expression of M2-specific genes

[23]. Although the levels of these interleukins were comparable between MSC CdM and Fib CdM, they may contribute to alternative macrophage activation in an orchestrated fashion, together with other components of MSC CdM. Fib CdM also contained higher amounts of the potent classical (M1) activator IL-6, while MSC CdM had higher levels of factors that have recently been shown to contribute to the promotion of an M2 environment. One of these factors is insulin-like growth factor-1 (IGF-1), which has been found to aid in creating an M2-favorable environment [7]. Another factor, adiponectin, that may exert similar M2-activating effects [43] was found in our MSCs CdM from both WT C57/BL6 and Balb/C mouse strains, but was undetectable in Fib CdM by ELISA [21].

Of the 96 different cytokines screened, MSC CdM contained markedly higher levels of potential protective factors compared to Fib CdM, suggesting that additional mechanisms could explain the therapeutic benefit of MSC CdM in this model. Stem cell factor (SCF) is increased almost 3-fold in MSC CdM compared to Fib CdM. SCF improves survival, proliferation, and differentiation of hematopoietic stem and progenitor cells and anti-apoptotic effects reported in kidney tubular epithelial cells [3, 12]. Another molecule, FcγRIIB, is increased 14-fold in MSC CdM compared to Fib CdM. FcγRIIB mediates the anti-inflammatory benefits of intravenous gammaglobulin (IVIG) in a murine model of immune thrombocytopenia [56]. We also found that IL-12p40, a natural antagonist

of IL-12, which is a cytokine responsible for the production of IFN-γ, was increased 7-fold in MSC CdM compared to Fib CdM. IL-12p40 has protective effects in bacterial pneumonitis in mice [18] and selectively inhibits airway hyperresponsiveness and peribronchial fibrosis in murine asthma [46]. A two-fold increase in soluble TNF-α receptor II (sTNFRII), an inhibitor of TNF-α and a possible regulator of inflammatory activity, was observed in MSC CdM compared to Fib CdM. sTNFRII may contribute to dampening of the activity of TNF-α in LPS injury [38].

In conclusion, our study provides direct in vivo evidence that MSCs exert their therapeutic benefit through a paracrine activity. Identification of pneumoprotective soluble factors in MSC CdM holds promise for the discovery of new pharmacological therapies for lung diseases. While MSCs may have a unique ability to monitor the microenvironment of injured tissues and respond appropriately, therapies with the proteins or cytokines produced by activated MSCs may be more practical than cell therapies [1, 32]. *In vitro* priming of MSCs to optimize the production of protective factors may combine the advantages of both cell and small molecule therapy.

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3.7. Figures

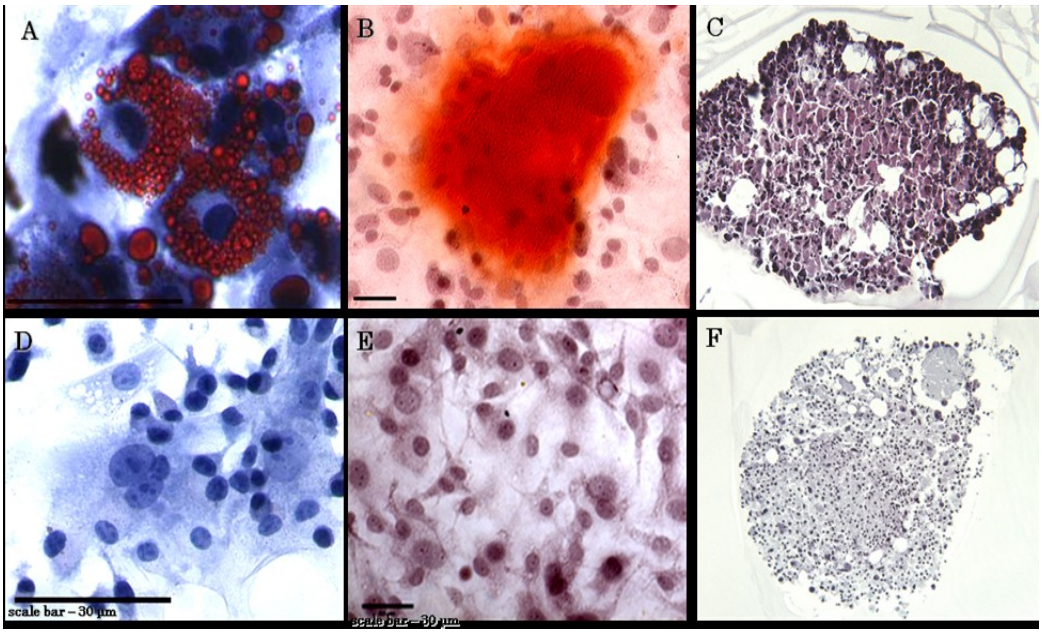

Figure 3-1. Mesenchymal lineage differentiation of C57BL/6 BM-MSCs. MSCs differentiated along adipogenic, osteogenic and chondrogenic lineages. Top row: differentiated MSCs; bottom row: control MSCs. Left to right panels: Oil Red O staining (adipocytes), Alizarin Red (osteocytes), Safranin O (chondrocytes). Size bar: 30 µm.

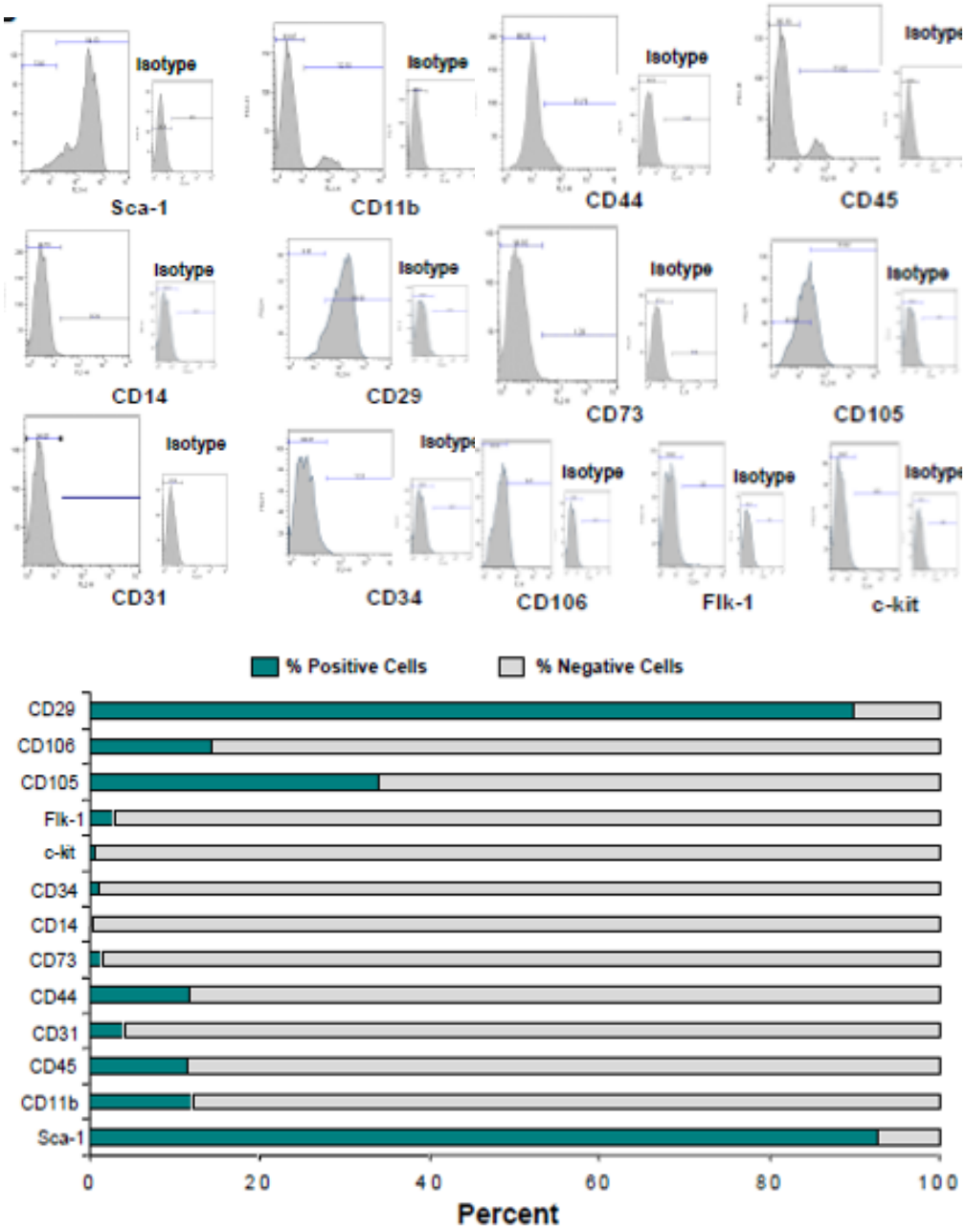

Figure 3-2. Flow-cytometric characterization of C57BL/6 BM-MSCs surface marker profile. Representative flow-cytometry histograms (**A**) and quantification of MSCs surface marker expression (**B**). Values are expressed as mean ± SEM.

