Human Lung Progenitor Populations in End-Stage Lung Disease and Transplantation

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Abstract

Bone marrow-derived progenitor cell populations have been implicated in tissue regeneration and also in human disease pathology. This thesis investigated the hypothesis that Clara Cell Secretory Protein positive (CCSP⁺) epithelial-like progenitor cells and circulating fibrocyte numbers are altered in human lung disease and injury, and aimed to determine the predictive value of these cell profiles. It was found that cystic fibrosis patients have an increased number of CCSP⁺ cells in their bone marrow and peripheral blood, while patients with bronchiolitis obliterans syndrome (BOS) have a decreased number. In addition, BOS and pulmonary fibrosis patients have increased circulating fibrocytes. In response to ischemia reperfusion injury, an increase in CCSP⁺ cells in the peripheral blood was found at 24 hrs following lung transplant. Lastly, in patients studied greater than 1-year from transplant, those diagnosed with BOS had a higher number of fibrocytes and a loss of CCSP⁺ peripheral blood cells when compared to patients with stable lung function, with increased fibrocytes being associated with time post-transplant. In these patients, the ratio of fibrocytes-to-CCSP⁺ cells was predictive of lung function.

Multiplex protein arrays were used to investigate corresponding patient plasma, aiming to elucidate key mediators of progenitor cell recruitment. While differences in various
cytokines were found between end-stage diseases, a specific relationship between Stem Cell Growth Factor-β and CCSP+ cells was identified and between Monocyte Chemotactic Protein-1 and fibrocytes. Conversely, response of CCSP+ cells following transplant appears to be mediated by known mobilizing factors SDF-1 and GM-CSF. Interestingly, in patients followed long-term after transplant, MCP-1 was associated with the number of CCSP+ cells, while SDF-1 correlated with fibrocyte numbers. These observations suggest common pathways acting on both populations that may be altered by the microenvironment, and may further suggest a common origin. This work contributes important information regarding changes in lung progenitor cells and their association with human disease and tissue repair, which could ultimately support future directions that directly advance therapy and improve patient care.
Acknowledgments

This work would not have been possible without the support and challenge provided by my mentor, Dr. Thomas K Waddell. I am truly grateful for his guidance, honesty, and continued faith in me. I am eternally thankful for the opportunity to learn from Dr. Waddell and know I am a better scientist and person for his efforts.

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“To give anything less than your best is to sacrifice the gift”
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<td>AZA</td>
<td>Azathioprine</td>
</tr>
<tr>
<td>BADJ</td>
<td>Bronchioalveolar Duct Junction</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalvolar Lavage</td>
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<tr>
<td>BASC</td>
<td>Bronchioalveolar Stem Cell</td>
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<td>BMC</td>
<td>Bone Marrow Cell</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BNP</td>
<td>Brain Natriuretic Peptide</td>
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<td>BOS</td>
<td>Bronchiolitis Obliterans Syndrome</td>
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<td>CCSP</td>
<td>Clara Cell Secretory Protein</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
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<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Receptor</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin Gene-Related Peptide</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
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<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DLCO</td>
<td>Diffusing Capacity of the Lung for Carbon Monoxide</td>
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<tr>
<td>ENaC</td>
<td>Epithelial Sodium (Na) Channel</td>
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<td>EPC</td>
<td>Endothelial Progenitor Cell</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<tr>
<td>FEV1</td>
<td>Forced Expiratory Volume in 1 Second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte/Monocyte Colony Stimulating Factor</td>
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<tr>
<td>GMP</td>
<td>Guanosine Monophosphate</td>
</tr>
<tr>
<td>HRCT</td>
<td>High Resolution Computed Tomography</td>
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<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
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<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>ILD</td>
<td>Interstitial Lung Disease</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IP-10</td>
<td>Interferon-Inducible Protein-10</td>
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<td>IPAH</td>
<td>Idiopathic Pulmonary Arterial Hypertension</td>
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<td>IPF</td>
<td>Idiopathic Pulmonary Fibrosis</td>
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<tr>
<td>ISHLT</td>
<td>International Society of Heart and Lung Transplantation</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LOS</td>
<td>Length of Stay</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
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<tr>
<td>MCT</td>
<td>Monocrotaline</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MIP-1</td>
<td>Macrophage Inflammatory Protein</td>
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<td>MMF</td>
<td>Mycophenolate Mofetil</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
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<tr>
<td>NEB</td>
<td>Neuroepithelial Bodies</td>
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<tr>
<td>NFκB</td>
<td>Nuclear Factor Kappa-B</td>
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<tr>
<td>PAH</td>
<td>Pulmonary Arterial Hypertension</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<tr>
<td>PFT</td>
<td>Pulmonary Function Test</td>
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<tr>
<td>PGD</td>
<td>Primary Graft Dysfunction</td>
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<tr>
<td>PH</td>
<td>Pulmonary Hypertension</td>
</tr>
<tr>
<td>PNEC</td>
<td>Pulmonary Neuroendocrine Cell</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End Products</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SP</td>
<td>Surfactant Protein</td>
</tr>
<tr>
<td>TARC</td>
<td>Thymus and Activation Regulated Chemokine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-alpha</td>
</tr>
<tr>
<td>Tx</td>
<td>Transplant</td>
</tr>
<tr>
<td>UIP</td>
<td>Usual Interstitial Pneumonia</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Chapter 1

Introduction
In this work, observations made initially in a murine system were further explored in humans with end-stage lung disease. We measured circulating progenitor populations in the bone marrow at the time of lung transplant and in the peripheral blood at various times during and after transplant. To understand the context of these studies, the various forms of lung disease and injury examined in the study populations will be first briefly reviewed, including current measures of disease and therapy.

1.1 End-stage lung disease

1.1.1 Introduction and Relevance

In 2004, almost 400,000 Americans died from lung disease, representing an age-adjusted death rate of 135.5 persons per 100,000 (1). Lung disease rates continue to rise as death rates due to other major causes of death, such as heart disease, cancer and stroke, are declining (2). In Canada, respiratory diseases are responsible for a large economic burden on our health care system, with almost 6.5% of total costs being attributed to lung diseases. This accounts for $5.70 billion spent on direct health care costs including hospitalization, physician visits, research and pharmaceuticals (3). End-stage lung disease is the final phase in progressive pulmonary diseases including cystic fibrosis (CF), interstitial lung disease (ILD), and chronic obstructive pulmonary disease (COPD). The value of research aimed to understand and treat these diseases is immense, as few therapies currently exist that have shown efficacy in preventing lung function decline and eventual respiratory failure.

Taken together, chronic lung diseases are commonly marked by excessive inflammation, tissue remodelling, and epithelial damage, which ultimately lead to a loss in function and organ failure. While the pathogenesis of many lung diseases may involve common elements, there remain many unknown factors and mechanisms. Although variability exists in the causes and manifestation of different lung diseases, common elements including tissue fibrosis and cell injury are often observed and may result from similar underlying mechanisms. Further research aimed at creating a deeper understanding of the processes contributing to lung disease pathology may ultimately be beneficial and could lead to novel therapeutic options for the thousands of people suffering from lung disease and receiving lung transplants. The opportunity to study lung injury across a spectrum of diverse diseases and in the context of transplantation provides a framework to elucidate common mechanisms associated with tissue repair or remodelling. An introduction of
the major lung disease groups studied will be presented below, followed by evidence supporting the importance of bone marrow-derived cell populations in lung injury and repair.

1.1.2 Cystic Fibrosis

Demographics

While cystic fibrosis (CF) was once almost exclusively a child's disease, most individuals with this disease are now living into their twenties and thirties (4). This changing face of cystic fibrosis has major implications for the health care system and the community at large (3). In 2002 there were almost 3500 Canadians with Cystic Fibrosis (5), making it the most common fatal genetic disease affecting children and young adults. The most recent summary from the Canadian Cystic Fibrosis Foundation patient database (2008) reported a median age of 19 yrs, with 49.3% of patients being over 18 yrs (6). There were 114 new diagnoses and 44 deaths at a median age of 28yrs, contributing to a median survival of 38.1 yrs. Of the 3,222 patients included, 95.4% were Caucasian. Based on the Canadian CF patient database, there were 40 lung transplants performed for CF in 2008, a number which continues to rise.

Pathogenesis

The underlying cause of CF pathology is linked to mutations in the Cystic Fibrosis Transmembrane Receptor (CFTR) gene. This genetic defect was first identified and cloned in 1989, consisting of a deletion of three base pairs resulting in the omission of a phenylalanine residue at the center of the first predicted nucleotide-binding domain reported (7). Today, over 1500 CFTR mutations have been discovered, although many are rare and the functional relationship to CF disease is uncertain for a majority. The original deletion of phenylalanine in position 508 (ΔF508) is homozygous in 49.2% of patients and 88.3% carry at least one ΔF508 mutation (6). Other mutations are much less common, with less than 10 mutations occurring with a frequency of more than 1% (8). The consequence of these mutations on the CFTR protein can include lack of synthesis, abnormal conductance, and accelerated degradation, which ultimately lead to loss of function in some capacity.

The primary function of the CFTR is as a chloride channel in apical membranes, pumping chloride ions from the intracellular to extracellular space, and acting to maintain ion and fluid homeostasis. Loss of ion transport disrupts the osmotic gradient leading to dehydration of the epithelial surface and accumulation of macromolecular secretions (9). CFTR may also regulate the
function of the epithelial sodium channel (ENaC) and the outwardly rectifying chloride channel (8). Loss of CFTR therefore causes excessive sodium and water absorption through the ENaC. Together, airway fluid disruption contributes to ciliary collapse and loss of mucociliary clearance (10). A depletion of airway surface liquid is also found in CF lungs, further contributing to abnormal mucociliary transport (11). The end result of this mucus retention is an ideal environment for bacterial colonization and the inability to clear these infections is characteristic of CF, leading to a self-perpetuating cycle of infection and neutrophilic airway inflammation.

**Measures of Disease**

Initial diagnosis of CF is traditionally made using a sweat test, measuring abnormal chloride concentration at >80 mmol per litre (12). With the implementation of newborn screening, infants are now commonly diagnosed before any symptoms are manifested, providing the opportunity to begin treatment at the earliest point and this initiative has been shown to increase longevity, decrease subsequent hospital stays, and further improves height, weight and lung function (13). Newborn screening often involves DNA testing performed on dried blood smears to identify the relevant gene mutations (14).

Disease monitoring and improvements are often quantified using markers of disease severity including body mass index (BMI) and Forced Expiratory Volume in 1-second (FEV1), where increases in both are associated with better outcomes. The frequency of pulmonary exacerbations can also be used to define disease severity (15). In addition to traditional clinical parameters, biomarkers have also been proposed as important components of disease monitoring. Lung secretions from patients with CF contain large concentrations of the proinflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8, which are all induced downstream of the transcription factor nuclear factor (NF)-κB and are activated by cellular interaction with bacteria (16). CF patients with chronic *Pseudomonas aeruginosa* pneumonia also have elevated serum concentrations of GM-CSF, which may be important in regulating migration of peripheral blood mononuclear cells (PBMCs) (17). Additionally, CF PBMCs also demonstrate altered cytokine production, namely reduced IFN-γ and increased IL-10 (18). Epithelial cells from CF patients express decreased levels of the chemokine RANTES (19). Many other reports of altered inflammatory markers are present in the literature, and while these observations speak to the
underlying disease pathology, no one marker has been found to be specific and sensitive enough to predict meaningful changes in disease.