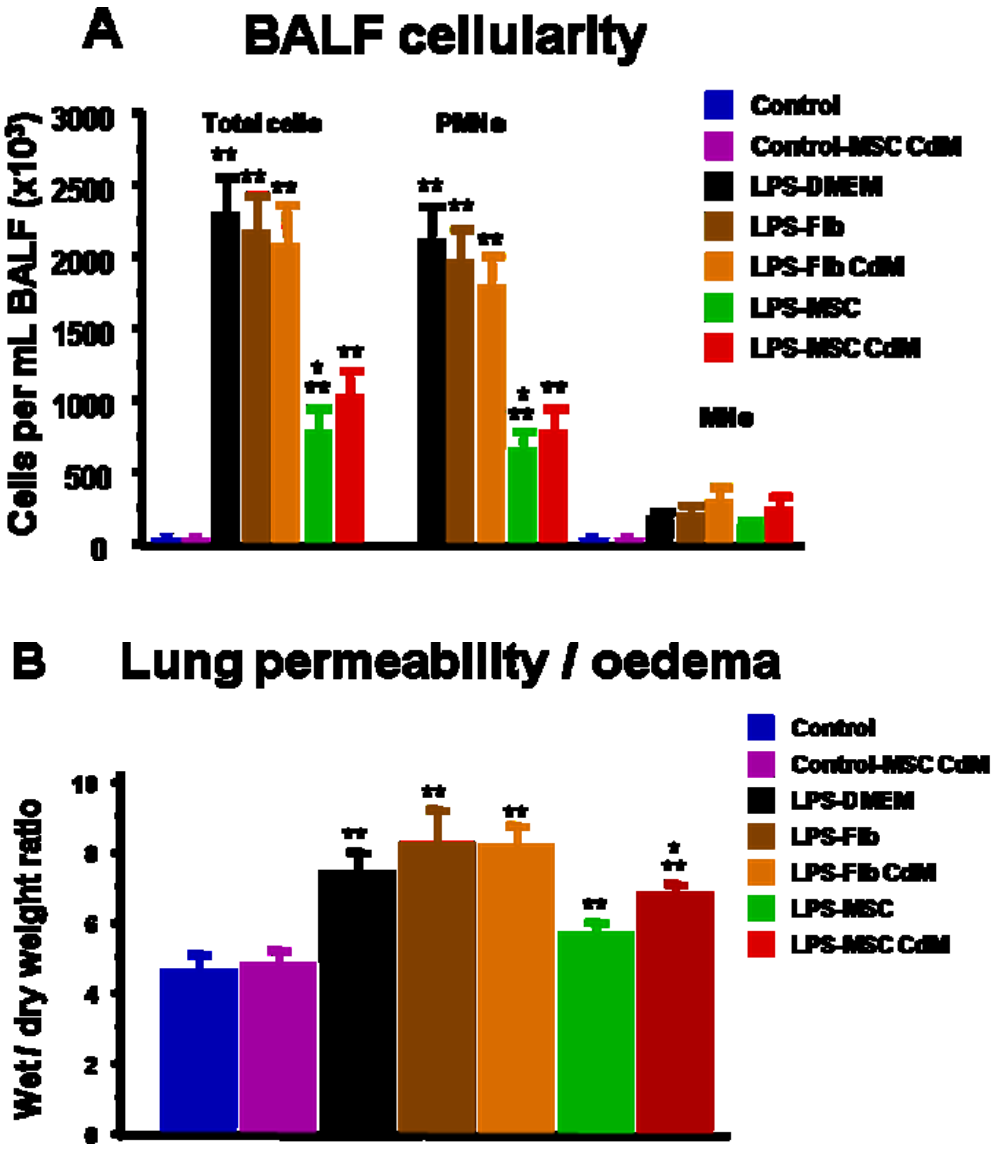

Figure 3-3. BALF and lung permeability analyses. (A) BALF total cell and neutrophil numbers. LPS-DMEM mice (n=10) had significantly more inflammatory cells than uninjured controls (n=5) and control-CdM (n=6) mice. Treatment with MSCs (n=5) or MSC CdM (n=10), but not Fib (n=5) or Fib CdM (n=5), significantly attenuated lung cells influx. BAL fluid of mice treated with DMEM had 2 times more PMNs than those treated with

MSC CdM. There were no differences in MN number. *p<0.05 control, control-CdM vs. LPS-MSC CdM; **-p<0.01 control, control-CdM vs. LPS-DMEM, LPS-Fib, LPS-Fib CdM, LPS-MSC; LPS-MSC vs. LPS-DMEM, LPS-Fib, LPS-Fib CdM. **(B) Lung permeability evaluation.** LPS-DMEM (n=5) lungs had increased wet / dry weight ratios compared to control (n=5) and control-CdM (n=5) lungs. LPS-MSCs (n=4) and LPS-MSC CdM (n=5), but not LPS-Fib (n=4) or LPS-Fib CdM (n=5), had significantly decreased wet / dry weight ratios compared to LPS-DMEM. *p<0.05 LPS-DMEM vs. LPS-MSC; LPS-Fib and LPS-Fib CdM vs. LPS-MSC CdM; **-p<0.01 control, control-CdM vs. LPS-DMEM, LPS-Fib, LPS-Fib CdM, LPS-MSC CdM; LPS-Fib and LPS-Fib CdM vs. LPS-MSC.

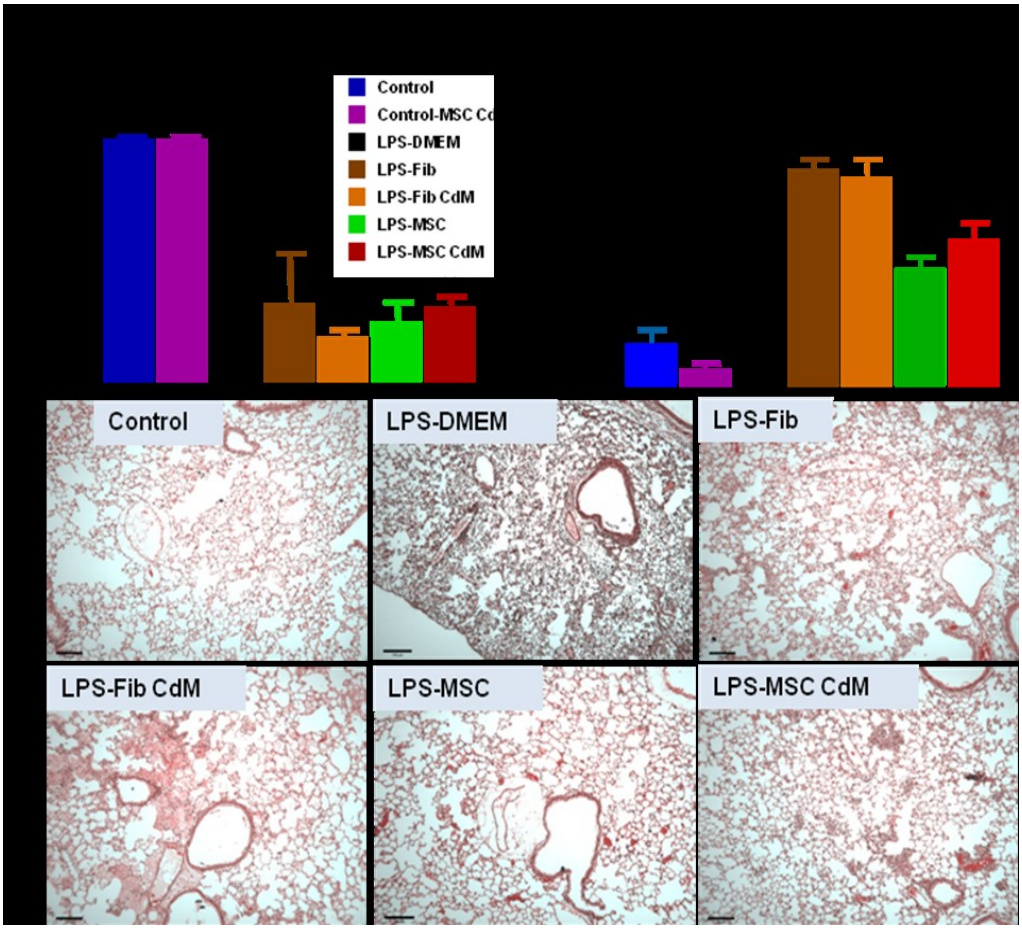

Figure 3-4. LPS-induced body weight loss and lung histological assessment. (A) Body weight loss following LPS-induced lung injury. No effect of treatment on LPS-induced body weight loss. Control (n=13), control-CdM (n=6), LPS-DMEM (n=36), LPS-Fib (n=9), LPS-Fib CdM (n=10), LPS-MSC (n=8), LPS-MSC CdM (n=35). *p<0.01 control groups vs. each LPS group. **(B) Lung injury score.** LPS-MSC (n=5) and LPS-MSC CdM (n=8) lungs had improved lung injury score compared to LPS-DMEM (n=8), LPS-Fib (n=5), LPS-Fib CdM (n=8). **p<0.01 control groups vs. each LPS group, LPS-MSC and LPS-MSC CdM vs. LPS-DMEM, LPS-Fib and LPS-

Fib CdM. **(C)** Representative images of lungs from experimental animals. Size bar: 130 μm.

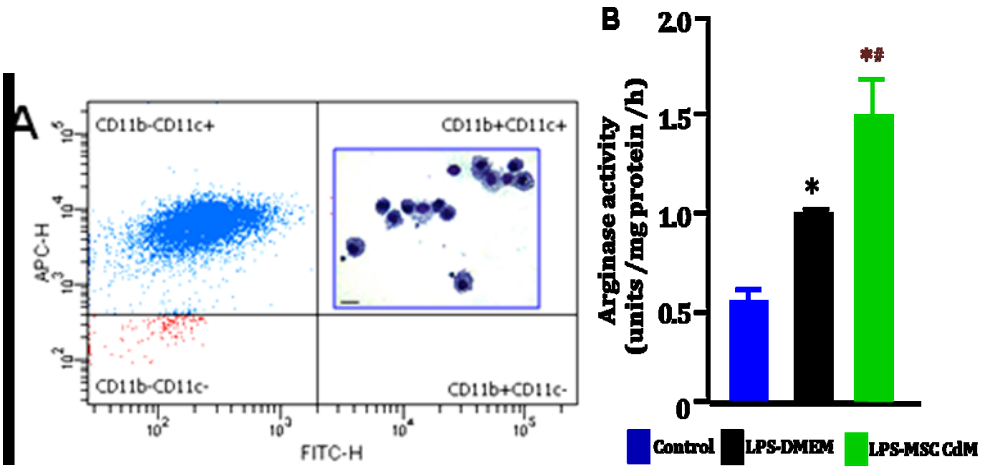

Figure 3-5. AM polarization following *in vitro* LPS exposure. (A) AMs were 98.2% CD11c+ and CD11b-. **Insert:** representative image of Hema3-stained AMs. **(B)** AMs exposed to LPS for 24 h had greater arginase activity compared to control AMs. MSC CdM enhanced arginase activity compared to DMEM (n=4/group). *p<0.05 control vs. LPS-DMEM and LPS-MSC CdM; LPS-DMEM vs. LPS-MSC CdM. *p<0.05.

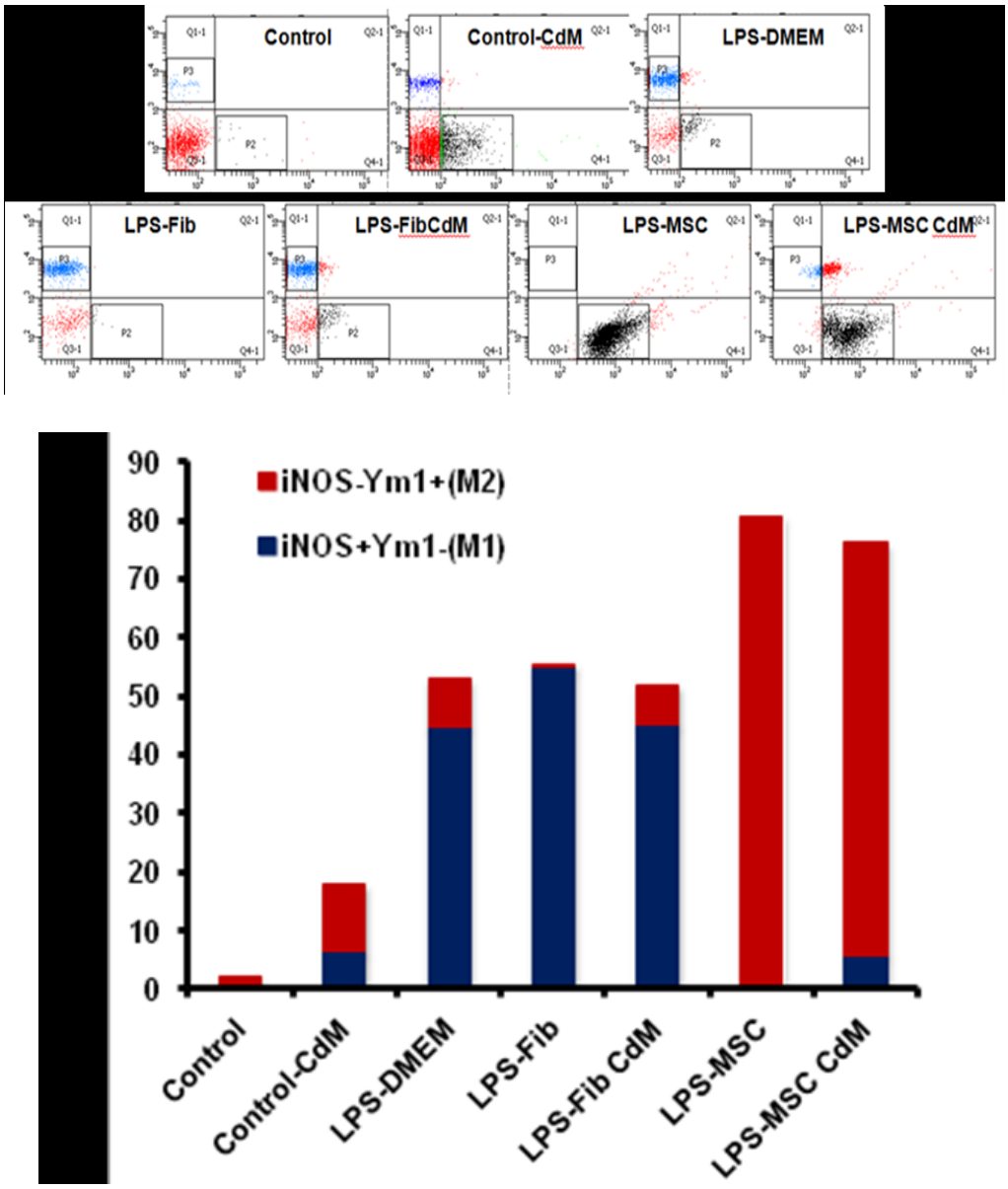

Figure 3-6. AM polarization following *in vivo* LPS administration.