Current Treatment

The current treatment for patients with CF can be summarized as aggressive, and this has resulted in increasingly better outcomes (6). An association exists between higher pulmonary function test (PFT) results and more frequent clinic visits, more frequent sputum cultures, and greater use of antibiotics (20), suggesting that greater treatment management is beneficial to patients but can also create a larger burden of care. Airway clearance regimens are widely recommended although no one clearance methods has been proven most effective, and additional aerobic exercise is also recommended (21). Therapy also frequently involves hypertonic saline nebulisation designed to replace the missing salt to the airways and assisting in mucus lifting from the epithelium (11). The nebulised agents can also include enzymes capable of digesting extracellular DNA released by infiltrating leukocytes, cleaving it to more easily cleared fragments (22). The treatment of infection is also complex but critical in CF management and frequent cultures are required to identify early infections. One of the most common infections in CF is P. aeruginosa, and treatment strategies often include aerosolized and oral antibiotics. Corticosteroids may also be used to treat chronic inflammation. A large study evaluating the use of oral prednisone found slower decline in lung function and fewer hospitalizations in the treated group, although there were a number of side effects (23) and at this time neither oral nor inhaled corticosteroids are recommended for routine use in CF patients due to adverse events and a general lack of proof of efficacy (24). Interestingly, oral ibuprofen has become a useful tool in CF care. Ibuprofen can inhibit neutrophil migration and decreases the activation of NFκB, making it a well-tolerated anti-inflammatory agent, and high dose treatment has been associated with fewer hospital days (25). Therapy for pulmonary exacerbations of CF often includes antibiotics, increase in airways clearance techniques, and supplemented nutrition (24).

Development of novel therapeutics for CF continues to be pursued. Many of these efforts have focused on gene therapeutics to correct the underlying defect. Many strategies have been attempted including adenovirus and adeno-associated virus platforms, and although initial results looked promising, problems involving vector expression, transgene longevity, and protein function remain (26). Alternatively, pharmacologic therapy has been explored aiming to alter CFTR
expression or function, although this approach would require greater patient specificity to target the individual mutation and defect (8).

1.1.3 Chronic Obstructive Pulmonary Disease (COPD)

Demographics

A reported 754,700 Canadians live with COPD (27), a progressive disease which includes both chronic bronchitis and emphysema. Worldwide, COPD is a leading cause of morbidity and mortality and represents a substantial economic and social burden (28). In 2004, COPD was a more costly disease than asthma, with over half of these costs attributed to exacerbations. Cigarette smoking is by far the most important risk factor for COPD worldwide. Other important risk factors include environmental and occupational exposures, socio-economic status and genetic predisposition such as $\alpha_1$-antitrypsin deficiency (29). COPD affects both genders and all races, with mortality rising fastest in women and African-Americans, a fact that reflects the changing epidemiology of cigarette smoking (30). Clinically, patients generally present with increased sputum production, cough, wheeze, and dyspnea (28). Diagnosis of COPD requires spirometric confirmation of irreversible airflow limitation defined by a post-bronchodilator FEV$_1$/FVC ratio of \( \leq 0.7 \) and an FEV$_1$ of less than 70% of predicted, as determine by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) Staging (31). In general, the more advanced the COPD-associated lung injury becomes, larger changes in FEV$_1$ relative to FVC are noted and these are important in assessing disease progression. The primary clinical recommendation is cessation of cigarette smoking, but therapy may also include the use of bronchodilators and anti-inflammatory glucocorticoids. Oxygen therapy and pulmonary rehabilitation may also be recommended. Pharmacologic therapy is useful in reducing or abolishing symptoms in many but not all patients, but does not effectively prevent progressive deterioration of lung function. In mild and moderate COPD, lung cancer and cardiovascular disease are the most common causes of mortality, while in severe cases of COPD respiratory disease is the leading cause of death (32). Again, lung transplantation is often the only available therapeutic option for end-stage COPD patients.

Pathogenesis

It has become evident that the pathogenesis of COPD begins decades before symptoms are manifest. Pathologic changes occur in four main compartments of the lung; central airways, peripheral airways, lung parenchyma and pulmonary vasculature, and each are variably affected
among individuals. COPD is commonly defined as a disease state characterised by airflow limitation that is not fully reversible (28), although underlying this fact is a range of pathological changes. In general, these changes include mucus hypersecretion, airway narrowing, and loss of alveoli (33). COPD physiology is associated with airflow limitation, abnormalities in gas exchange and lung hyperinflation (33).

It is generally believed that COPD results from an initial injury to the airways (often cigarette smoke), which initiates an inflammatory process. If unresolved, the pattern of tissue injury and inflammation chronically activates cellular and biochemical pathways of injury and repair. Inhaled cigarette smoke can damage the epithelial cells of the airways and alveoli, leading to cell death and release of inflammatory cytokines and chemokines. Inflammatory mediators are also released in the formation of granulation tissue and the process of repair. Inhaled toxins also stimulate a humoral immune response and induce activation of the complement cascade, particularly the C5a component, which is a known chemoattractant (34). Infiltrating neutrophils further contribute to the inflammatory milieu, and additionally produce elastase, serine proteases, and matrix metalloproteinases (MMPs) that contribute to tissue damage and remodelling (35). Neutrophil elastase also stimulates mucus production and induces goblet cell metaplasia (36). The importance of elastase balance in tissue maintenance is further underlined by the genetic disorder alpha-1 anti-trypsin deficiency, which results in liver and COPD-like lung disease (37). Oxidative stress is also important in COPD pathogenesis. An imbalance of the oxidative burden and antioxidant capacity, as well as free radical generation orchestrates further inflammation and epithelial damage (38). Chronic remodelling leads to fibrosis and resulting airway narrowing, a process mediated by TGF-β activation and matrix deposition (39).

**Measures of Disease**

FEV$_1$ is the primary outcome measure in COPD and is generally believed to be a reliable representation of airflow obstruction, when normalized for FVC. However, it is has also been shown that spirometry is a poor reflection of patient symptoms or quality of life (40). Other indicators of disease used to assess physiological abnormalities include blood gas measurements of oxygen and carbon dioxide, as well as measurements of forced vital capacity (FVC) at rest or exercise. The diffusing capacity of the lung for carbon monoxide (DLCO) is a measure of carbon monoxide transfer from the lung to the capillaries, and this measure has also been used to assess COPD status and response to therapy (41). Dyspnoea scores may also be useful in the
Secondary measurement of symptoms or indicators of airflow limitation may not accurately define the state of the underlying causes. Imaging techniques such as computed tomography (CT) allow for more precise characterization of the parenchymal disease and airway remodelling. Several studies have found that densitometric scores are a more accurate indicator of progression than pulmonary function tests or health status (43, 44).

As with many chronic lung diseases, the use of biomarkers to better predict outcomes and monitor disease has been widely explored. Biomarkers are generally defined as elements measured in biological material that can indicate status of a disease or condition and may be used to monitor disease progression or response to therapy. A systematic review summarizing research into markers of disease severity for COPD found that sputum neutrophil counts and IL-8 concentration, as well as serum TNF-α and C-Reactive Protein (CRP) showed a trend toward separation between COPD stages. Other measures such as cigarette exposure (as measured by pack-years) and lung-specific quality of life (as measured by St. George's Respiratory Questionnaire) only distinguished between diseased and disease-free states (45). Bronchial biopsies have been studied as an indicator of changes in the smaller airways. A pattern of infiltrating CD8⁺ T-lymphocytes was found, and a CXC chemokine expression pattern representative of a type-1 response was further identified (46). This lymphocyte infiltration is also found during exacerbations of disease, where eosinophils and neutrophils increase and further express the chemokines RANTES and CXCR5 (47). Analysis of BAL samples provides an available source of cellular and protein biomarker identification. Inflammatory markers are routinely measured as increased in COPD BAL, including IL-8 (CXCL8) and leukotriene B4 (48). Serum measurements further describe the inflammatory status of COPD patients, with changes in systemic CRP, fibrinogen, leukocytes, TNF-α, IL-6 and IL-8 being reported (49). Much of the current biomarker literature is confused or clouded by underpowered and improperly controlled studies, making the determination of an accurate and sensitive biomarker for COPD difficult. As the COPD patient population is quite variable in terms of symptoms, status, and pattern of pathology, it is particularly important to use a matched-patient design in outcomes and response-to-treatment studies.
1.1.4 Pulmonary Fibrosis

Demographics

Idiopathic pulmonary fibrosis (IPF) is a clinical, physiologic, radiographic, and pathologic entity that can take many names and forms. It is the most common of the fibrosing lung diseases, and the pathologic description has therefore been term Usual Interstitial Pneumonia (UIP) (50). The pattern of UIP is also observed in other systemic fibrotic diseases including scleroderma and rheumatoid arthritis. The current prognosis for UIP/IPF is poor, with a median survival of approximately 5 years (51) and current therapy is largely ineffective (52). Conclusive demographic data is lacking, although incidence rates are currently estimated at 10.7 cases per 100,000 per year for males and 7.4 cases per 100,000 per year for females (53). Approximately two-thirds of patients with IPF are over the age of 60 at the time of presentation, with a mean age at diagnosis of 66 yrs (52). No predisposition has been associated with geographic location or ethnicity. Risk factors including cigarette smoking (54), exposure to environmental factors such as metal and wood dust (55), and chronic aspiration secondary to gastroesophageal reflux (56) have also been proposed but not fully proven. No specific genetic markers have been identified to predict IPF (57).

Pathogenesis

Originally believed to be primarily an inflammatory disease, new concepts in the pathogenesis of IPF have been proposed, stemming mainly from the observation that anti-inflammatory therapy is generally ineffective. Although BAL from IPF patients is enriched for neutrophils and eosinophils when compared with normal individuals, this is now believed to be caused by changes in lung architecture and altered inflammatory cell trafficking (58), suggesting that this phenomenon is a result rather than a cause. Cellular injury and apoptosis is also an important component of disease pathogenesis, and this has been particularly documented for the alveolar type II cell population (59). Pro-apoptotic protein expression and increased oxidative stress also contribute to the disease process. Supporting this hypothesis, inhibition of epithelial cell apoptosis has been shown to abrogate the development of fibrosis in the experimental bleomycin model (60). Transforming growth factor-beta (TGF-β), a key mediator in fibrosis, has also been shown to induce epithelial cell death in models of fibrosis (61). Tumour necrosis factor-alpha (TNF-α) is another potential mediator of alveolar epithelial injury (62). Accordingly, mice lacking TNF-α receptors are resistant to bleomycin-induced fibrosis (63) and TNF-α expression is increased in IPF patients (64). Loss of basement membrane integrity is another important feature
of IPF pathology, which may result from loss of the alveolar epithelial barrier (58). Epithelial cell regeneration is subsequently inhibited as detachment from an intact basement membrane prevents cell proliferation. Many factors are thus induced to combat this effect including TGF-β, PDGF, FGF-2, and KGF, further perpetuating the fibrotic environment. As these growth factors accumulate they also act on local fibroblasts, stimulating proliferation and matrix production. An imbalance of matrix production and degradation is an essential feature of fibrosis. Inhibitors of matrix degradation (TIMPs) may also be over-produced in IPF lungs (65). Matrix degradation products may further stimulate inflammatory gene expression, contributing to an ongoing fibrotic milieu. Fibroblasts in fibrotic lungs ultimately adopt a unique phenotype characterized by altered proliferation (66), matrix production, and conversion to a contractile myofibroblast that expresses α-smooth muscle actin (67). Currently no unifying theory explains the pathogenesis of pulmonary fibrosis in its entity, but ongoing research promises new insights and ultimately new targets for therapy. Recently, a genome-wide association approach was used to scan linkages in idiopathic pulmonary fibrosis and familial interstitial pneumonia (68). A region of interest on the p-terminus of chromosome 11 was identified within the area encoding gel-forming mucins. Specifically, a minor allele of the single nucleotide polymorphism rs35705950 in the promoter of Muc5 gene was present at a frequency of 38% among IPF patients and 34% among familial cases, resulting in an odds-ratio for disease of 6.8 and 20.8 for heterozygous and homozygous individuals respectively. These results identify a common heritable element that may lead to the development of pulmonary fibrosis and identified an important role for increased Muc5 in the development of fibrotic disease. It has also been identified that heterozygous germ-line mutations leading to shortened telomerases in leukocytes and in alveolar epithelial cells are associated with familial cases of IPF (69) and are a risk factor for both IPF and cryptogenic liver cirrhosis (70).