(A) Representative scatterplots of 4-color stained AMs from experimental lungs. (**B)** AMs from LPS-DMEM, LPS-Fib, LPS-Fib CdM lungs displayed an iNOS+Ym1- phenotype. AMs from LPS-MSC, LPS-MSC CdM showed an iNOS-Ym1+ phenotype (n=5/group).

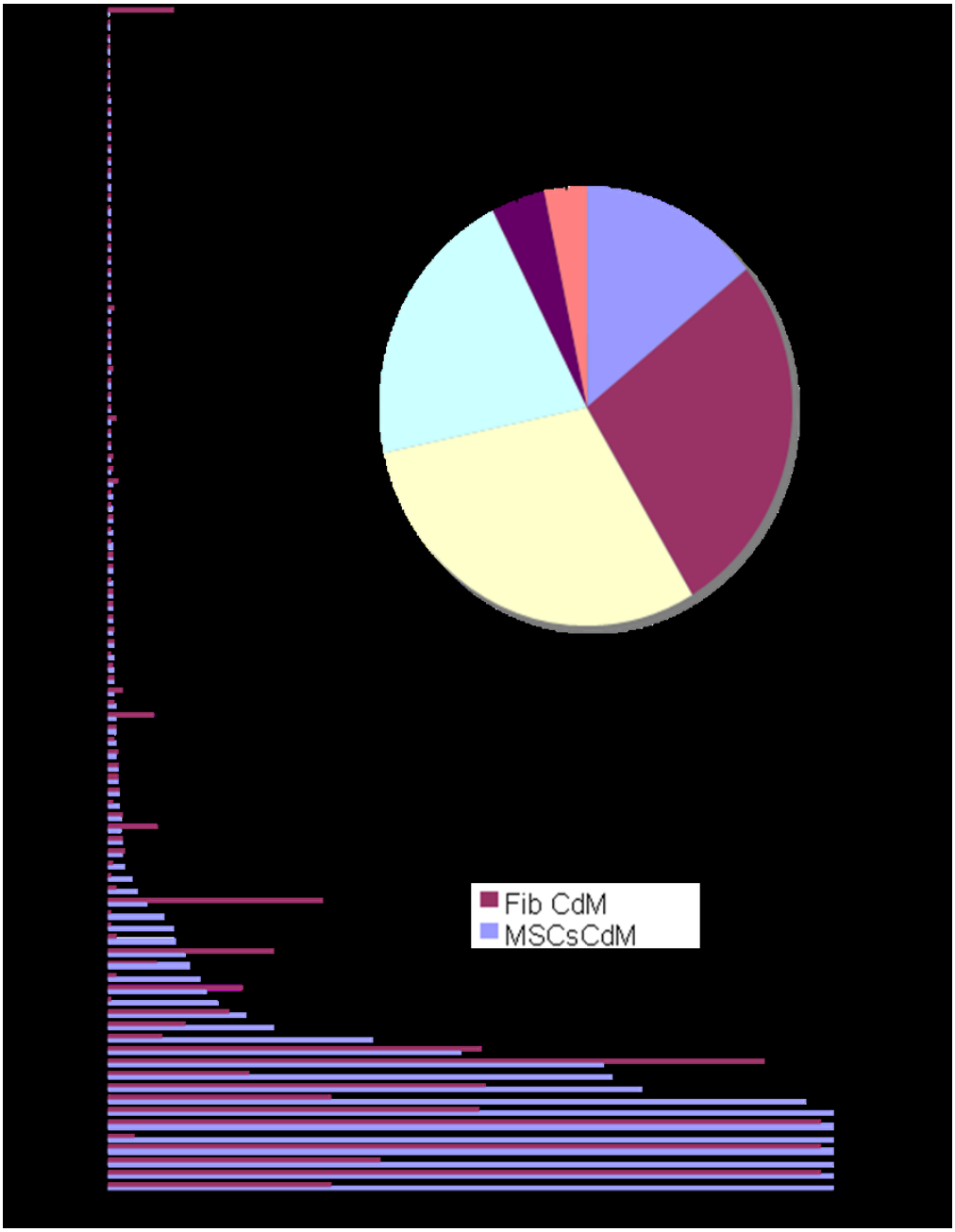

Figure 3-7. MSC secretome analysis. Antibody array-based comparison between MSC CdM and Fib CdM.

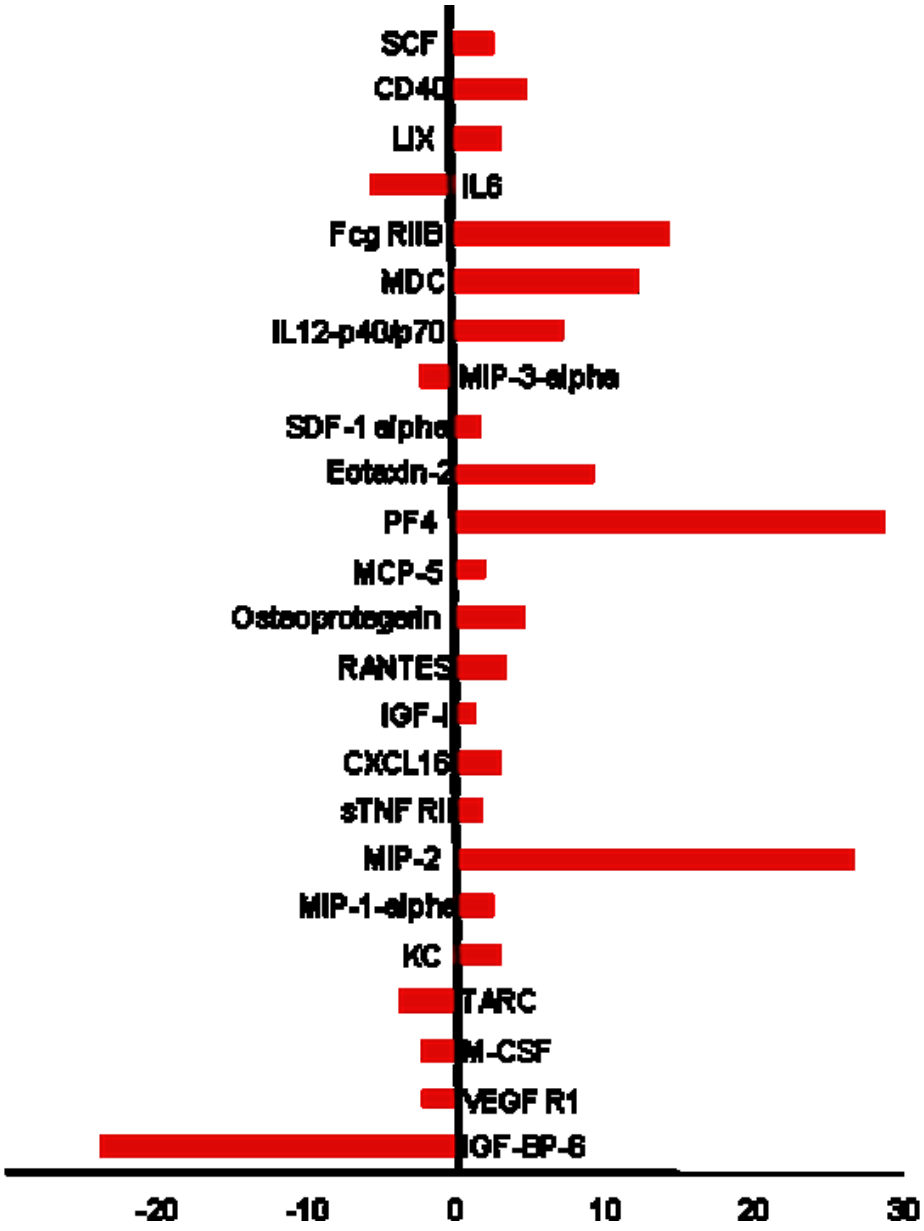

Figure 3-8. Factors up- or downregulated in MSC compared to LF secretome. Significant differences in MSC- vs. Fib CdM levels of factors highlighted.

CHAPTER 4 – GENERAL DISCUSSION AND CONCLUSIONS

This chapter was written by LII and edited by BT. Fragments of this chapter have been published as part of:

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4.1. Overview

Since the initial observations that stem cells engraft into the lung [15, 16], stem cell-based therapies (using mostly whole BM-derived cells or MSCs) have been studied extensively in various animal models of lung diseases. An overwhelming proportion of these studies showed the ability of these cells to prevent lung injury, despite a low rate of cell engraftment. The current hypothesis is that stem cells act via a paracrine mechanism to protect resident lung cells from injury, rather than through engraftment and cell replacement. Recent insight is now constructing an emerging view of cell-to-cell communication mediated by transfer of exosomes/ microvesicles or whole organelles. Microvesicles are small membrane fragments enriched in bioactive molecules [29]. Since the observations that ESCs can donate exosome-"packaged" mRNA and proteins to reprogram hematopoietic progenitors [28], increasing evidence indicates that MSCs donate microvesicles [27] and mitochondria [1, 6] *in vitro* and *in vivo* to protect against ALI [11] and acute and chronic kidney injury [10]. The microvesicles could contain a variety of molecules, including microRNA [7] and factors involved in cellular processes such as proliferation, migration, adhesion and morphogenesis [14]. The identification of factors produced and released into the local microenvironment or directly transferred to neighboring cells by MSCs

may yield new therapeutic avenues for lung diseases that currently lack efficient treatment strategies, thereby alleviating the potential risks associated with whole-cell delivery.

The studies presented highlight the ability of murine adult BM-derived stromal cells to prevent:

(i) allergic airway inflammation, AHR and remodeling in an OVA-induced mouse model of asthma;

(ii) lung inflammation in an LPS-induced mouse model of ALI

through paracrine-mediated actions on two immune cell effector types: a "flexible" subset of regulatory T cells and lung macrophages.