**Measures of Disease**

While patient history and physical examination are often the first clues to the existence of disease, lung biopsy and high resolution computed tomography (HRCT) scanning are required for accurate diagnosis. Disease progression monitoring and evaluation of therapy requires sensitive measure of disease to reliably predict outcomes. Many indicators of disease severity have been reported. A composite scoring system that includes clinical, radiological and physiological (CRP) measures has been shown to correlate with the extent and severity of the important histopathologic features of IPF (71). Evaluation of the predictive value of changes in clinical and
physiologic variables on survival time also identified six-month changes in dyspnea score, total lung capacity, thoracic gas volume, FVC, FEV₁, diffusing capacity of carbon monoxide, partial pressure of arterial oxygen, oxygen saturation, and alveolar–arterial oxygen gradient as useful measures, suggesting that multiple parameters must be evaluated to properly define disease progression (72). Changes in distance walked and quantity of desaturation during a six-minute-walk test (6MWT) may also add prognostic information (73).

Current Treatment

No effective therapies are currently available for IPF. Conventional therapy typically involves high-dose corticosteroids and/or immunosuppressive/cytotoxic agents (52). These may be administered alone or in combination, yet response rates remain low, leading to questions of benefit versus harm of long-term immunosuppression. Anti-fibrotic agents that alter collagen synthesis such as colchicine or o-penicillamine have been proposed but their value remains unproven. Novel biological therapies targeting specific cytokines or growth factors are also an attractive option, although trials evaluating inhibition of TGF-β signalling by pirfenidone remain controversial (74), and IFN-γ therapy has shown no benefit (75).

1.1.5 Pulmonary Hypertension

Demographics

Pulmonary Hypertension (PH) can result as a consequence of another underlying disease or as the primary cause of symptoms, and is generally defined as abnormally high blood pressure in the arteries of the lungs. Specifically, pulmonary arterial hypertension (PAH) is defined as a sustained elevation of pulmonary arterial pressure greater than 25 mmHg at rest or 30 mmHg following exercise, with a mean pulmonary-capillary wedge pressure (an indirect measure of left atrial pressure) of less than 15 mmHg (76). PAH is commonly associated with congenital heart disease, connective tissue disease, HIV infection, and as a side effect of some drugs and toxins, but can also be associated with many other disorders (77, 78). The NIH registry estimates the median survival for PH at 2.8 years, with 1-, 3-, and 5-year survival rates of 68%, 48%, and 34%, respectively, although this is influenced by underlying aetiology (79). Idiopathic pulmonary hypertension (IPAH) is a rare disorder with a female/male ratio of 1.7:1 and a mean age at diagnosis of 37 years and a prevalence of about 6 per million (80).
Pathogenesis

In general, pulmonary hypertension is characterized by restricted flow through the pulmonary arterial circulation and a parallel increase in pulmonary vascular resistance, ultimately resulting in right heart failure (78). The histological appearance of vessels in PH is characterized by intimal fibrosis, increased medial thickness, blockage of arterioles in the lung, and plexiform lesions (76). The functional consequences have been attributed to many underlying processes at both the cellular and molecular level. Increased vascular resistance is commonly attributed to increased proliferation and decreased apoptosis of pulmonary artery smooth muscle cells, leading to occlusion of vessel lumen. Endothelial cell damage contributes to this process though reduced production of anti-proliferative and vasodilator substances such as prostacyclin and nitric oxide and increased pro-proliferative and vasoconstrictive agents such as thromboxane A2 and endothelin-1 (81). Given these observations, the initiating cause of the pathogenic changes is not fully understood. There is some evidence that genetic mutations may augment this process, as has been documented for transforming growth factor β (TGF-β) receptor BMPR2 and the serotonin transporter (82, 83). In general it is believed that a multi-hit process is required to initiate and maintain the cellular changes leading to the vasoconstriction and elevated pulmonary arterial pressure and vascular resistance seen in PH patients (84). In the case of familial PAH (FPAH), a disease which affects two or three individuals per million per year and is most common among young women, is highly associated with mutations in BMPR2, with the relative risk in the order of $10^5$ compared to a carrier of the wild-type allele. Still, many patients who carry the disease gene will not be affected by the clinical disorder and there is some evidence that a ‘second-hit’ is required to fully manifest the disease (85).

Measures of Disease

Initial diagnosis of pulmonary hypertension includes history, physical examination, chest x-ray, and electrocardiogram to determine anatomic and arrhythmic problems. Echocardiogram is also important to determine functional and morphological cardiac conditions that might contribute to the development of PH (86). The diagnosis of PAH commonly requires confirmation with a complete right heart catheterization. PH is a complex disease and accurate monitoring of patients frequently requires a diagnostic algorithm to fully track progression. Evaluation of therapy often utilizes the 6-minute walk test as an endpoint. Time to clinical worsening may represent an alternative, more sensitive measure of response (87).
The biomarker brain natriuretic peptide (BNP) has been shown to increase in proportion to the degree of right ventricular dysfunction and could predict survival in PH patients (88). Serum uric acid levels were also shown to be a prognostic marker in PH, which increase in proportion to clinical severity and are independently associated with long-term mortality (89). Serum cardiac troponin T, a specific marker of myocyte injury, has also been proposed as a biomarker and had been reported as an independent marker of mortality (90). Cellular biomarkers may also be important determinants of disease, as well as potential avenues for therapy. Endothelial progenitor cells (EPCs) have been quantified in PH patients based on the hypothesis that this population may contribute to endothelial dysfunction and disease progression. In patients with Eisenmenger syndrome and IPAH a reduced number of EPCs was measured (91). In parallel, therapeutic treatment with prostacyclin increased the number of EPC colonies in children with IPAH (92).

**Current Treatment**

Advances in the treatment of PH have been made due to an increased understanding of the underlying pathobiology. Pharmacologic therapy primarily targets the known biological processes associated with PH pathology. Prostacyclin (epoprostenol) therapy has shown anti-proliferative and platelet anti-aggregatory effects in IPAH patients (93). Treprostinil, a prostacyclin analog, has also been approved for use and has proven efficacious based on 6-minute walk test results (94). Antagonists of the endothelin receptor, expressed on smooth muscle cells (A type) and endothelial cells (B type), have been developed to block the vasoactive peptide. Treatment with bosentan, a non-specific endothelin receptor inhibitor, initially produced significant improvements in 6-minute walk distance and also improvements in pulmonary hemodynamics (95). Bosentan is currently the most widely prescribed pulmonary hypertension therapy, and subsequent long-term follow-up studies demonstrated a 2-year survival of 89% (96). Inhibitors of cyclic guanosine monophosphate (GMP) degradation have shown efficacy by decreasing the metabolism of this second messenger and increasing its vasodilator and anti-proliferative effects. Phosphodiesterase-5 inhibitors (sildenafil) have also been developed for this purpose and tested in PH patients, showing statistically significant improvements in 6-minute walk distance and pulmonary hemodynamics (97). Anticoagulant and diuretic therapies are often also recommended.

Novel therapies are also being investigated. The most exciting advance in recent years is the use of cell therapy for PH. In the PHACeT (Pulmonary Hypertension: Assessment of Cell
Therapy) trial treatment with EPCs that were genetically engineered with a DNA vector containing the gene for endothelial NO-synthase (eNOS) was assessed. As the eNOS enzyme is needed for nitric oxide synthesis, a process that is reduced in PH patients, this study aimed to stimulate both angiogenesis and tissue repair by cell therapy. Initial results found that the cells were well tolerated by patients and the first three patients all showed a nearly 50% reduction in total pulmonary vascular resistance over the 3-day cell delivery period, an outcome which might result from NO release within the pulmonary microcirculation (98). These results were supported by preclinical studies using the Monocrotaline (MCT) model of PH, which showed that EPCs delivered up to 3 weeks following MCT treatment can prevent PH development (99).

1.2 Lung Transplantation

1.2.1 Lung Transplant and Early Complications

It is well known that lung transplantation is often the only viable therapy for patients with chronic end-stage lung disease. The lack of effective treatment for many respiratory disorders emphasizes the essential nature of this complex and technically challenging procedure and highlights the value of research in this field.

The first successful isolated human lung transplant was performed in 1983 by Dr. Joel Cooper in Toronto on a recipient with pulmonary fibrosis (100). This was followed by the first en bloc double lung transplant in 1988 (101), although the primary technique performed today is sequential bilateral single lung transplantation. Donor lungs can originate from brain-dead individuals, non-heart beating donors, or living donors, although this is quite rare. While height and size matching may be considered, lung recipients are not HLA-matched to the donors, although screens for reactive circulating antibodies are often done to prevent hyperacute rejection (102). Hyperacute rejection occurs within minutes of the anastomosis and clamp removal, but is a rare event in lung transplantation, and generally only affects allosensitized recipients with pre-existing antibodies against donor antigens (103).

Primary graft dysfunction (PGD) is a form of acute lung injury that occurs within 72 hours (104), and is one of the major complication following lung transplantation (105). Complete and prolonged depletion of blood flow from the donor lung is unavoidable in the transplantation process, and can result in severe damage upon reperfusion, despite advances in preservation, surgical technique and peri-operative care. This injury is characterized by increased microvascular
permeability, increased pulmonary vascular resistance, pulmonary edema, alveolar damage, and impaired oxygenation. These complications are observed in approximately 15% of lung recipients (106), leading to severe graft failure in 20% of those cases. The current grading scheme proposed by the ISHLT defines PGD based on oxygenation and chest x-ray data.

Adapted from (107):

Table 1.1 - Recommendations for Grading of Primary Graft Dysfunction (PGD) Severity

<table>
<thead>
<tr>
<th>Grade</th>
<th>PaO$_2$/FiO$_2$ Ratio</th>
<th>Radiographic Infiltrates Consistent with Pulmonary Edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt; 300</td>
<td>Absent</td>
</tr>
<tr>
<td>1</td>
<td>&gt; 300</td>
<td>Present</td>
</tr>
<tr>
<td>2</td>
<td>200-300</td>
<td>Present</td>
</tr>
<tr>
<td>3</td>
<td>&lt; 200</td>
<td>Present</td>
</tr>
</tbody>
</table>

Several intrinsic donor factors including female gender, African American ethnicity and age have been shown to independently influence the development of PGD (108), although the mechanism of these observations has not been ascertained. Acquired donor characteristics such as cause of death, prolonged mechanical ventilation, aspiration, pneumonia, trauma, multiple blood transfusions or hemodynamic instability are also predictive of PGD (109), yet these clinical markers are not accurate enough to reliably predict graft dysfunction.

Biological markers have also been studied to determine their utility and predictive power. Measurement of pre-implantation cytokine levels in donor lungs by PCR found that IL-6, IL-8, TNF-alpha and IL-1beta were risk factors for mortality and IL-10 and IFN-gamma were protective factors. Additional logistical regression modeling demonstrated that the ratio of IL-6/IL-10 was the most predictive for 30-day mortality, whereas in this study no clinical factors were predictive in the same group of patients (110). More advanced donor lung gene expression profiling was accomplished using microarray analysis. Distinctive expression signatures were identified patients who subsequently developed severe PGD when compared with case-matched donor lung samples from those who had a favourable outcome without PGD. Four significantly upregulated genes (ATP11B, FGFR2, EGLN1 and MCPH1) were identified in the PGF group, which may be useful for refining lung assessment and selection criteria (111).

Assessment of plasma cytokines in PGD patients has also identified patterns of expression associated with lung injury. Higher levels of monocyte chemotactic protein-1 (MCP-1) and
interferon-inducible protein (IP-10) were measured in cases of PGD, implicating the importance of leukocyte recruitment (112). Measurements of the receptor for advanced glycation end products (RAGE) are also higher in patients developing post-transplantation PGD (113). This marker of lung epithelial injury was also associated with cardiopulmonary bypass and intraoperative red cell transfusion. Assessment of inflammatory cytokine expression in lung tissue by Enzyme-Linked Immunosorbent Assay (ELISA) further found an increase in TNF-α, IFN-γ, IL-10, IL-12, and IL-18 during tissue ischemia, whereas IL-8 predominantly increased after reperfusion, and significantly correlated with early graft function during the first 24 postoperative hours (114).