The targeted approach used in the first study indicates that the therapeutic effects of BALB/c BMCs CdM in Th2 allergic airway inflammation are mediated by IL-10 and APN. IL-10 has been previously shown to alleviate airway inflammation [23] and to contribute to anti-inflammatory properties of BM-derived stromal cells [22]; the present study proposes that exogenous IL-10 acts on IL-10-induced and –secreting Tr1 to determine additional, endogenous production of IL-10; APN also contributes to the generation of endogenous IL-10 through APN-reprogrammed local macrophages. Overall, two anti-inflammatory factors contribute through different effectors to amplify and sustain the local production of one of these factors.

In the context of LPS-induced inflammation, a wider approach (CdM screening) of C57Bl/6 BM-MSCs CdM was adopted in order to investigate the CdM-mediated reprogramming of local macrophages. Several factors with potentially relevant immunomodulatory activity were detected in MSCs CdM at significantly different levels compared to Fib CdM. Independent from the 96-factor screening, APN, which has also recently been shown to determine the alternate (M2) activation of macrophages [24], was also found to be present in C57Bl/6 MSCs CdM at levels comparable to those in BALB/c BMCs CdM. APN was not detected in Fib CdM. Fib CdM was used in both studies to determine whether the observed effects of BMCs and MSCs CdM were indeed stem cell-specific; minor improvements were observed following Fib CdM administration in asthmatic mice, in line with previous similar findings [22].

The effects of BM-MSCs CdM in LPS-induced ALI also largely reproduce the effects of whole-cell therapy. Although previous reports indicate comparable effects of CdM and whole-cell therapy in other lung injury models [4, 19], these findings may be considered at odds with a previous report in which BM-MSCs CdM administration was insufficient for macrophage reprogramming [22]. The method used here for preparing CdM, however, differs from this report, in which cells were cultured in complete medium for 3 days and the resulting supernatant was further diluted 1:1 with fresh medium for experiments. The presented studies

employed serum-starvation of MSCs for 24 hours and the CdM was prepared by concentrating the supernatants 25-fold for all the experiments. This suggests that the concentrations of various factors responsible in macrophage reprogramming in our CdM could be different from (if not higher than) the concentrations in the study mentioned.

4.2. Study Limitations

The same method was used to isolate the BM-derived stromal cells from experimental animals. Although the method used ensured minimal cell population variability amongst batches within each mouse strain, the innate differences amongst various mouse strains is well-documented [5, 11, 25, 26, 30]. This partially accounts for the current lack of consensus with respect to defining mouse MSCs in a fashion that would mirror the set of established criteria for human MSCs [8]; also, this accounts for one of the inherent limitations of studies involving the use of mouse MSCs. Both BALB/c BMCs and C57Bl/6 BM-MSCs met the plastic-adherence and mesenchymal (tri-lineage) differentiation criteria, but differed in terms of surface marker expression profile in a manner that has been previously reported [26, 30]. For instance, the low amount of Sca-1 expression by BALB/c BMCs compared to C57Bl/6 BM-MSCs has been described in direct comparison studies [26]. Notably, whereas BALB/c mice are

traditionally considered “Th2-biased” [9, 11, 13, 32] and therefore widely employed for generation of murine asthma models, the C57Bl/6 strain is used on a larger scale and the BM-MSCs from this strain conform to a greater extent to established MSCs criteria for human MSCs. These criteria [8] have often been used as a substitute tool to compare the molecular phenotype of different BM-derived stromal cell populations.

The “Th2 bias” of the BALB/c mouse strain and the more frequent use of C57Bl/6 mice for the development of disease models also partially justified a larger-scale approach to potential beneficial factors in C57Bl/6 MSCs CdM. One of the study-specific limitations derives from the relative scarcity of commercially available multi-factor screening arrays, which narrows the relevance of this array to a relatively limited number of known anti-inflammatory factors.

Another general limitation stems from the fact that murine models of asthma reproduce only a subset of features found in human asthma [18, 21].

The use of genetically engineered animals in which a gene has been constitutively modified is another general limitation. The absence of a single factor may often impact the levels of expression of numerous related factors. Our study involving the use of APN KO BMCs sought to partially account for possible additional consequences of innate lack of

APN by investigating the effects of recombinant APN administered alone in OVA-exposed animals.

4.3. Conclusions and Future Perspectives on Lung Regenerative Therapies

Recommendations for future directions for lung stem cell biology have been previously summarized [31]. The future aims and perspectives of this research fall within the general projections for the field. Stem cell biology has been developing exponentially over a relatively short period of time. The cell replacement paradigm was originally at the core of stem cell research; this may still hold promise for genetic diseases like cystic fibrosis, where a degree of CFTR functionality of no more than 5-10% of normal would alleviate CF symptoms; it is reasonable to estimate that a similar engraftment rate may be safely achievable and may ensure this degree of improvement. Stem cells engineered to express the corrected gene could be differentiated *in vitro* or *in vivo* and confer sufficient gene function; however, whole-cell administration calls for careful consideration of risks associated with cell transplantation. The dawning insight into the intimate mechanisms of cellular communication based on exosomal transfer indicates that preconditioning MSCs with lung-derived microvesicles before transplantation may enhance acquisition of AT2

phenotype by transplanted MSCs [2]; however, further research is mandatory, given that routine maintenance of MSCs in culture can also expose these cells to foreign (rodent, bovine) microvesicles [17]. In recent years, the change of focus toward the paracrine activity of stem cells has partially attenuated concerns linked to whole-cell therapy; whereas it is not impossible that the CdM by itself may still carry insufficiently characterized pro-tumorigenic, pro-apoptotic and pro-inflammatory factors that may become predominant in certain conditions, determination of specific beneficial factors would eventually lead to preparations containing only these factors.

The need to characterize the secretory profile of stem cells extends to the need to link this profile to the molecular phenotype of these cells. Definitive characterization of both endogenous and exogenous stem cell population will certainly be a natural addition to the field. While much more needs to be learned about the mechanisms of normal lung development, injury and repair, stem cell biology and the long term efficacy and safety of stem cell-based therapies, the promising animal and sparse clinical observations suggest that it might be time to initiate clinical trials using stem cell-based approaches for devastating lung diseases that currently lack effective therapies. There is ample precedent in medicine of established treatments for which the mechanism of action is still not fully

understood. Carefully conducted trials for patients in desperate need for improvement may teach us more than additional pre-clinical studies.

The projected developments of iPSC research will facilitate further investigation of cell-based approaches for therapeutic purposes but also for understanding of disease processes and drug testing. The challenge of lung regeneration, e.g. repair of established lung damage, relevant for lung fibrosis and emphysema, for example, remains and may require additional strategies in combination with stem cell-based approaches to “rebuild” the lung. It is hoped that insight into lung stem cell biology will facilitate and expand bioengineering approaches for lung regeneration.

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APPENDIX 1 - INDUCED PLURIPOTENT STEM CELLS (IPSC) DIFFERENTIATE INTO ALVEOLAR EPITHELIAL CELLS *IN VITRO* AND PREVENT HYPEROXIA-INDUCED LUNG INJURY *IN VIVO*

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ABSTRACT

Bronchopulmonary dysplasia (BPD) is the chronic lung disease that follows mechanical ventilation and oxygen therapy in about 30% of premature infants with birth weight < 1000g. The delivery of supplemental oxygen saves the lives of these infants, but arrests lung development in the late canalicular stage, resulting in decreased alveolarization and impaired angiogenesis characteristic of BPD. The lung is a complex structure composed of more than 40 types of cells with different embryologic origin. Hyperoxic injury of alveolar epithelial type 2 (AT2) cells, the putative distal lung progenitors and the sole source of pulmonary surfactant, can lead to arrested lung development and permanent respiratory dysfunction. Recent evidence indicates that administration of embryonic stem cell (ESC)-derived AT2 may benefit lung repair; ethical and immunological concerns that have so far limited the clinical relevance of ESC may be alleviated with the use of induced pluripotent stem cells (iPSC), "ESC-like" cells engineered from adult somatic cells. Increasing evidence suggests that iPSC may differentiate *in vivo* and restore the functionality of damaged organs such as heart and liver.

We hypothesized that iPSC differentiate into AT2 cells *in vitro* and alleviate hyperoxia-induced lung damage *in vivo*.

Mouse ESC and fibroblast-derived iPSC cultured as embryoid bodies with activin A and small airway growth medium acquired lung cell-specific markers: prosurfactant protein C (AT2) and aquaporin 5 (AT1), as indicated by immunofluorescence and flow-cytometry.

In an *in vivo* model of BPD, newborn mice exposed to 95% O_2 from postnatal day (P) 4 to P14 received cells (ESC, iPSC or lung fibroblasts - LF) intratracheally at P4 (prevention) or P14 (rescue), followed by lung function testing and histological analysis at P28. ESC and iPSC could prevent, but not rescue, hyperoxia-induced decreases in lung resistance and elastance. ESC and, to a lesser extent, iPSC, prevented, but did not rescue, arrested alveolar growth, while LF had no effect.

In vitro evaluation of potential paracrine actions revealed that ESC and iPSC conditioned medium (CdM) significantly accelerated wound closure rate in confluent AT2 compared to LF CdM. iPSC and ESC CdM also protected against AT2 hyperoxic injury while LF CdM had no effect (MTT assay).

In summary, mouse iPSC differentiate into alveolar epithelial cells, accelerate wound healing and prevent hyperoxic injury in vitro, while improving lung function in a mouse model of BPD. The possibility of using readily available iPSC to generate AT2 holds great promise for the future of cell-based therapy for lung injury.

INTRODUCTION

Preterm delivery is a major and growing health care problem [10], affecting 10% of all births and accounting for more than 85% of all perinatal complications and death [35]. Recent advances in perinatal care favored the survival of premature infants born at increasingly earlier stages of gestation [11], which is synonymous to earlier stages in lung development. The immature lungs of extremely premature neonates (born at < 28 weeks of gestation) require ventilator support and sustained delivery of high concentrations of oxygen (O_2); however, these infants are at high risk for long-term injury to lung, brain and eyes [39] due to this support in itself. Each year, ~10,000 babies suffer from bronchopulmonary dysplasia (BPD) [5], a chronic lung disease that follows ventilator and O_2 therapy for acute respiratory failure after premature birth [26]. BPD is characterized by an arrest in lung development, which encompasses simplification of alveolar structure and blunting of lung capillary growth. BPD has long term respiratory [1] and neuro-developmental [6, 18] consequences that reach beyond childhood and result in increased health care costs [23]. Structural abnormalities closely mimicking human BPD (fewer and larger airspaces and decreased capillary density) have been demonstrated in a newborn rat model of BPD

caused by exposure to hyperoxia during their first 14 days of postnatal life [30, 33, 43].

Among the resident lung cell populations, type II alveolar epithelial cells (AT2) are the sole source of pulmonary surfactant and differ both phenotypically and functionally from their type I counterparts. AT2 cells proliferate and generate AT1 cells following injury and have therefore long been considered the putative AT1 progenitors [31]. AT1 have also been shown to differentiate into AT2 *in vitro* [9], which suggests that AT1 and AT2 may be alternate progenitor cells depending on the type of lung injury. Hyperoxic injury of AT2 can lead to permanent, life-threatening respiratory dysfunction. Recent evidence indicates that administration of embryonic stem cell (ESC)-derived AT2 may benefit lung repair; however, ethical and immunological concerns limit the clinical relevance of ESC. These concerns may be alleviated with the discovery of induced pluripotent stem cells (iPSC) [36, 41], "ESC-like" cells engineered from adult somatic cells. iPSC-derived AT2 may have therapeutic benefits in diseases such as BPD and there is increasing evidence that iPSC may differentiate *in vivo* and restore the functionality of damaged organs such as heart [27], brain [42] and liver [7, 20]; moreover, there are reports indicating that pluripotent stem cells cultured in specific directing conditions can give rise to definitive endoderm lineages-committed cells

[4, 8, 12, 17, 32], including cells with liver- [16] and lung- and thyroid- [21, 24] specifications.