The molecular mechanisms of PGD and ischemia-reperfusion injury are an active area of research. Animals model of lung ischemia have demonstrated an induction of reactive oxygen species (ROS) production by macrophages and endothelial cells (115), leading to injury of the pulmonary endothelium and epithelium. Pro-inflammatory cytokines are also activated (summarized below), contributing to up-regulation of adhesion molecules on lung endothelium (106).

Adapted from (106).

Table 1.2 - Source and Function of Cytokines Implicated in Reperfusion Injury Post-Lung Transplant

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Main Cell Source</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Macrophages, lymphocytes</td>
<td>Proinflammatory</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Lymphocytes</td>
<td>Proinflammatory</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Immune cells, lung epithelial cells</td>
<td>Macrophage chemotaxis</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Macrophages, fibroblasts</td>
<td>Proinflammatory</td>
</tr>
<tr>
<td>IL-2</td>
<td>Lymphocytes</td>
<td>T cell proliferation</td>
</tr>
<tr>
<td>IL-6</td>
<td>Macrophages, endothelial and epithelial cells</td>
<td>Proinflammatory</td>
</tr>
<tr>
<td>IL-8</td>
<td>Macrophages, epithelial cells, and fibroblasts</td>
<td>Neutrophil chemotaxis</td>
</tr>
<tr>
<td>IL-10</td>
<td>Macrophages, lymphocytes</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>IL-12</td>
<td>Macrophages</td>
<td>T cell activation</td>
</tr>
<tr>
<td>IL-18</td>
<td>Macrophages</td>
<td>T cell activation</td>
</tr>
</tbody>
</table>

In addition, endothelins can accumulate in the reperfused lung, contributing to vasoconstriction and further stimulating cytokine production by monocytes/macrophages and assisting in neutrophil recruitment (116). Pulmonary edema can result in poor lung function and gas exchange (105). As a consequence of alveolar and epithelial cell damage, surfactant imbalance
is also observed in ischemia-reperfusion injury, resulting in reduced lung compliance (117). Donor-derived passenger leukocytes, including macrophages, T cell, and NK cells, might also play a role in the injury process (118).

1.2.2 Acute Rejection following Transplant

Acute rejection remains common, with 36% of patients experiencing at least 1 episode in the first year, according to data compiled from July 2004 through June 2008 (119). Acute rejection is diagnosed histopathologically by the identification of perivascular infiltrates, and is graded from A0 (no rejection) to A4 (severe rejection) [112(120]. Patients often present with dyspnoea, cough, or sputum production.

This alloimmune response contributing to acute rejection is predominantly driven by recognition of foreign major histocompatibility complexes (MHC) by T cells directly stimulated by donor dendritic cells in the graft. As these donor derived antigen-presenting cells are eliminated, recipient dendritic cells also process antigen and present it as part of the indirect pathway (121). Other important processes likely include innate immune stimulation by tissue injury and infection, as well as by autoimmune recognition driven by cryptic self-antigen exposure in the damaged tissue (122). These fundamental mechanisms highlight the essential need for immunosuppression and the inhibition of T-cell mediated immunity for successful graft survival.

Risk factors shown to be predictive for acute rejection include the level of HLA mismatching between donor and recipient (123), viral infections (124), and pharmacogenetic variability including a multidrug-resistant (MDR1 C3435T) genotype (125) or variant Toll-like Receptor-4 (TLR4) (126).

Numerous studies have identified episodes of acute rejection as the strongest predictor of chronic allograft dysfunction (127).

1.3 Chronic Allograft dysfunction

1.3.1 Definition and Demographics

Chronic lung allograft dysfunction in the form of Bronchiolitis Obliterans Syndrome (BOS) remains a most challenging problem, limiting the long-term success of lung transplantation. This complex disorder affects 30% and 75% of patients by 2.5 and 10 years post-transplant respectively and is the leading cause of death in patients greater than one year after transplant (119). Based on
data collected from January 1994 through June 2007, survival rates were 89% at 3 months, 79% at 1 year, 64% at 3 years, 52% at 5 years, and 29% at 10 years, with lower survival rate noted for recipients over 50 years of age. Among patients surviving at least 1 year, original diagnoses of CF, IPAH, sarcoidosis, and \( \alpha \)-1 antitrypsin deficiency had significantly better survival at 10 years after transplantation compared to those with COPD and IPF.

Histologically, BOS is defined as a chronic inflammatory and fibrotic process affecting the small airways, resulting in partial or complete luminal occlusion (128). The fibro-obliteration is often associated with destruction of the smooth muscle of the airway walls, and frequently with fibrointimal thickening of the pulmonary arteries and veins (129). Functionally, BOS is defined by post-transplant pulmonary function using the forced expiratory volume in 1 second (FEV\(_1\)) as the primary parameter (130). To accurately define a loss of pulmonary function, for each lung transplant recipient a stable post-transplant baseline FEV\(_1\) is determined and defined as BOS Stage 0. More specifically, baseline is defined by the 2 highest (not necessarily consecutive) measurements obtained at least 3 weeks apart. From there, progressive stages of BOS, from 1 to 3 are defined in patients who experience a decrease in FEV\(_1\), according to the magnitude of the decrease. The current guidelines set out by the International Society of Heart and Lung Transplantation (ISHLT) also recommends the diagnostic use of mid-expiratory flow rate (FEF\(_{25-75}\)) measurements to define airflow obstruction, and although large intra-subject variability can occur, others have reported a high sensitivity for this test (131). The full diagnostic criteria are stated below.

Adapted from (130):

*Table 1.3 - Current Classifications of BOS*

<table>
<thead>
<tr>
<th>Classification</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOS 0</td>
<td>FEV(<em>1) &gt; 90% of baseline and FEF(</em>{25-75}) &gt; 75% of baseline</td>
</tr>
<tr>
<td>BOS 0-p</td>
<td>FEV(<em>1) 81-90% of baseline and/or FEF(</em>{25-75}) ≤ 75% of baseline</td>
</tr>
<tr>
<td>BOS 1</td>
<td>FEV(_1) 66-80% of baseline</td>
</tr>
<tr>
<td>BOS 2</td>
<td>FEV(_1) 66-80% of baseline</td>
</tr>
<tr>
<td>BOS 3</td>
<td>FEV(_1) 66-80% of baseline</td>
</tr>
</tbody>
</table>

Acute rejection has been reported as a probable risk factor for BOS, particularly when the acute rejection is long lasting, high grade, or repeated (132, 133). Lymphocytic bronchitis/bronchiolitis, as detected by transbronchial biopsy, is another probable risk factor for
BOS (134). Other factors such as medication non-compliance and cytomegalovirus (CMV) infection have been suggested as risk factors but conclusive evidence of this link is not currently available.

Pathologically, BOS is primarily targeted at the respiratory epithelium (135). Initially a lymphocytic infiltration of the airways is observed, accompanied by epithelial cell necrosis and denudation of the mucosa. A secondary cytokine-mediated inflammatory response then attracts neutrophils to sites of injury and further stimulates local fibroblast and myofibroblasts, resulting in the formation of intraluminal granulation tissue. This process results in a fibrous scarring and eventual obliteration of the airway lumen (130, 136). In some cases, this process is accompanied by vascular sclerosis of the pulmonary arteries and veins (137).

1.3.2 Immunological Considerations in the Development of BOS

The clinical manifestations of BOS are often considered to result from repeated alloimmune-mediated injury to the lung allograft, mediated by both humoral and cellular immunity. This statement is supported by evidence that increasing number of human leukocyte antigen (HLA) mismatches between donor and recipient is associated with increased risk of BOS (138). It has been shown that airway epithelial cell can express MHC class II molecules and that this expression is up-regulated during chronic allograft rejection (139). Furthermore, lysis of airway epithelial cells by bronchoalveolar lymphocytes isolated from lung transplant patients BAL was enhanced during episodes of acute rejection when compared with non-rejection periods (140). Antibodies targeted to airway epithelium have also been measured in patients with de novo BOS (141). Extrapolating from this observation, it was further found that development of these de novo anti-MHC class I antibodies is a predicting factor for the development of BOS and decreased survival after lung transplantation (142).

Also contributing to the rejection process, donor MHC class I and II molecules are recognized by recipient CD8+ and CD4+ T cells, respectively. It is thought that the chronic process of rejection may be predominantly mediated by CD4+ T cells activated through the indirect recognition of donor MHC molecules processed and presented by recipient antigen presenting cells (143). These alloreactive CD4+ T cells may also provide the cytokines required for the generation and expansion of alloreactive B-cells and CD8+ cytotoxic T cells (144, 145). Patients diagnosed with
BOS have been found to have an oligoclonal CD4\(^+\) T cell expansion not present in patients without BOS (146).

Autoimmunity is emerging as an important component of the immune response to the graft and the development of BOS. As a consequence of chronic tissue remodelling, it has been found that expression of Collagen Type V (Col-V) located within the lung interstitium is enhanced (147). Prospective monitoring of the immune recognition to Col-V and Col-II found that lung transplant recipients but not healthy controls were Col-V reactive (148). These responses were dependent on recognition of Col-V by CD4\(^+\) T cells and monocytes, and were mediated by IL-17, TNF-\(\alpha\), and IL-1\(\beta\). In this study, a strong immune response to Col-V was associated with substantially increased incidence and severity of BOS. Interleukin 17 is an important promoter of mucosal immunity and is associated with autoimmunity in human and animal models through action of Th-17 autoreactive helper T cells (149), which are in turn maintained by a cytokine milieu rich in IL-1\(\beta\), TGF-\(\beta\), IL-6, IL-23 and IL-21 (149). IL-17 is also a chemoattractant for neutrophils. Anti-Col-V autoreactivity may also play a role in primary graft dysfunction (PGD) as it has been shown that both cellular and humoral anti-Col-V responses are associated with higher incidence of PGD, further predisposing patients to BOS (150). Further supporting evidence comes from the observation that pre-transplant IPF patients have a higher incidence of Col-V autoreactivity than non IPF patients. Taken together, this evidence supports the use of strategies aimed at inducing tolerance to Col-V prior to transplantation.

1.3.3 Biomarkers and Surrogate Markers of BOS

The current diagnostic criteria for BOS are based on changes in secondary functional measurements resulting from the underlying pathologic processes. The identification of biomarkers that could accurately predict these changes at the tissue and cellular level prior to changes in spirometry would be very useful and could lead to earlier or more targeted treatment options.

Measurable markers in the peripheral blood would provide a fast, non-invasive test. Cellular components of the blood include immune effectors such as activated T cells, as discussed above. Additionally, regulatory T cell subsets may also provide insight into immune status of transplant recipients. T-regulatory cells are an important population in allograft rejection and have been demonstrated to have suppressive properties in alloimmunity and in promoting
unresponsiveness to organ transplants (151). It has been found that the CD4+CD25+ T-regulatory cell subset are lower in BOS patients when compared to stable lung transplant recipients greater than 3 years from transplant (152), and this decrease was independent of immunosuppressive regimen used (153). Humoral responses targeted against endothelial cells and the bronchiolar microvasculature may also be important in the rejection process. Anti-endothelial cell antibody deposition has been identified within the bronchial wall microvasculature in BOS lung biopsy sample, and positive anti-endothelial cell antibodies have also been detected (154). Protein markers isolated from the peripheral blood may also be useful. Elevated soluble CD30 levels, a marker of activated T-cells, has been correlated with the development of BOS (155). Serum levels of TARC (CCL17), the thymus and activation regulated chemokine, are also significantly lower in patients at 1 month post-transplant who go on to develop BOS (156). Interestingly, Clara Cell Secretory Protein (CCSP) protein levels are also lower in BOS patients following allogeneic stem-cell transplantation (157) and in lung transplant recipients (158), highlighting the important functions of this protein (discussed below, see section 1.5.2).

Given the advantage of direct access to the allograft, surrogate markers of rejection have also been identified in the easily obtained BAL. Initially, differential cell counts are a useful indicator of graft inflammation and BAL neutrophilia in the absence of infection is suggestive of BOS in patients beyond 6-months from transplant (159). Protein markers in BAL are also potential biomarkers. The chemokines MCP-1 (CCL2), RANTES (CCL5), and IL-8 (CXCL8) have all been reported as elevated in BALF of patients who developed BOS compared with stable controls (160). Additionally, levels of the important biological mediators TGF-β (161), VEGF (162), and IFN-γ (163) are also altered in BAL from BOS patients. Elevated serum levels of the chemokine MCP-1 are also associated with chronic rejection, a process likely mediated by mononuclear cell recruitment and CCR2 expression (164).

1.4 The Contribution of Bone Marrow to Lung Disease

1.4.1 Introduction of regenerative medicine

The National Institutes of Health define regenerative medicine as “the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage, or congenital defects” (165), but the techniques and approaches used to accomplish this task are neither simple or standardized. Advances in stem cell biology and technologies have
generated much interest in harnessing this knowledge to develop novel therapies and treatments for myriad diseases. These approaches are particularly attractive for diseases which currently have no successful treatment options, such as many chronic lung diseases. The promise of bone marrow-derived cellular therapies to treat these diseases is based on data suggesting that the bone marrow harbours a stem cell population capable of contributing to the adult tissue. This concept and its applicability to the lung is currently the topic of much research and debate.

1.4.2 Bone marrow cell contribution to the lung

There are hundreds of papers reporting that bone marrow-derived cells (BMCs) can adopt the morphology and protein expression patterns of epithelial cells in various organs, including the lung. The seminal report of bone marrow contributing to mature lung tissue was published in 2001 (166). In this study a single hematopoietic long-term repopulating cell was transplanted from a male donor to a lethally irradiated female recipient mouse. Assessment of epithelial engraftment by immunohistochemistry and Y-chromosome detection found a surprisingly high level, with up to 20% of the lung parenchyma being derived from the donor. This observation was quickly disputed by a study that reported no donor cell contribution to non-hematopoietic tissue following transplantation of a single GFP labelled HSC, although robust reconstitution was observed for the peripheral blood leukocyte lineages in this model (167). It is possible that these conflicting results may be due to variations in experimental methodology including differences in the animal models and controls used. To explore this question, mice receiving various fractions of bone marrow in combination with total body irradiation were analyzed for epithelial engraftment. In this study, it was determined that increasing doses of radiation were associated with increasing donor-derived cells with a pulmonary epithelial phenotype (168). This result highlights the underlying need for injury in exogenous cell recruitment and retention. The concept that a specific threshold of injury is required for retention has been proposed. A comparison of various myeloablative radiation regimens found that after low dose irradiation, little or no lung injury is apparent, and in parallel there were no marrow-derived pneumocytes observed despite high levels of hematopoietic chimerism. In contrast, with higher levels of irradiation and resulting lung tissue damage, marrow-derived type II pneumocytes were present reported (169). These data indicate a critical relationship between lung injury and the phenotypic change from bone marrow to lung epithelial cells.
Following from these initial reports, several groups challenged the observations and questioned the true level of engraftment. Contributing to the ongoing controversy, a transgenic mouse model was generated where the human SP-C promoter drives expression of ubiquitous LacZ or eGFP. When bone marrow from these animals was transplanted into wild-type mice, engraftment was not detectable by histology or flow cytometry, and all putative marrow-derived pneumocytes resulted from an overlapping fluorescent signal from an endogenous pro-SP-C⁺ type II pneumocyte and a donor-derived eGFP⁺ cell (170). In agreement with these data, the same animal model was used to examine the contribution of dead cells and autofluorescence to microscopic analysis of cell engraftment. This work concluded there was detectable reconstitution of lung alveolar epithelial cells by unfractionated bone marrow or purified hematopoietic stem cells after transplantation (171). Taken together, it appears that the ability of bone marrow cells to become functional lung tissue might be much lower than levels than previously suggested and opinions vary as to the clinical applicability of this phenomenon. To further address the clinical relevance of bone marrow-derived cell therapy to correct gene defective lung epithelium, a CFTR⁻/⁻ mouse was studied. When bone marrow-derived cells from wild-type animals were systemically administered to the deficient mice, only very low retention of CFTR⁺ cells was found up to 3 months after delivery, and retention did not dramatically increase with naphthalene injury (172).

In transplantation of wild-type whole bone marrow to CFTR⁻/⁻ mice no donor-derived airway epithelial cells expressing CCSP or CFTR were detected by FISH immunofluorescence in the airways of chimeric mouse lungs. Assessment of the functionality of these rare cells was addressed by measuring the restoration of CFTR-dependent chloride secretion in transplanted CFTR⁻/⁻ mice. The authors of this study report a modest restoration of CFTR function in the GI tract and nasal epithelium (173). These results highlight the need for further research, both technical and mechanistic, in order to realise the full therapeutic potential of bone marrow cells for gene correction.

Several models of lung injury have been used to further explore the ability of bone marrow to become lung tissue. Injury by LPS (174), detergent (175), elastase (176), radiation (177), and paracetamol (178) have all been used to show that bone marrow cells can be retained within the lungs and have the ability to adopt various markers of an epithelial lineage. A model of acute respiratory infection by *Pseudomonas aeruginosa*, a pathogen of specific importance in cystic fibrosis patients, also found engraftment of bone marrow-derived cells within the airway.
An increasing number of groups have addressed this phenomenon by studying human lung tissue recovered from lung and bone marrow transplant recipients. Micro-dissection of epithelial tissue from explanted lung allografts demonstrated integration of recipient-derived cells in the bronchial epithelium, in type II pneumocytes and in the seromucous glands, and an increased level of chimerism was seen in areas of chronic injury (181). Samples of peripheral lung from patients who had undergone sex-mismatched lung transplantation have been examined by Y chromosome in situ hybridization and donor-derived cells were found within the alveolar epithelium, as well as within the macrophage and endothelial lineage, suggesting extensive differentiation potential of recruited extrapulmonary cells (182). In the context of allogeneic hematopoietic stem cell transplantation, one study reported between 2-6% of Y-chromosome positive cells within the lung also expressed cytokeratins and surfactant proteins typical of an epithelial lineage (183). Conversely, another study found very low levels of male cells within female HSC transplant recipients, calculating the contribution to the type II pneumocyte lineage to be approximately 1.75% in one patient and absent in 3 other patients studied (184). Low study numbers and patient-to-patient variability likely explains some of this discrepancy. While this evidence does support the observation that bone marrow cells can be found within the tissue it does not directly address the function of these cells once found.

Circulating cells have also been suggested as progenitors of the lung epithelium. PCR-based techniques have been used to quantify the level of cytokeratin-5 (CK-5) within the peripheral blood leukocyte fraction, immediately following lung transplant and serially thereafter (185). A drastically decreased level of CK-5 mRNA was measured in lung transplant recipients compared to healthy controls. In addition, a correlation between CK-5 level and improved lung function was found, suggesting these measurements may serve as a surrogate biomarker for clinical outcome. Cells positive for intracellular pan-cytokeratin have also been identified in peripheral blood cells isolated from lung transplant recipients with stable lung function (186). Additionally, cells isolated from bronchial brushings performed on sex-mismatched lung recipients were found to be of recipient origin by PCR and FISH. These lung-retained cells were positive for both cytokeratin and
the Y-chromosome. These studies provide further evidence of stem or progenitor cells that have the ability to be recruited to and retained within the lung.

Cells modified in vitro and subsequently reintroduced to the recipient are an attractive source of lung precursors for cell therapy. Mesenchymal stem cells (MSCs) have been reported to have the capacity to be retained and contribute to healing with the lung. MSCs delivered intravenously to wild-type mice following bleomycin injury were shown to engraft within the lung parenchyma and adopt a phenotype comparable to a type I pneumocyte, but not that of type II pneumocytes (187). In addition, the plasticity of cultured bone marrow cells was questioned, as both cultured and fresh marrow aspirates were found to express type I pneumocyte markers, T1a and aquaporin-5. This observation was extended to studies of MSCs derived from bleomycin-resistant mice that were delivered via jugular vein injection to bleomycin-sensitive mice. Administered MSCs were found to make very little contribution to the total lung DNA in control-treated mice, while in bleomycin injured mice, donor cell engraftment increased 23-fold and was preferentially localized to sites of injury. Administration of MSCs following bleomycin injury was also found to significantly reduce inflammation and attenuate collagen deposition (188), suggesting this population has the ability to inhibit proliferation of mesenchymal cells and reduce matrix production/deposition, likely through secreted paracrine factors. Experiments altering the ability of the bone marrow to respond to lung injury have also been used to study their role in tissue repair. Myelosuppresion has been shown to increase susceptibility to bleomycin injury, whereas protection was associated with the mobilizing factors G-CSF and GM-CSF (189). Cells isolated from injured lung were also found to produce soluble factors capable of stimulating MSC proliferation and migration.

It is clear that the mechanisms by which bone marrow cells transition to non-hematopoietic lineage is not yet understood and that further studies are certainly required.

1.4.3 Controversies

The increasing amount of research suggesting bone marrow cells are capable of contributing to lung tissue has not yet managed to resolve the associated controversies. Many questions still exist regarding the true function of these bone marrow cells identified within the lung.
An ongoing semantic debate continues regarding the best terminology to describe the observed phenomenon. It is important as scientists to use words that accurately portray our observations. For example, a majority of the initial reports claimed engraftment of cells within epithelial tissue. At what point is engraftment defined? What functional requirements must be met to qualify for use of this term? A shift towards the term retention has been made; a word which may more accurately define the process observed. Underlying this debate is the need for rigorous experimental methodology that fully support the claims made and a requirement for more studies to directly address questions of exogenous cell contribution to adult tissue homeostasis or repair.

Contributing to this debate is the variability in experimental conditions used to reach a common conclusion. Variable animal models, techniques used to detect cells, and paradigms used to assess lineage have created conflicting data. Although many groups have described the localization of bone marrow-derived cells within the lung epithelium, the exact number, function, and phenotype reported varies. Two major controversies exist regarding identification of cells; cell fusion and inaccurate microscopy. Replying on microscopic techniques to define cell lineage and origin is associated with many potential problems. The high standard of methodological control must be met, including high quality images, appropriate positive and negative controls, confocal microscopy to rule out cell overlay, and the use of specific and correct antigenic markers (190). Even in the best controlled experiments, cell fusion has been proposed as an explanation for apparent cell plasticity (191). In sex-mismatched hematopoietic stem cell transplant recipients it was found that a vast majority of marrow-derived hepatocytes possessed both male and female sex-chromosomes (192). Observations such as these highlight the need for genetically tagged models to properly assess lineage.

It has also been proposed that cell plasticity may be altered by the microenvironment. To test this, cells isolated from radiation-injured lung was cultured together with marrow cells but separated by a 0.4μm cell-impermeable membrane (193). Co-cultured marrow cells subsequently analyzed for lung specific markers demonstrated a propensity to express epithelial markers. Analysis of the cultured media identified microvesicles containing pulmonary epithelial specific mRNA capable of entering bone marrow cells and ultimately directing cell fate. This work contributes further evidence to the increasingly complex mechanisms governing cell plasticity and
fate, and that secretion of paracrine factors capable of acting on endogenous lung cells may provide a valuable mechanism by which exogenous cells can contribute to tissue repair.