MATERIALS AND METHODS

All procedures were approved by the Animal Welfare Committee of the University of Alberta.

ESC and iPSC Culture Maintenance

iPSC were generated from CD-1 mouse embryonic fibroblasts using the EOS lentiviral vector selection system [14, 15]. ESC and iPSC were initially cultured on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs, The Jackson Laboratory) and gradually adapted to feeder-free conditions in 6-well plates coated with a solution of 0.1% gelatin in deionized water (Millipore, Billerica, MA). Cells were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% ES-qualified fetal bovine serum (FBS), L-glutamine, non-essential amino-acids [NEAA], sodium pyruvate, beta-mercaptoethanol (all Invitrogen, Burlington, ON) and leukemia inhibitory factor (LIF, Millipore). Culture medium was replaced daily. For iPSC selection, puromycin (1 μg/mL, Invitrogen) was added to the culture medium. ESC and iPSC were grown on gelatin-coated plates for at least 4 weeks prior to assessment of *in vitro* experimental endpoints or *in vivo* administration.

Conditioned Medium (CdM) Preparation

For *in vitro* studies CdM from ESC, iPSC, MSC and LF was prepared by culturing the cells in serum-free medium for 24 hours. The resulting CdM was stored at -80 °C until use.

Wound Closure (Scratch) Assay

Alveolar epithelial cells (RLE-6TN cell line, ATCC) were seeded at 25,000 cells / well in a 24-well plate and allowed to reach confluency. The cell monolayer was scratched in a straight line using a P200 pipette tip [19]. Cells were rinsed with PBS, complete culture medium (F12, Invitrogen) as control or serum-free DMEM or cell-derived CdM were added. The surface area of the wound was recorded at the time of scratch (T0) and monitored at 4, 8 and 12 hours after scratch (OpenLab software).

Cell Viability (MTT Assay)

Alveolar epithelial cells (RLE-6TN) were seeded as specified above. Upon reaching confluency, cells were exposed to 95% O_2 for 48 hours (XVivo, Biospherix). Following hyperoxic culture, cells were washed twice with PBS and a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen) (0.5 mg/mL in serum-free DMEM) was added. Cells were incubated for 2 hours at 37°C. MTT was removed and 200 µL of dimethyl sulfoxide (DMSO, Sigma) was used

to dissolve the resulting formazan crystals. Samples were transferred to a 96-well plate and a colorimetric plate reader was used to measure absorbance at 550 nm, a direct indicator of cell number [22].

In Vivo Murine BPD Model and Cell Administration

Newborn CD-1 mice were exposed to 95% O_2 from postnatal day (P) 4 to P14. On P4 prevention protocol) or P14 (rescue protocol), the animals received a single intratracheal injection of cells (iPSC, ESC, MSC or lung fibroblasts, LF – 1.2 x 10^5 cells / mouse in 25 µL DMEM). Control animals received an equal volume of DMEM.

Lung Function Testing (LFT)

LFT was performed at P28 using a computer-controlled small animal ventilator (flexiVent, Scireq, Montreal, QC) as previously described.

Lung Histological Analysis

Lung fixation and histological analysis were performed as previously described [37].

In Vitro Differentiation Protocol

For differentiation, mouse ESC and fibroblast-derived iPSC were transferred to low-adherence culture plates (Corning) on day 0 of

differentiation protocol and grown as embryoid bodies (EBs) for the entire duration of the protocol. All EBs were initially cultured in EB medium (high-glucose DMEM with 15% regular FBS, L-glutamine, NEAA, sodium pyruvate and beta-mercaptoethanol); half of the EBs received supplementation with 100 ng/mL activin A for 4 days. EBs were then maintained in EB medium (control) or transferred to EB medium supplemented with decellularized lung matrix powder (DLMP, 2 mg / mL) or small airway growth medium (SAGM, Lonza) for an additional 14 days. SAGM was supplemented with 30 ng/mL recombinant human keratinocyte growth factor [38] (KGF, R&D Systems) between days 14-18. On day 18, EBs were dissociated using 0.05% trypsin (Invitrogen) and plated overnight for immunofluorescence staining or homogenized for protein and RNA extraction.

Lung Decellularization and DLMP Preparation

Lung decellularization followed a previously published protocol [28]. Briefly, adult (8-10 weeks) CD-1 mice were anesthetized using sodium pentobarbital (65 mg/kg i.p.) and sternotomy was performed. The trachea was cannulated, the cannula secured using surgical thread (Prolene) and the lungs and heart were mobilized from the thorax. Thoracic aorta and pulmonary artery were cannulated and successively perfused at a constant pressure of 20 mm Hg with: heparinized PBS (15

min), 0.1% sodium dodecyl sulfate (SDS, Sigma-Aldrich, Oakville, ON) in deionized water (120 min), deionized water alone (15 min), 1% Triton-X-100 (Sigma) in deionized water (10 min), PBS supplemented with 100 U/ml penicillin G, 100 U/ml streptomycin, amphotericin B (Invitrogen) (72 h). Complete decellularization was confirmed using hematoxylin & eosin (H&E) staining and immunoblotting. Decellularized lungs were lyophilized and the resulting DLMP reconstituted in EB medium at a concentration of 2 mg/mL.

Immunofluorescence Staining

EBs were dissociated as a single-cell suspension by treatment with trypsin (0.25%, Invitrogen) followed by passage through a 1000 μL pipette tip and a 23-gauge syringe needle. The resulting cells were plated onto poly-L-lysine (Sigma)-coated glass coverslips (Fisher) and allowed to adhere overnight. Cells were stained with fluorescence-labeled antibodies (Abcam) against murine prosurfactant protein C (pro-SP-C) and aquaporin 5 (AQP5). Slides were examined using an inverted microscope (Leica) running Openlab software (Improvision, Coventry, UK).

Immunodetection (Western Blot)

EBs were homogenized using a Dounce homogenizer and the protein content extracted by centrifugation at 14,000 g for 15 minutes.

Immunoblotting was performed using pro-SP-C, AQP5 and beta-actin (Abcam) antibodies with corresponding secondary antibodies (Abcam).

Fluorescence-Activated Cell Sorting

EBs were dissociated into a single-cell suspension and the cells stained with pro-SP-C or AQP5 antibody and corresponding secondary antibodies. Data acquisition and analysis were carried out using the FACSCanto II system running FACSDiva software (Becton-Dickinson).

RESULTS

iPSC CdM Accelerates *In Vitro* Wound Closure

ESC and iPSC CdM, as well as MSC CdM, significantly accelerated wound (scratch) closure rate in confluent alveolar epithelial cells compared to LF CdM (**Fig. 1A**).

iPSC CdM Protects Alveolar Epithelial Cells Against Hyperoxic Injury

ESC and iPSC CdM significantly improved cell viability (as assessed by the MTT assay) following 48 hours of sustained hyperoxia compared to LF CdM (**Fig. 1B**)

iPSC Prevent But Fail To Rescue O_2-Induced Functional Lung Damage

LFT revealed that administration of iPSC at P4 restored O_2-impaired lung function, as assessed by measuring whole-lung resistance and elastance at P28 (**Fig. 2**). iPSC prevented the significant decrease in lung resistance and elastance observed in O_2-exposed animals treated with DMEM alone (O_2-DMEM). Administration of iPSC at P14 failed to rescue lung function (**Fig. 3**).

iPSC Prevent But Fail To Rescue O_2-Induced Structural Lung Damage

Histological analysis indicated that iPSC administration at P4 preserves alveolar structure in O_2-exposed animals (**Fig. 4**). The apparent improvement in mice treated with iPSC at P14 compared to O_2-DMEM or O_2-LF was not found significant by lung morphometric analysis (**Fig. 5**)

Tumor Development Following iPSC Administration

At 18 months post-cell administration at P14 (n=5), 3 iPSC recipients developed abnormal growths with apparent infiltration in kidney, spleen and colon (**Fig. 6**).

Lung Decellularization for DLMP-Medium Preparation

Complete lung decellularization was confirmed by histological analysis and immunoblotting. H&E staining did not reveal remaining cell nuclei or nuclear fragments. Immunoblotting confirmed absence of pro-SP-C in decellularized constructs along with preservation of elastin (**Fig. 8**).

ESC and iPSC Express Pro-SP-C and AQP5 Following Culture in Lung Cell-Directing Conditions

Immunofluorescence staining revealed that ESC (**Fig. 9A**) and iPSC (**Fig. 9B**) can acquire pro-SP-C and AQP5 expression following culture as

EBs in lung lineage-specification conditions: AA (100 ng/mL) + SAGM. Flow-cytometric analysis indicated that ASK yielded higher proportions of pro-SP-C or AQP5-expressing iPSC (15% and 25%, respectively) than DLMP-based medium (10% and 22%, respectively) (**Fig. 10**).

DISCUSSION

In the present study, we sought to assess the differentiation and therapeutic potential of iPSC within the framework of BPD: impaired alveolar structure [30, 33, 43] with a corresponding functional impairment [13]. There is a large amount of evidence for the benefits of MSC administration of in numerous organ injury models, including hyperoxic lung injury [3, 38]; however, these benefits cannot be accounted for by *in situ* cell differentiation and engraftment. There are only sparse reports indicating the potential of ESC to acquire lung cell-specific markers *in vitro* and to engraft into the lung to a very limited extent following lung injury; a very recent study confirms the potential of iPSC to give rise to lung- and thyroid-marker-expressing progenitor cells [21]. Another recent report indicates that intravenous administration of iPSC prevents endotoxin-induced lung injury [44]; however, the therapeutic impact of iPSC administration in hyperoxic lung injury has never been investigated.

We tested the paracrine effects of iPSC *in vitro* by screening the iPSC CdM against ESC, MSC and LF CdM in a wound closure assay and in an *in vitro* hyperoxia exposure model. iPSC CdM (and generally stem cell-derived CdM, but not LF CdM) accelerated wound closure and preserved cell viability throughout 48-hour O_2 exposure, suggesting that iPSC may

have beneficial properties relevant to our model that are mediated by paracrine factors.

We assessed the therapeutic potential of iPSC in a mouse model of BPD by administering a single i.t. injection of iPSC before (prevention) or after (rescue) 10-day neonatal O_2 exposure. We controlled for iPSC-specific effects by comparing iPSC effects to LF, ESC and MSC administration, as MSC have been previously shown to pose therapeutic benefits in rodent models O_2-induced lung injury [3, 38]. iPSC prevented the development of BPD-reminiscent functional and structural impairment, but were unable to rescue lung function or structure after establishment of injury.

We also report a possible long-term effect of iPSC administration at P14 in normoxic subjects (healthy). With the tumorigenic potential of pluripotent stem cells in mind [3], we monitored ESC- and iPSC-treated mice for two years after i.t. injection of cells. Three subjects that had received iPSC and one ESC recipient developed tumors that were macroscopically localized on the limbs. We show a characteristic synovial sarcoma-like tumor (the earliest, developed by an iPSC recipient 18 months after cell administration). The tumor was accompanied by infiltration of the spleen (with splenomegaly), kidney (with bilateral nephromegaly) and colonic wall (**Fig.6**). It is important to note that teratoma formation is usually employed to certify cell pluripotency *in vivo*;

although there are major differences in route of administration and timing of tumor formation between our study and the usual cell administration - teratoma formation test, possible reactivation of the proto-oncogene *c-myc* used in generating the iPSC used in this study could form the basis for malignant tumor (teratocarcinoma) formation in iPSC recipients [3]. Whereas methods for reprogramming somatic cells without the use of *myc* have been proposed [25, 45], these tumorigenesis-related concerns may be alleviated with the administration of pure iPSC-derived AT2.

We sought to differentiate iPSC into alveolar epithelial cells by employing a few general differentiation principles: 1) removal of LIF from the culture medium; 2) aggregation of iPSC as EBs and 3) culture in the presence of Activin A (AA), a factor that promotes endodermal differentiation [8, 12, 17], for 4 days. Following AA exposure, EBs were transferred to SAGM or DLMP-based medium for two weeks. Previous reports indicate the ability of SAGM, as part of a multi-step differentiation protocol, to determine lung marker-expression in ESC. Differentiation of iPSC was assessed using lung cell-specific markers: pro-SP-C (intracellular AT2 marker) and AQP5 (AT1 surface marker). Immunofluorescent staining-based screening of iPSC-derived EBs on day 18 indicated that lung specification conditions (AA + SAGM) determined pro-SP-C and AQP5 expression.

In the quest to maximize the yield of iPSC-derived, pro-SP-C-expressing cells, we treated the EBs with 30 ng/mL KGF during SAGM exposure; recent evidence suggests that KGF promotes the proliferation of ESC-derived alveolar epithelial cells in vitro [38]. However, the timing of KGF supplementation (days 4-8, 9-13 or 14-18 of the differentiation protocol) did not seem to impact the proportion of pro-SP-C- or AQP5-expressing cells, as assessed by FACS (data not shown).

The successful decellularization of the lung, with preservation of alveolar structure, has been described recently [28, 29]. We confirmed lung decellularization with absence of cell residues, including pro-SP-C, and prepared DLMP-based culture medium by addition of lyophilized lung scaffolds (2 mg/mL) to the regular EBs culture medium. This preparation was able to induce pro-SP-C and AQP5 expression in iPSC, but the yield was lower when compared to iPSC cultured in ASK conditions.

In summary, we found that mouse iPSC differentiate into alveolar epithelial cells, accelerate wound healing and prevent hyperoxic injury *in vitro*, while improving lung function when administered preventively in an oxygen-induced mouse model of BPD. Since the initial reports that three-germ-layer-competent iPSC can be derived from terminally differentiated cells, iPSC have often been referred to as “ESC-like” cells. However, the biology of reprogramming is far from being understood. From the very point of reprogramming, different approaches have already been used in

terms of gene “cocktails” or small molecules and transduction methods [25, 34, 45]. Efficiency and “completeness” of reprogramming are still subject to effervescent debate, as well as the risks of cell therapy in general and iPSC in particular. While there is still much to be learned about the biology of iPSC, the possibility of using readily available pluripotent stem cells to generate AT2 holds great promise for the future of cell-based therapy for lung injury.

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FIGURES

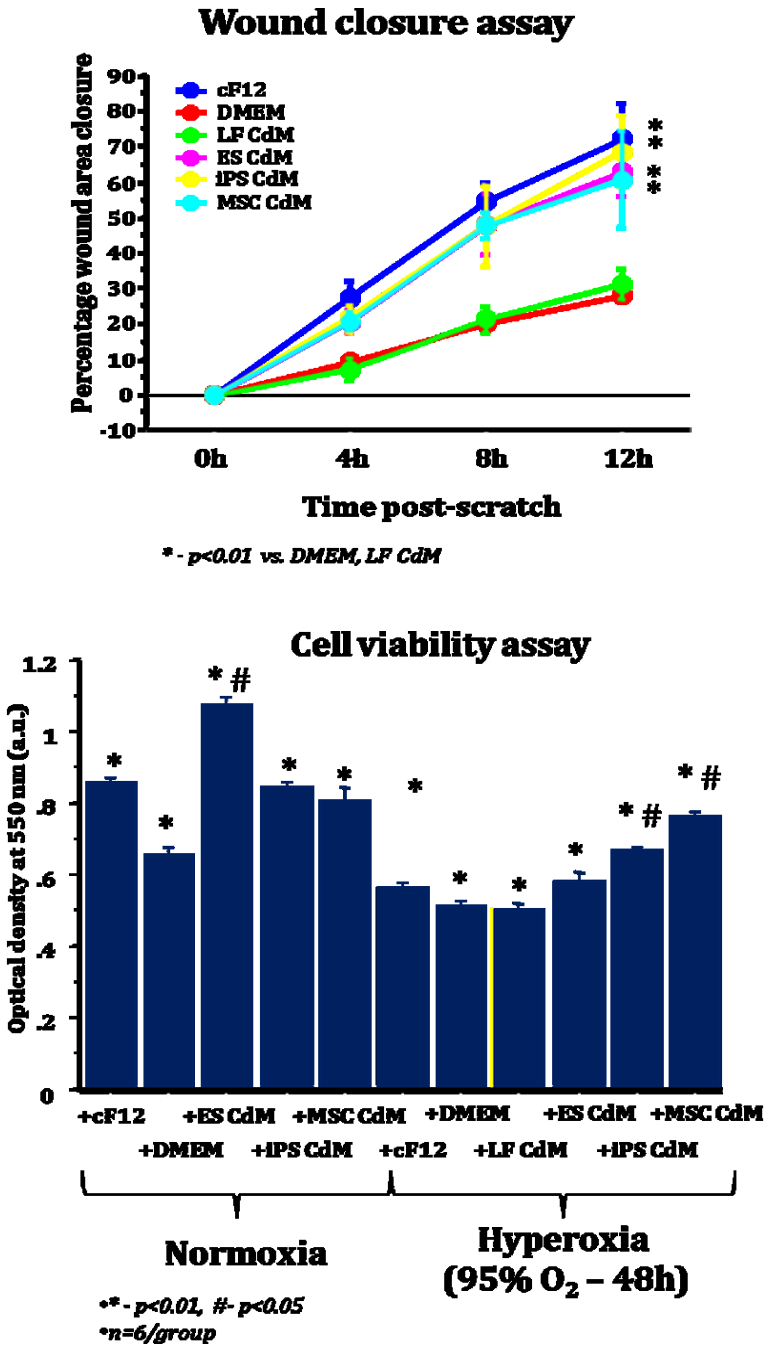


Figure 1. iPSC CdM accelerates AT2 wound closure (upper panel) and preserves AT2 viability during hyperoxic exposure (lower panel).

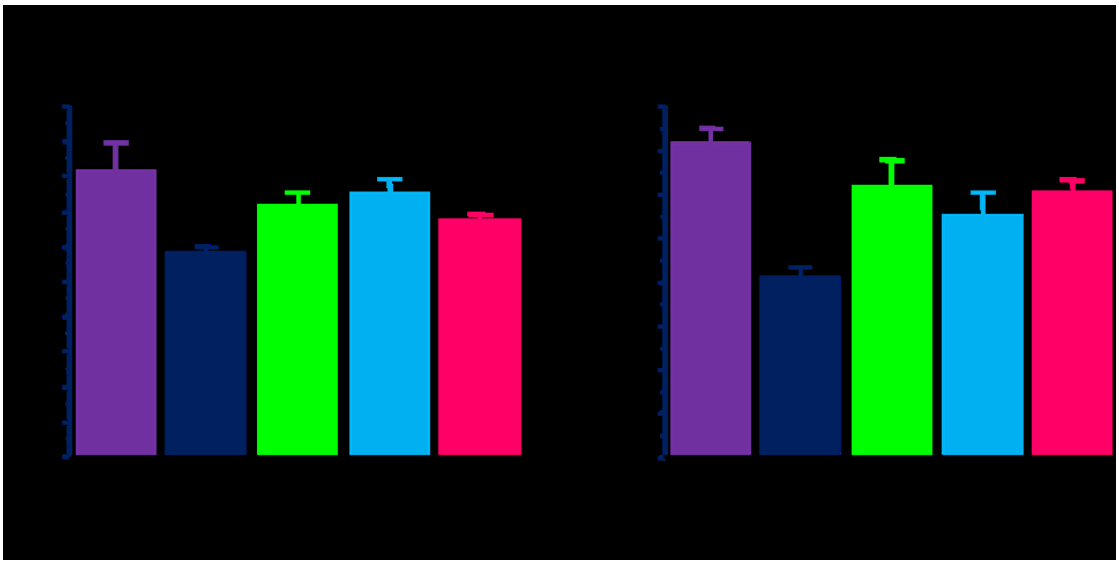
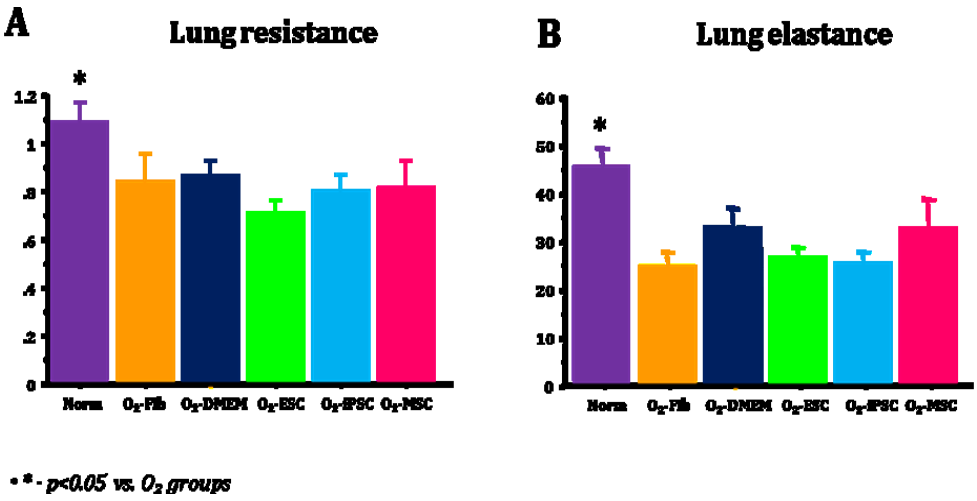


Figure 2. iPSC prevented lung function impairment in hyperoxia-induced lung injury.



* - $p<0.05$ vs. O_2 groups

Figure 3. iPSC failed to rescue lung function in hyperoxia-induced lung injury.