1.5 Clara Cell Secretory Protein and CCSP⁺ Epithelial Progenitors

1.5.1 Clara Cells

Clara cells were first described by the German anatomist Max Clara in 1937 based on his work with human and rabbit bronchioles (194). Since this time Clara cells have been shown to have an important function in airway homeostasis, protection, and repair. These cuboidal, non-ciliated cells are localized to the terminal (11 ± 3%) and respiratory bronchioles (22 ± 5%) in humans, with a substantial number of these cells proliferating (195), while in mice Clara cells constitute the majority of the distal airway epithelium (196).

Clara cells possess dense ovoid granules which function to house secretory products. Clara cells have been shown to produce components of airway surfactants, including glycosaminoglycans of the hypophase (197). Of note, mucus over-production is commonly observed in obstructive airway diseases including COPD and CF (198). Clara cells further function to protect the airway epithelium by metabolizing inhaled toxins through high levels of cytochrome P450 in their smooth endoplasmic reticulum (199) and expression of p450 mono-oxygenases (200). The expression of cytochrome P450 also conveys a susceptibility to the toxin naphthalene, a property that has been exploited in a murine model of lung injury for specific Clara cell ablation (201). The major secretory product of Clara cells is the aptly named Clara Cell Secretory Protein (CCSP), a 16-kDa homodimeric protein (202), which is most abundantly expressed protein in mammalian airways. This protein is also known as CC10, CC16, Uteroglobin, Blastokinin, and Secretoglobin 1A. While CCSP is mainly used as a marker of lung Clara cells, cells expressing CCSP or its homologues have also been reported within the kidneys (203) and uterus (204). Many functions associated with CCSP have been attributed to a hydrophobic pocket created by the homodimeric structure, which serves as a binding site for lipophilic ligands such as steroids and toxins, aiding in the clearance of potentially harmful substances (205).

Transgenic animals generated to be deficient in CCSP (CCSP⁺⁻) have been used to further investigate the physiologic function of this protein. While the overall configuration of Clara cells in the bronchioles of CCSP⁺⁻ mice is not significantly altered, a loss of secretory granules and a >95%
reduction in rough endoplasmic reticulum is found in CCSP\textsuperscript{−/−} mice (206). It was initially reported that CCSP\textsuperscript{−/−} mice possess no overt phenotypic consequences associated with CCSP deficiency maintained in the absence of environmental stress, and appear healthy and fertile (205).

Another CCSP deficient mouse line was generated (reported as Ug\textsuperscript{−/−}, and referred to as such hereafter), which has been characterized by severe renal disease associated with massive glomerular deposition of predominantly multimeric fibronectin (207). This appears to result from the loss of fibronectin-uteroglobin/CCSP binding that normally prevents fibronectin aggregation and tissue deposition. In contrast to the initial CCSP\textsuperscript{−/−} mice these animals have normal kidney function and no fibrosis or fibronectin accumulation (208). The Ug\textsuperscript{−/−} mice was also been shown to be highly susceptible to developing bleomycin-induced pulmonary fibrosis, potentially through the suppression of pro-inflammatory T-helper 2 cytokines and TGF-beta (209).

The differences in phenotype observed between knockout animal models may be due to differential gene targeting, as the Stripp CCSP\textsuperscript{−/−} mouse (205) possesses a neo insertion in exon1 leading to truncation of the protein coding sequence, while the Mukherjee Ug\textsuperscript{−/−} mouse (207) targets the CCSP gene segment in exon2. The differences in associated genetic material that is altered in either mouse may have relevant consequences in terms of epigenetic control of gene expression and regulation of downstream genes. Also, as CCSP is the founding member of the Secretoglobin (SCGB) superfamily (210), and is known as SCGB1A1, it is possible that the other known family members (SCGB3A1, SCGB 3A2) that remain intact may have compensatory effects that vary between the stains of mice, contributing to the differences in resulting knockout phenotype.

In a model of hyperoxia exposure, CCSP\textsuperscript{−/−} mice demonstrated increased lung edema and inflammatory cytokine expression, as well as reduced survival following lung injury when compared to wild-type mice (211). High density cDNA arrays have been further used to profile gene expression in CCSP\textsuperscript{−/−} mice. This work identified differential expression of immunoglobulin A (IgA), which localized to the peribronchial lymphoid tissue, suggesting alterations to peribronchial B-lymphocytes in CCSP\textsuperscript{−/−} mice and suggests an immunomodulatory role for CCSP (212).

In one study, CCSP\textsuperscript{−/−} mice demonstrated displayed increased sensitivity to ozone and hyperoxia injury, but no altered phenotype following lipopolysaccharide (LPS) endotoxin challenge, responding with nearly identical inflammatory and chemokine gene expression profiles compared
to control animals (213). In contrast, it was reported that CCSP−/− mice challenged with intratracheal *Pseudomonas aeruginosa* infection responded with enhanced pulmonary inflammation and an increase in the proinflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (214). Further proteomic analysis of CCSP−/− mice identified a shift in the isoelectric point of the immunomodulatory protein annexin A1 (ANXA1) to more acidic isoforms, while mRNA levels and protein localization were similar between CCSP−/− mice and wild-type (215). In the lung, ANXA1 is expressed specifically by ciliated cells of the conducting airway (216) and can act to antagonize neutrophil extravasation (217). This work suggests a complex cross-talk between the immune system and cells of the respiratory epithelium. A recent study further tested the hypothesis that Clara cells regulate the lung response to inflammatory stimuli. In this work, while overall gene expression was similar, CCSP−/− mice were found to have an enhanced LPS-elicited inflammatory response in terms of the macrophage response. Macrophages isolated from knockout animals showed increased TNF-α production and greater cell surface Toll-like receptor (TLR)-4 expression than wild-type animals (218). These studies support the hypothesis that that Clara cells can attenuate the inflammatory response through regulation of macrophage behaviour, and that a reduction in CCSP may directly contribute to lung inflammation.

### 1.5.2 Function of CCSP and Value as a Biomarker

Several reports have demonstrated alternations in CCSP levels in association with various forms of lung injury. These observations are linked to the anti-inflammatory or protective functions reported for CCSP and serve to propose CCSP as an important biomarker for lung injury. From a diagnostic perspective, many studies have aimed to quantify the lung epithelial integrity based serum assessment of CCSP. CCSP can also be measured in other biological fluids including BAL, sputum, and even human amniotic fluid.

In healthy individuals, baseline levels of serum CCSP have been reported to be between 10-15µg/L (219). Changes in CCSP concentration have been linked to many environmental factors including LPS, chlorine, nitrogen oxides, and particulate matter (219). It has been shown that exposure to ambient ozone (O3) during periods of exercise result in a significant increase in serum CCSP level (220). In addition, an increase in CCSP was measured in firefighters resulting from only 20 minutes of smoke exposure, an observation that was found to be more sensitive than conventional spirometric testing (221). Conversely, chronic cigarette smokers have been found to have a dose-dependent decrease in serum CCSP concentration, with an average of 15% decrease
for each 10-pack-years smoked (222). This decrease had been attributed to a parallel and progressive loss of bronchiolar Clara cells.

Acute lung injury is also associated with changes in CCSP concentration. An increase in BAL CCSP levels was found in patients following cardio-pulmonary bypass and in those who develop ARDS (223). It has been further shown that CCSP concentration is inversely related to oxygenation as measured by the PaO2/FIO2 ratio in patients with acute respiratory failure (224). In an experimental model of ventilator-induced lung injury, CCSP−/− mice suffered from increased lung injury, further illustrating the protective role of CCSP in lung injury (225).

Modulation in CCSP levels has also been associated with chronic lung diseases. A polymorphism in the 5′-untranslated region of exon 1 of the CCSP gene is associated with an increased risk of childhood asthma (226), and is also linked to a significant decrease in serum CCSP concentration (227). Patients with COPD have been reported to have lower CCSP levels in BAL and serum compared to healthy controls (228). In a complementary study, expression of CCSP in the bronchial epithelium of COPD patients was also significantly decreased, particularly within the small airways (229). A decrease in BALF CCSP has also been found in IPF patients, which was associated with a corresponding increase in the proinflammatory enzyme phospholipase A2 (230), which was previously been shown to be inhibited by CCSP/Uteroglobin (231). The observed decrease in CCSP protein levels may be due to loss of endogenous Clara cells or to a decrease in gene expression and protein production by these or other cells.

CCSP has also been studied in patients with bronchiolitis obliterans syndrome. In a prospective longitudinal study of patients following lung transplantation, 162 serum samples from 19 patients and 191 BAL samples from 22 patients were analyzed for CCSP. In both BAL and serum, CCSP levels were consistently lower in patients who developed BOS versus those who did not. In addition, the percentage of neutrophils negatively correlated with CCSP in BALF (158). Further work from the same group demonstrated that patients with BOS following stem-cell transplantation had significantly lower CCSP levels compared to non-BOS patients or those with GVHD (157). In contrast, a study aimed to independently replicate these results measured serum protein concentrations over 2 years and found no predictive value of these measurements in terms of BOS development. On average, a lower CCSP level was measured once BOS was diagnosed, but no differences was found for surfactant protein (SP)-D, an alternate marker of respiratory epithelial
integrity (232). A recent report has found a link between high-grade primary graft dysfunction (PGD) and plasma levels of CCSP. The median CCSP level 6-hours after transplant was significantly higher in patients with PGD than in patients without PGD (233). Taken together, this work supports the hypothesis that measurement of CCSP may serve as a surrogate marker of airway epithelial damage and altered alveolar permeability in BOS patients once established but may not have significant predictive power. These results further demonstrate the importance of cells expressing CCSP for lung health, which themselves may also be a useful predictor of lung damage or repair.

Changes in CCSP concentration may be caused by several biological mechanisms including decreased protein production, loss or damage to Clara cells, or impaired renal clearance. Leakage of protein from the lung into the circulation is tightly linked to epithelial permeability. In addition, CCSP concentration gradients may be an important driving force of trans-epithelial transport, which would be enhanced following lung injury due to low baseline levels of CCSP in serum (234). Unfortunately, changes in CCSP concentration lacks both sensitivity and specificity, with changes not directly associated with any one form or mechanism of injury, limiting its use as a biomarker at this time, despite high biologic relevance.

Several lines of evidence have attributed an immunoregulatory function to CCSP. The observed changes in protein concentration associated with lung injury and disease may be associated with changes in the immunosuppressive properties of CCSP. Mixed lymphocyte reaction experiments have been used to demonstrate the suppressive functions of CCSP, reporting a downregulation of the inflammatory cytokines IFN-γ, IL-1 and TNF-α by stimulated leukocytes following CCSP treatment (235). After acute adenovirus infection, lung inflammation and cellular infiltrates were increased in mice lacking CCSP (236). Augmented levels of IL-1 and IL-6 were also noted, as well as increased chemotactic factors MIP-1α and MCP-1, further highlighting the anti-inflammatory function of CCSP. These finding were subsequently recapitulated in a model of respiratory syncytial virus infection (237).

1.5.3 Clara Cells as Endogenous Progenitors of Lung Epithelium

A series of early studies initially proposed that the Clara cells are capable of self-renewal and function as the progenitor cell for itself and for ciliated cells of the airway, indicating multilineage differentiation. Rats exposed to nitrogen dioxide or ozone showed that at 1-hour following
injury, proliferating cells were exclusively non-ciliated, while by 4-days one-third of the proliferating cells were ciliated, suggesting that the non-ciliated cells could as act a progenitor (238). Subsequent pulse-chase experiments using $[^3]H$-thymidine further defined this mitotically active cell as the facultative Clara cell progenitor population (239). These proliferating cells, initially termed Type A cells, lack typical ultrastructural features, including smooth endoplasmic reticulum and secretory granules, which are characteristic of differentiated Clara cells. Upon restoration of normal physiological conditions, the mitotic capacity of Clara cells diminishes and they return to a quiescent state, essentially defining their reparative capacity as conditional.

Pulmonary neuroendocrine cells (PNECs) localized within neuroepithelial bodies (NEB) at airway branch points have also been identified as airway progenitors (240) and have also been implicated in the development of small cell lung carcinoma (241). These cells are essential in lung development, being the first cell type to form and differentiate within the airway epithelium (242). Neural peptides secreted by pulmonary neuroendocrine cells have also been shown to be epithelial mitogens (243). Following naphthalene treatment proliferating Clara cells and PNEC cell both localize to the NEB during airway repair in mice (244, 245). Cells dual-positive for CCSP and calcitonin gene-related peptide (CGRP), a marker of PNEC cells, were identified within the NEB, representing approximately 10% of the NEB-associated cells. This intermediate phenotype had previously been observed only in the developing lung, and suggests a progenitor cell function with multilineage differentiation capacity. In addition, a variant Clara cell (vClara cells) that is resistant to naphthalene was observed within the NEB. These cells express CCSP but lack Cytochrome P450 CYP2F2. Taken together, it is believed that the NEB microenvironment is critical for maintenance of a progenitor population following acute lung injury. A transgenic model of conditional Clara cell ablation that utilizes herpes simplex virus thymidine kinase expression controlled by ganciclovir delivery was used to further investigate the contribution of NEB-associated CCSP-expressing progenitor cells in airway repair (240). Following the selective loss of CCSP$^+$ cells, proliferation and hyperplasia of PNE cells was observed in the absence of detectable proliferation by any other airway epithelial cell populations, suggesting that Clara cells within the NEB are not essential for PNE cell hyperplasia. This model of Clara cell ablation was used to further investigate the progenitor cell function within NEBs. As with the naphthalene studies, it was found that while CGRP$^+$ PNECs proliferated after Clara cell depletion, they were unable to repopulate the depleted airways. These results support the hypothesis that vClara cells are critical for epithelial
maintenance, while PNECs are not sufficient for epithelial renewal but likely represent a critical component of the stem cell niche (246). An additional study utilized conditional Clara cell depletion by diphtheria toxin A expression to further investigate the necessity of Clara cells in tissue regeneration. Results from both acute and chronic Clara cell depletion provided additional evidence of the critical progenitor function of Clara cells. In this model, metaplasia of ciliated cells was found following Clara cell depletion and normal reepithelialisation was inhibited, leading to a peribronchiolar fibrosis (247). Proliferation of residual progenitor cells was observed at multiple sites along the airway and was not confined to the NEB region.

More recent studies have identified the bronchioalveolar duct junction (BADJ) as a second airway stem cell niche. The population of interest within the BADJ has been termed the bronchioalveolar stem cell (BASC), and it marked by dual expression of both surfactant protein-C (SP-C) and CCSP and lack of CGRP expression (248, 249). This relatively rare population is restricted to the BADJ and not found in the trachea, large airways, bronchiole or alveoli. BASCs have been shown to be resistant to both naphthalene epithelial-specific injury, as well as to induced bleomycin alveolar-specific injury, and proliferate during airway repair. In culture, FACS-sorted BASCs form colonies when grown on a feeder cell layer and also exhibit extensive self-renewal. BASCs have also been implicated in lung tumourigenesis, a process mediated by phosphatidylinositol 3-kinase (PI3K) activation of oncogenic K-ras, and subsequent expansion of the BASC pool (250). These studies further emphasize the importance of the niche in lung stem cell biology and highlight the increasing complexity required for tissue homeostasis and repair.

The requirement for lung stem or progenitor cells in the steady-state maintenance of airway epithelium has been disputed. Using a transgenic lineage tracing model, it has been reported that specific stem cell niches and the unique populations they harbour may function only in the context of injury and that single, randomly distributed cells are responsible for normal homeostasis (251). Conversely, following naphthalene injury, large patches of proliferating cells associated with the BADJ and NEB microenvironments were noted, suggesting robust clonal expansion of lung stem cells is only observed following severe injury. To directly test the role of CCSP+ cells in homeostasis and repair, lineage tracing experiments were utilized to study their contribution. In confirmation of previous studies, in all conditions studied, bronchiolar Clara cells were found to both self-renew and differentiate into ciliated cells (252). However, when the putative SP-C+CCSP+ BASC population was specifically analyzed, no contribution of this specialized
cell type was found in the context of lung growth, homeostasis, or repair following injury, and that CCSP+ cells did not significantly contribute to the alveolus.

A recent study addressed the question of human endogenous lung stem cells by examining surgical lung tissue specimens. The isolation of a distinct c-kit-positive stem cell population from normal adult human lungs was reported, which after clonal expansion, was capable of regenerating an injured mouse lung by creating complete respiratory units in vivo within 14 days, including conducting airways and vessels (253). This study contributes important information for lung regenerative medicine and bioengineering, and supports the concept of endogenous lung cells capable of multi-lineage differentiation and repair following injury.

Many cellular signals are likely required for stem cell maintenance and differentiation. SOX2 has been shown to be required for the differentiation of ciliated, Clara, and goblet cells lining the bronchioles of the lung (254). When SOX2 is specifically deleted in Clara cells bronchiolar cell proliferation drastically declines and a loss mature epithelial cell markers, including CCSP, Foxj1, α-tubulin, and MUC5A is noted. Wnt signalling through β-catenin is a key pathway in intestinal epithelial cell biology (255), and has been proposed as an important pathway in the more slowly renewing lung epithelium. β-catenin signalling is essential for embryonic lung development and it has been shown that constitutive β-catenin signalling within the developing lung endoderm can arrest epithelial maturation and lead to an expansion of the BASC pool in adult airways (256). This expansion was found to be due to attenuated differentiation rather than an altered proliferation capacity, suggesting that stabilization of β-catenin can lead to expansion of tissue stem cells. In contrast, Cre-mediated deletion of β-catenin had no impact on expression of Clara cell differentiation markers, mitotic index, or sensitivity to naphthalene (257). In addition, airway repair in β-catenin–null epithelium was not altered when compared to wild-type animals.

The ability of Clara cells to act as a progenitor population and the critical functions of its major product, CCSP, suggest a potential use in novel therapeutics aimed to stabilize or enhance these two elements in treatment of chronic disease or acute lung injury.

1.5.4 Identification of a Novel CCSP-expressing Bone Marrow Population in Mice

With abundant evidence suggesting that cells from the bone marrow can be recruited and retained in the lung, it is reasonable to question both the phenotype and lineage of the populations best able to contribute to lung regeneration. With the specific aim of enhancing
epithelial regeneration following injury, experiments were done using the naphthalene model of Clara cell ablation (258). Two days following injury, a dose of $1 \times 10^6$ plastic adherent bone marrow cells cultured for 7-days were trans-tracheally delivered to C57Bl/6 mice. Using the fluorescent label CMTMR, bone marrow-derived cells were localized within the distal airway and alveoli of injured mice, while no cells were found in uninjured lungs. This observation was noted as early as 1-day following cell delivery and persisted for at least 14-days. In comparison studies, cells delivered via intra-vascular jugular vein injections resulted in a significantly lower number of labelled cells found within the lungs. A greater specificity of cell localization following injury was also observed. While intra-vascular delivery resulted in cells found within the liver, spleen, and bone marrow, this was not observed following trans-tracheal delivery.

Labelled cells localized within the lung were found to be positive for expression of CCSP as early as one day following delivery, and these double-positive bone marrow-derived cells were noted as morphologically similar to native airway Clara cells. To enhance these conclusions, a cell lineage-specific transgenic mouse reporter system was next used. Bone marrow from transgenic mice expressing GFP under the regulatory control of the human K18 promoter (K18GFP) was used as the source of delivered cells. Analysis of genomic DNA by PCR found donor-derived K18GFP expression could be noted in naphthalene injured animals up to 120 days following cell delivery. In addition, K18GFP mRNA expression was found to increase within the lungs of experimental animals over the same time period. No cytokeratin expression was found in freshly isolated bone marrow cells.

The observation of early CCSP expression by delivered bone marrow-derived cells within the lungs lead to further questions regarding the original phenotype of this population. freshly isolated marrow was analyzed for CCSP$^+$ and a small percentage of surface-positive cells were found by flow cytometry (259). In addition, adherent bone marrow cells cultured for 7-days had a significantly increased CCSP$^+$ population. Intracellular cell staining for CCSP found an even greater percentage of positive cells. Given the novel nature of this finding, several measures were taken to ensure accuracy and reproducibility of the conclusions. The initial observation was confirmed with several CCSP specific antibodies. Western blotting of fresh and cultured bone marrow cells confirmed an 11-kD band, matching that of the CCSP protein. Real-time PCR experiments further confirmed expression of CCSP in fresh and cultured marrow, while no CCSP expression was detected in CCSP knock-out mice. FACS sorting was used to separate CCSP-expressing cells from
CCSP-negative cells, and CCSP mRNA expression was restricted to the CCSP\(^+\) population, confirming that antibody based separation could truly identify two unique bone marrow populations. It should be further noted that the sorted CCSP\(^-\) population did not give rise to CCSP\(^+\) cells once placed back into culture.

To address the species specificity of this observation, bone marrow aspirates from 5 human volunteers were analyzed for CCSP expression. Detectable levels were measured by flow cytometry and real-time PCR for all samples analyzed. This observation laid a critical foundation for the work presented in this thesis.

Phenotyping of the CCSP\(^+\) population in 7-day murine bone marrow cultures was accomplished by sorting the bulk culture and then re-plating the positive and negative populations for 4 additional days. Subsequent immunofluorescent staining identified that the majority of CCSP\(^+\) cells are positive for CD45 and CD34, few expressed CD31 or CD133, and the majority expressed mesenchymal markers CD73, CD90, and CD105. CCSP\(^+\) cells were negative for von Willebrand factor and collagen type 1. Freshly isolated bone marrow cells sorted by CCSP expression further confirmed a CD45\(^+\), CD90\(^+\), CD105\(^+\), Sca-1\(^+\), CD31\(^+\), CD73\(^{low}\), CD34\(^{low}\) phenotype. Epithelial ion channel (ENaC and CFTR) and tight junction protein (E-cadherin) mRNA was detected in CCSP\(^+\) bone marrow cells but not CCSP\(^-\) cells by real time PCR analysis. Patch clamping studies further determined that while the CFTR is likely not functional, ENaC is indeed functional in these cells.

To investigate the differentiation capacity of CCSP\(^+\) cells, 7-day cultured bone marrow cells were sorted for CCSP expression, re-cultured for 2 further weeks and then placed in air-liquid interface culture for 4 weeks. It was found that most cells lost CCSP expression but could give rise to cells positive for SP-C\(^+\), AQP5\(^+\), K14\(^+\), or acetylated \(\alpha\)-tubulin\(^+\). PCR analysis was used to confirm the expression of epithelial markers following air-liquid interface culture. These results demonstrate the multi-lineage potential of CCSP\(^+\) cells and suggest their function as a progenitor cell of the respiratory epithelium.

Many studies of cell therapy protocols have indicated that tissue injury is essential for therapeutic cell retention and subsequent regeneration. Using a strategy similar to the whole bone marrow delivery studies, CCSP\(^+\) cells were given via trans-tracheal administration 2 days following naphthalene injury. Retention within the injured lung was significantly greater for CCSP\(^+\) cells compared to CCSP negative. To assess the response of endogenous bone marrow cells to lung
injury, the percentage of CCSP+ cells was determined in the bone marrow and peripheral blood of mice following naphthalene treatment. A transient increase in both bone marrow and blood was found to peak at 2-days post injury, remained elevated for 4-days, and then returned to baseline by 20-days.

A bone marrow transplantation model was utilized to investigate the migration potential of CCSP+ bone marrow cells. Following lethal irradiation, whole wild-type bone marrow or CCSP+ cells mixed with bone marrow from CCSP-null mice was transplanted into CCSP-null recipients. After 30-days of reconstitution, half of the mice were treated with naphthalene and then sacrificed 4 or 30 days later. Bone marrow from mice who received CCSP+ cell containing transplants had a measureable level of CCSP+ cells after 30-days, further suggesting the progenitor capacity of this population. Furthermore, mice receiving CCSP+ cell containing bone marrow showed increased CCSP+ cells in the bone marrow and respiratory epithelium following naphthalene injury, compared to mice receiving CCSP-null bone marrow. The CCSP protein level was not detectable the of BAL of CCSP knock-out mice reconstituted with CCSP+ bone marrow, as determined by ELISA.