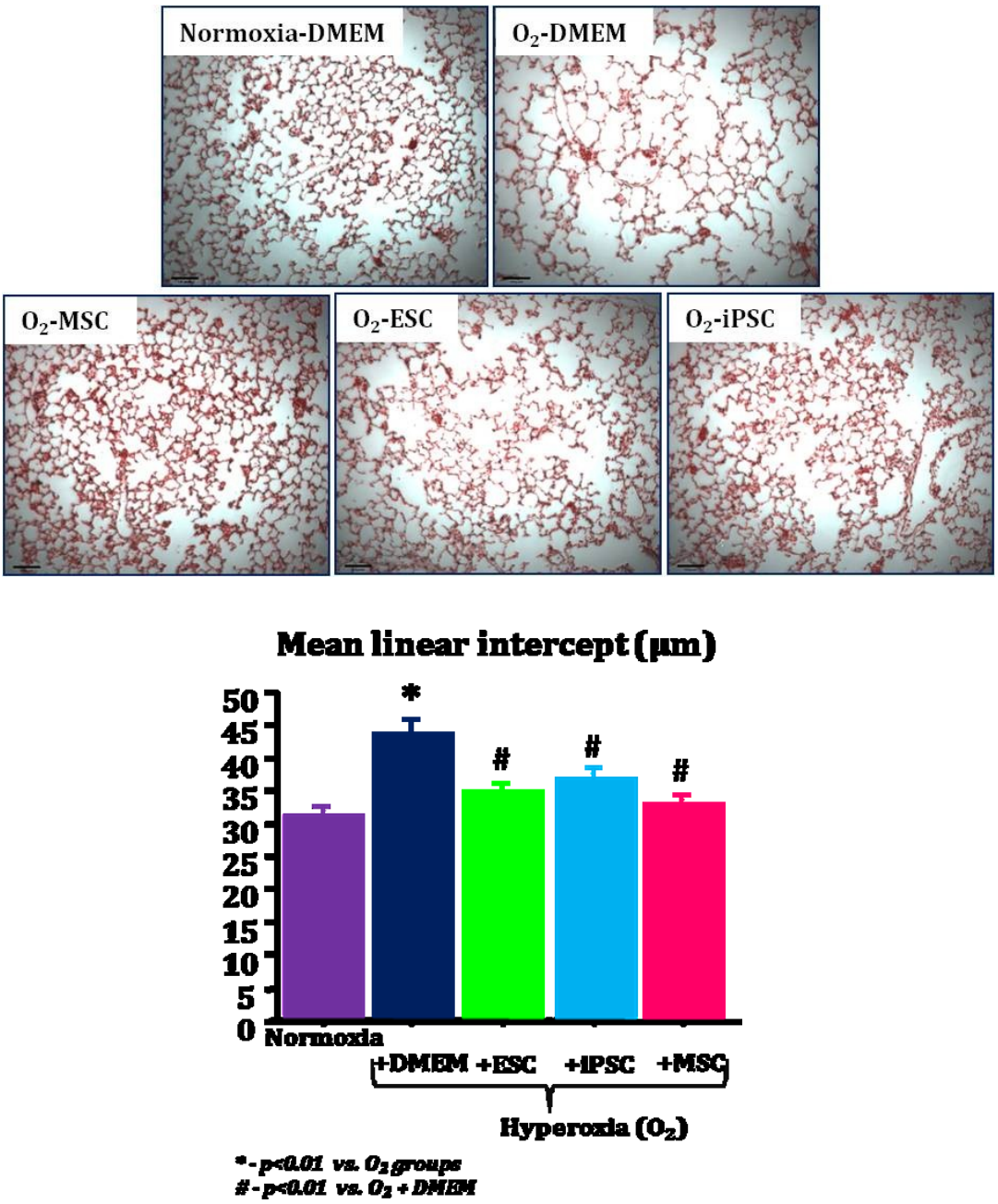
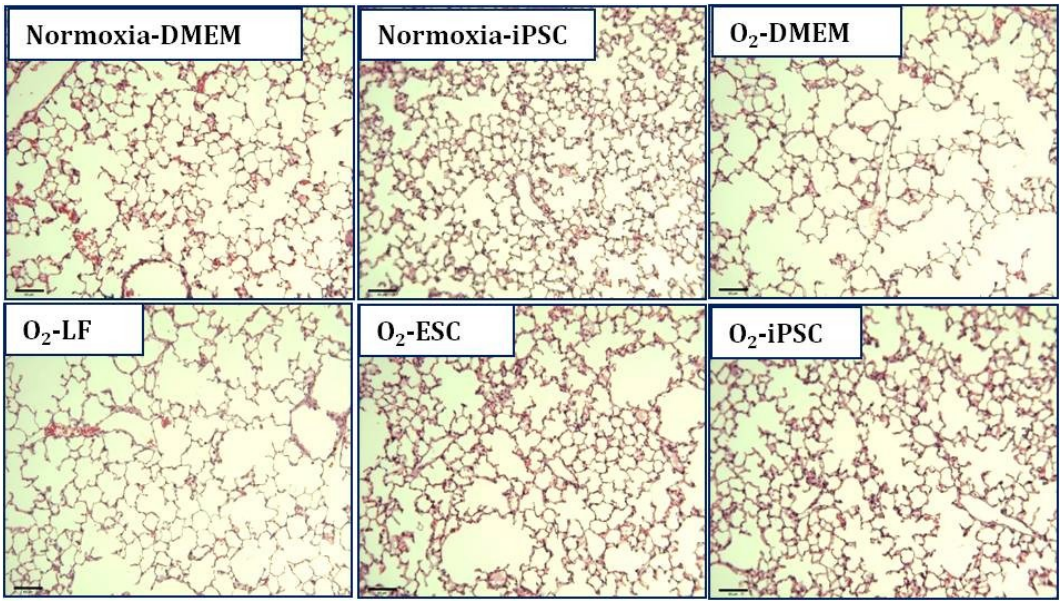


Figure 4. iPSC preserved lung structure in hyperoxia-induced lung injury (prevention protocol).



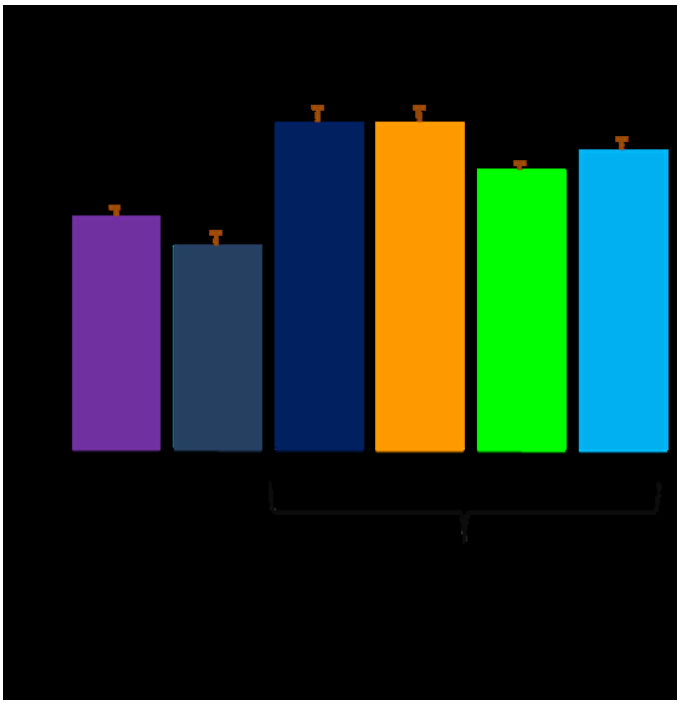


Figure 5. iPSC did not rescue lung structure following hyperoxia-induced lung injury (rescue protocol).

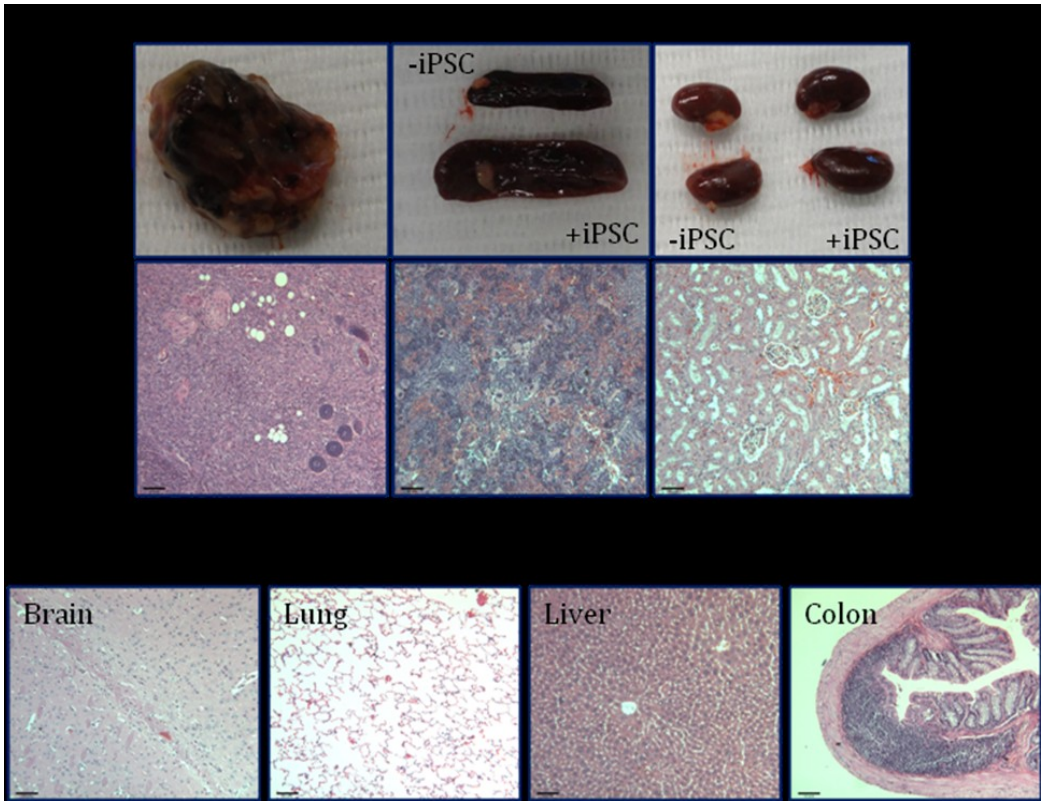


Figure 6. Potential long-term effects of iPSC administration.

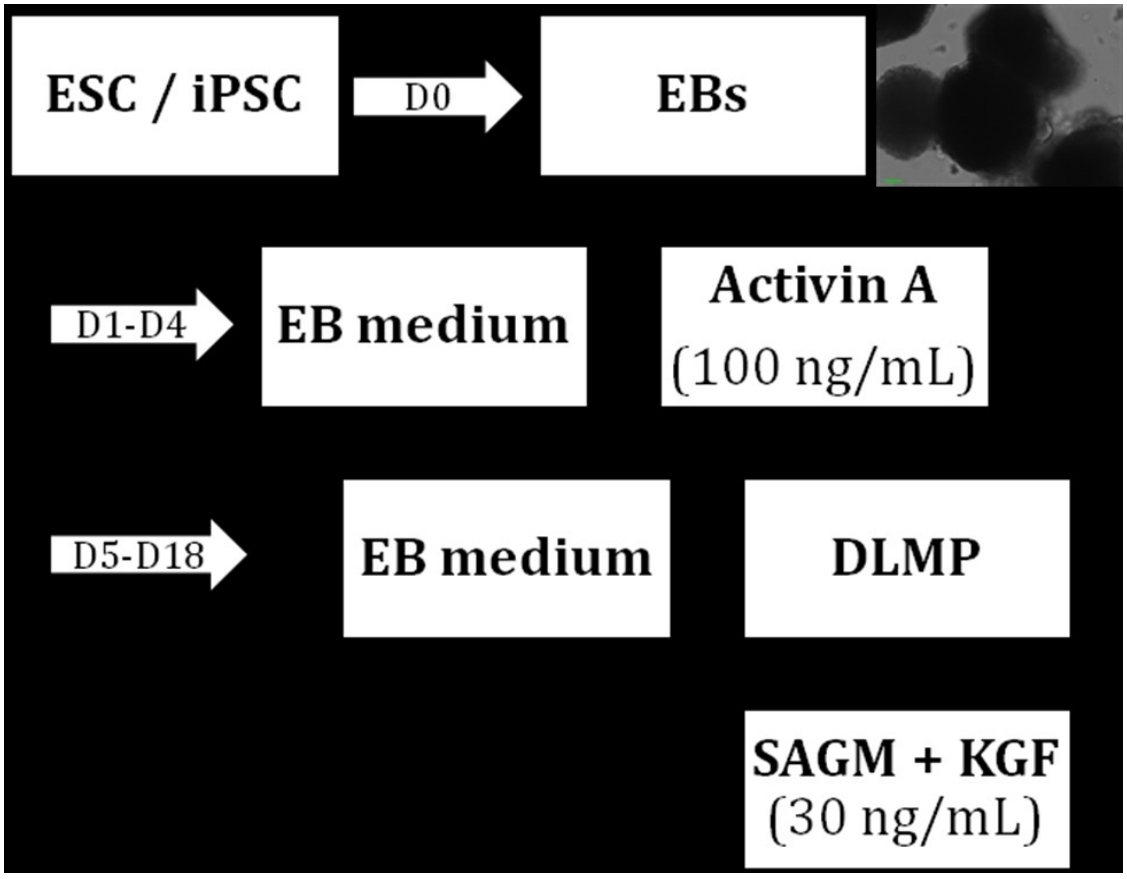


Figure 7. Differentiation protocol.

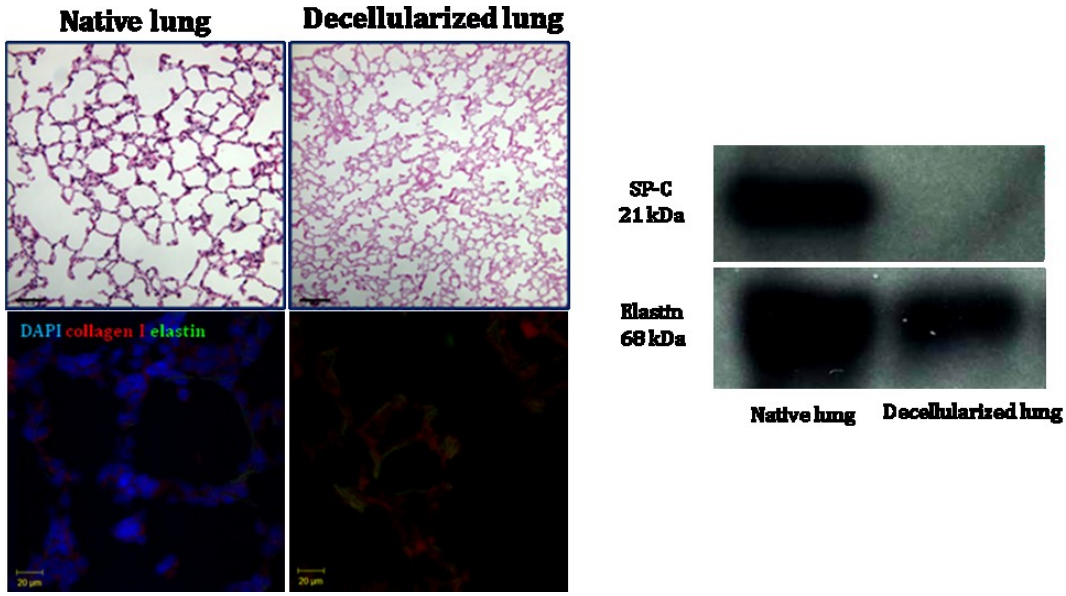


Figure 8. Decellularized lungs did not contain nuclear fragments or pro-SP-C residues.

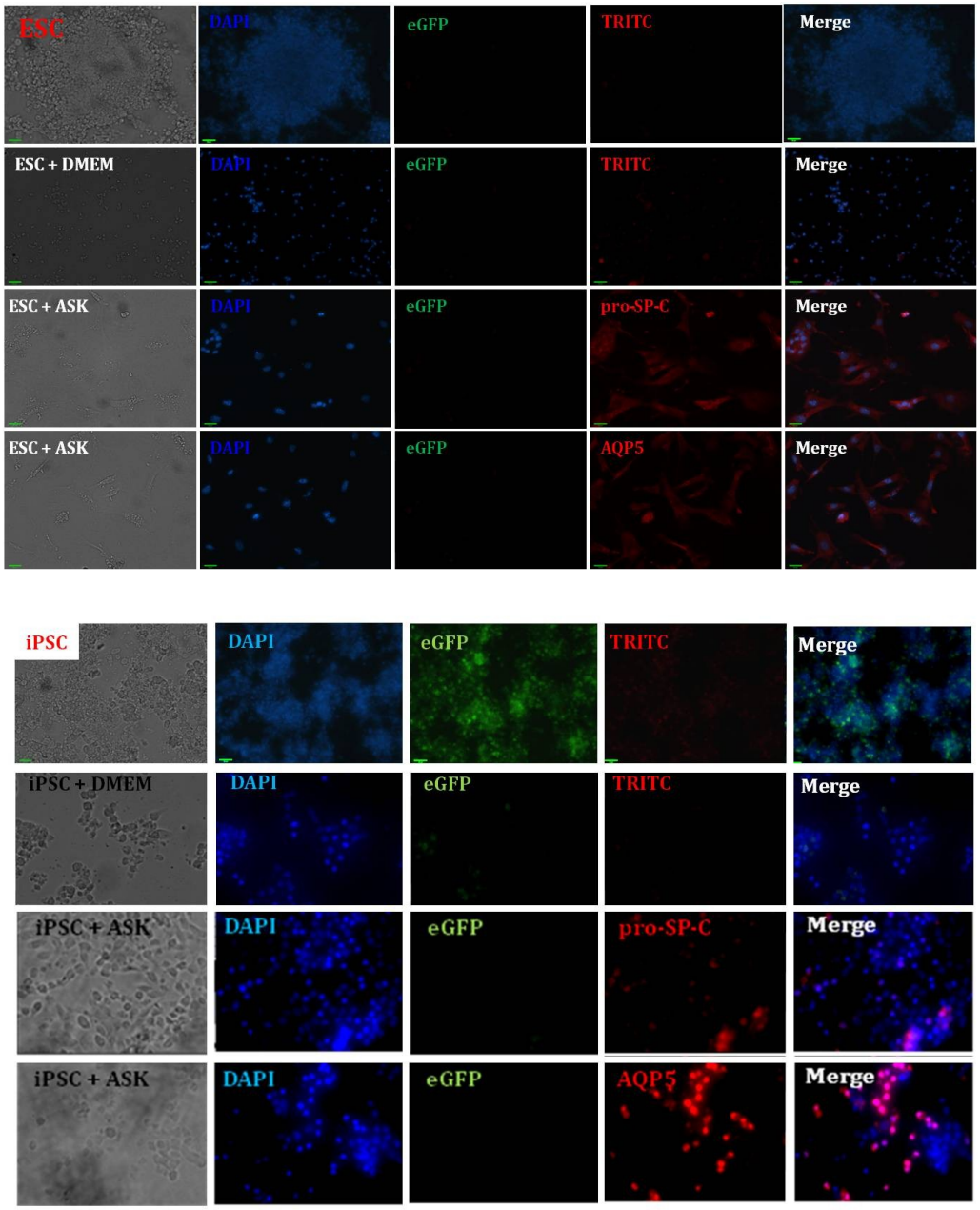


Figure 9. Immunofluorescence staining of plastic-adherent single-cell EBs preparations.

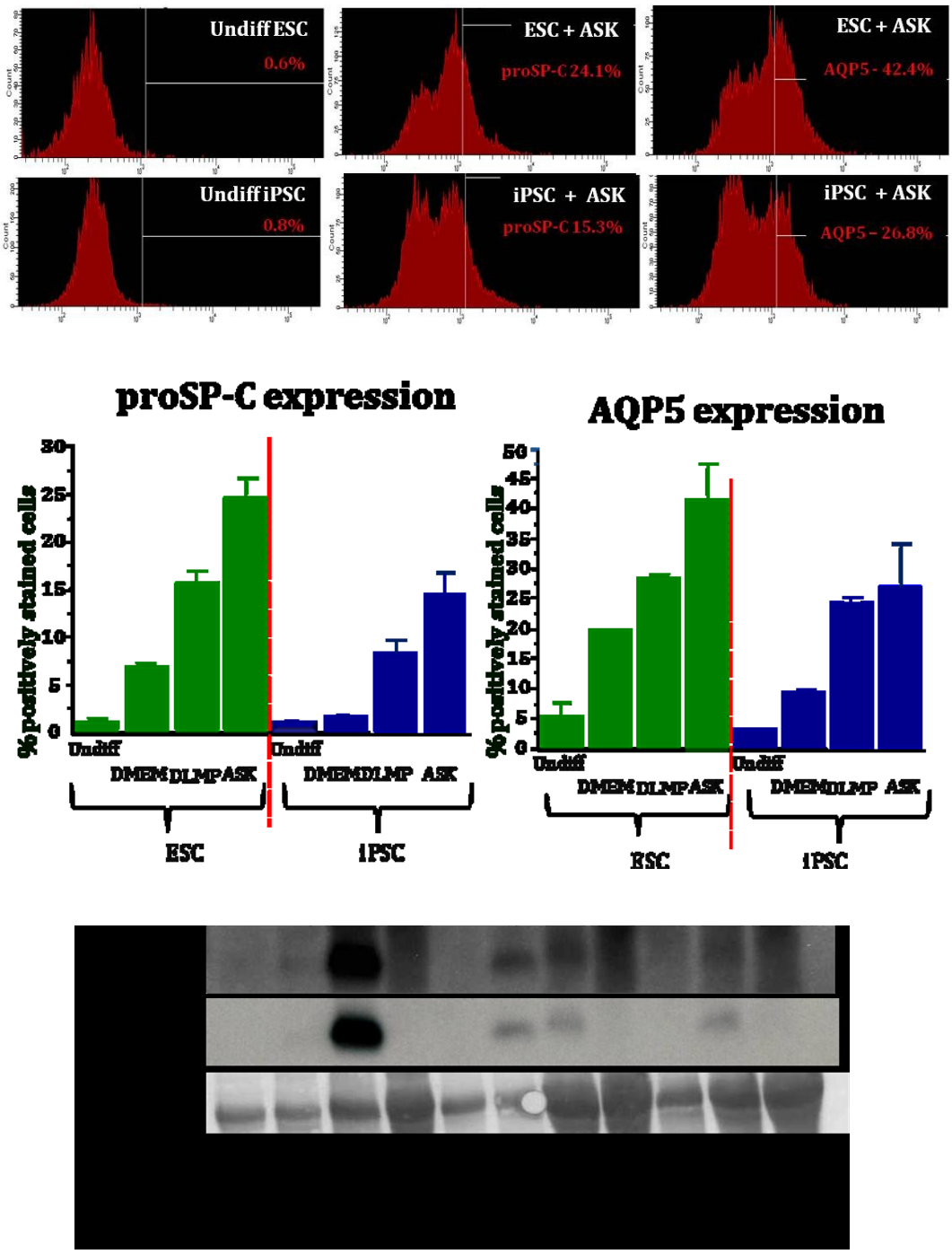


Figure 10. FACS and immunoblotting indicated that ESC and iPSC acquired alveolar epithelial cell markers.