The mechanism of CCSP+ cell recruitment following lung injury was investigated. It was initially noted that approximately one quarter of CCSP+ cells co-express CXCR4. Furthermore, protein levels of the CXCR4 ligand SDF-1 was found to be increased in naphthalene injured lungs. In vitro migration assays determined that CCSP+ cells could migrate in response to SDF-1 stimulus, which was partially inhibited by anti-CXCR4 blocking antibody. Significant migration was not observed using CCSP- cells.

Further phenotypic characterization of the 7-day cultured bone marrow cells identified a subset of CCSP+ expressing cells that co-express the stem cell marker Sca-1. The Sca-1+ population constituted approximately 10% of the CCSP+ population, which corresponds to 2% of the total cultured cells. In fresh marrow, approximately 0.1% of the cells co-express CCSP and Sca-1. In vitro assays of the clonogenic potential of the CCSP+Sca-1+ subpopulation demonstrated an expansion of approximately 40% from a single cell. A smaller subset co-expressed CCSP with pluripotency markers including OCT4, Nanog, or SSEA1.

The functional properties of the CCSP+Sca-1+ subpopulation were further investigated using the naphthalene injury model in unpublished studies. It was found that the CCSP+Sca-1+ population increased in both the bone marrow and peripheral blood following lung injury, peaking
at 2-days post injury and sustaining this increase up to 20-days post injury. The CCSP\(^+\)Sca-1\(^+\) population was further used in cell therapy models, as previously described. The double positive population did not show a selective advantage for retention when compared to mixed bone marrow cell delivery. In contrast, when mice were challenged with a second dose of naphthalene 30-days after the first, an increase in donor-derived cells was found in the lungs of mice receiving CCSP\(^+\)Sca-1\(^+\) cells. This suggests that the CCSP\(^+\)Sca-1\(^+\) cells may be resistant to naphthalene and may proliferate once resident within the lungs, contributing to regeneration and repair.

1.5.5 CCSP\(^+\) Cells in a Transgenic Model of Cell Ablation

The specific contribution of CCSP\(^+\) cells to lung repair and regeneration was further investigated using a transgenic model to specifically eliminate all cells expressing the CCSP protein, regardless of function or location. Ablation of the CCSP\(^+\) cell population was accomplished using a transgenic mouse that expresses the Herpes simplex thymidine kinase suicide gene under the regulation of the mouse CCSP promoter (HSV-CCtk) (240). Chronic administration of ganciclovir is expected to result in selective ablation of all CCSP-expressing cells, including the bone marrow and peripheral blood CCSP\(^+\) progenitor cells, as well as lung Clara cells, naphthalene resistant variant Clara cells, and the CCSP\(^+\)SP-C\(^+\) BASC population. Previous studies have shown that this model results in a dramatic decline in both CCSP and CYP2F2 mRNA expression by 6-days of treatment. Mice further experience extensive lung injury, inflammation, matrix deposition, and rapid mortality (240, 260).

In unpublished work done in our lab, female HSV-CCtk mice were treated with ganciclovir for 10 days, followed by trans-tracheal administration of CCSP\(^+\) or CCSP\(^-\) bone marrow cells from wild-type donor mice. Male, donor-derived cells were quantified in the lungs of recipient mice by measurement of Sry genomic DNA by PCR. An 8.9-fold increase in Sry DNA in the group of mice administered with CCSP\(^+\) cells was found compared to mice administered CCSP\(^-\) cells. Using immunofluorescent analysis of lung tissue, an increased number of fluorescently labelled CCSP\(^+\) bone marrow-derived cells was found compared to the CCSP\(^-\) cell treatment group. These results suggest that CCSP\(^+\) cells are more efficiently retained in the airway epithelium following injury. Delivery of CCSP\(^+\) cells also prolonged survival of ganciclovir treatment HSV-CCtk mice compared to mice receiving CCSP\(^-\) cells. Furthermore, CCSP\(^+\) cells trans-tracheally delivered cells were found to express epithelial markers CFTR and pan-cytokeratin, the ion channel protein ENaC, the type 2 pneumocyte marker Sftpc, and the ciliated cell marker Foxj1. Upon histological analysis, an overall
preservation of host airway epithelium was noted in mice receiving CCSP\(^+\) cells. In addition, total BAL cell infiltrate was reduced in CCSP\(^+\) cell treated mice, suggesting an immunomodulatory function of these cells. When proliferative capacity of the delivered CCSP\(^+\) cells was investigated by immunofluorescent Ki67 staining, no significant difference in proliferative index was noted between CCSP\(^+\) and CCSP\(^-\) cells. This observation suggests that the beneficial effects of CCSP\(^+\) cell therapy may manifest via paracrine factors acting on the host epithelium.

In continuation of this work, bone marrow transplant models were utilized to study the effect direct contribution of cell originating from the bone marrow in lung injury. In experiments where either CCTk-derived ganciclovir sensitive bone marrow or FVBn wild-type bone marrow was transplanted to CCTk recipients mice, detection of CCSP in the lung following 60 days of engraftment and 15 days of ganciclovir was significantly increased in mice receiving wild-type marrow. Mice receiving wild-type marrow also experienced a preservation of airway epithelium cells following ganciclovir treatment. In parallel experiments, wild-type marrow recipients treated with the MIF inhibitor ISO-1 exhibited a survival advantage compared non-treated wild-type recipients and to CCTk marrow recipients. In the reverse experiments CCTk or wild-type bone marrow was transplanted to wild-type recipients, followed by ganciclovir treatment in combination with naphthalene injury. In this model, CCSP expression was increased in the peripheral blood 12-20 days post-injury in the wild-type marrow recipients, while no expression was measured for CCTk marrow recipients. An improvement in PaO2/FiO2 and a decrease in BAL cell count was also noted for wild-type marrow recipients. CCSP expression in the lung was higher in wild-type marrow recipients at 15-days following ganciclovir treatment, suggesting as early-phase response of bone marrow cells to lung regeneration, while CCSP expression levels in the lung were equal by day 20 between wild-type and CCTk marrow recipients, further suggesting a later-phase response of endogenous lung progenitor cells. Immunocytochemistry identified donor-derived Y-chromosome positive cells within the lung that were positive for both CCSP and cytokeratin. Taken together this work further supports the hypothesis that CCSP\(^+\) bone marrow-derived cells play an important role the response to lung injury and subsequent epithelial repair, and that inhibition or loss of this population is detrimental. Based on these concepts, we hypothesize that a chronic deficit in CCSP\(^+\) progenitor cells contributes to the pathology of human lung disease.
1.6 Circulating Fibrocytes

1.6.1 Identification and Phenotyping of Fibrocytes

The circulating fibrocyte population was first identified in 1994 using mouse models of tissue repair (261). Following subcutaneous surgical implantation of wound chambers into mice, a rapid influx of peripheral blood cells was observed; including neutrophils, monocytes, and lymphocytes. Within the infiltrate, an unexpected population of cells was observed that was adherent in culture and adopted a spindle-shaped morphology reminiscent of tissue fibroblasts. Initial analysis indicated this novel population was positive for collagen-1 and vimentin, and negative for non-specific esterases, excluding them from the monocyte lineage. Based on the assumption that these infiltrating cells must originate from a circulating origin, further examination of human peripheral blood was performed to characterize this fibroblast-like population. Total blood leukocytes separated by ficoll centrifugation and placed into serum-supplemented culture demonstrated a loss of non-proliferating monocytes and the appearance of expanding colonies of adherent colonies similar to those observed from the wound chamber infiltrates.

Phenotypic characterization of both the mouse and human fibrocyte population indicated positivity for mesenchymal markers vimentin, collagen-1, and collagen-3, hematopoietic markers CD34 and CD45, and adhesion molecules CD11b and CD18. Fibrocytes were negative for epithelial, endothelial, and smooth muscle specific markers (261).

To further test the hematopoietic origin of fibrocytes, sex-mismatched bone marrow chimeric mice were implanted with wound chambers and the cellular infiltrates analyzed. While the majority of donor-derived aspirated cells possessed a CD34+Collagen-1− phenotype typical of classical inflammatory cells, in these studies the CD34+Collagen-1− population did not originate from the Sry+ donor marrow. It was hypothesized that the aspirated CD34+Collagen-1+ cells may originate from a radioresistant marrow stromal population. Additional histological analysis of scar tissue isolated from wound chambers implanted for 7-56 days identified a CD34+ cell population with fibroblast morphology, and this population was found to increase with age of the scar tissue. Human cutaneous scar tissue isolated from cadavers possessed a similar CD34+ population (261).

These initial experiments provided the first evidence of a circulating fibroblast-like progenitor that participates in wound repair, and led to the hypothesis that these cells could contribute to both physiologic tissue homeostasis, as well as fibro-proliferative scar formation.
A number of subsequent studies aimed to elucidate the function of fibrocytes. A report from Chesney et al. investigated mRNA expression of collagen-1 and various inflammatory cytokines by fibrocytes purified from both murine wound chambers and from human peripheral blood (262). Collagen-1 expression was reported for CD34+ fibrocytes, but not CD14+ monocytes or CD90+ T cells. Fibrocytes were also found to express high levels of the cytokines IL-1β, IL-10, PDGF-A, TGF-β1, M-CSF, MIP-1α/β, MIP-2, and MCP-1. Treatment of purified, cultured human fibrocytes with IL-1β further induced protein expression of chemokines, hemopoietic growth factors, and the fibrogenic cytokine TNF-α, while overall the collagen-1 level decreased, suggesting IL-1β may modulate the transition between repair and fibrogenesis.

The same group also reported an antigen presenting function for fibrocytes and their expression of class II major histocompatibility complex molecules (HLA-DP, -DQ, and -DR), the co-stimulatory molecules CD80 and CD86, and the adhesion molecules CD11a, CD54, and CD58 (263). Murine fibrocytes have been shown to migrate to proximal lymph nodes following with HIV-proteins p24 or gp120 antigen pulsing, and porcine fibrocytes were further shown to stimulate CD4+ T-cells following IFNγ activation and induce proliferation of virus-specific CD8+ T-cells (264).

Fibrocytes have been further implicated in the process of angiogenesis through the secretion of pro-angiogenic factors MMP9, VEGF, bFGF, and PDGF. Fibrocytes were reported to induce endothelial cell migration and proliferation, and to promote neovascularization in the murine model of subcutaneous Matrigel™ implantation in vivo (265).

Human fibrocytes have been further demonstrated to serve as adipocyte progenitor cells under specific conditions. In culture with adipogenic media, cells transform to a rounder shape with intracellular lipid accumulation and up-regulation of mature adipocytes genes and proteins, including FABP4, leptin, and PPARγ (266). These results were supported by evidence from a model using subcutaneous injection of Matrigel™ embedded with human fibrocyte-derived adipocytes into SCID mice, and the consequent development of human adipose tissue formation and neovascularure.

Vascular remodeling in the context of chronic hypoxic pulmonary hypertension is also believed to involve fibrocytes. An observed perivascular accumulation of fibrocytes was observed in the neonatal rat and calf models of hypobaric hypoxia, when compared to control animals. The blood origin of these cells was confirmed by liposome-delivered in vivo immunofluorescent labeling
of leukocytes and subsequent monitoring for specific fluorescence and fibrocyte markers in tissues following hypoxic exposure (267).

1.6.2 Recruitment of Bone Marrow-Derived Fibrocytes

The initial observation of a bone-marrow derived mesenchymal progenitor cell quickly lead to hypotheses about the role of this cell in tissue fibrosis. Questions regarding the intrinsic stimulus and the mechanism of recruitment were also raised.

As previous discussed (section 1.1.4), Idiopathic Pulmonary Fibrosis (IPF) is a progressive interstitial lung disease involving inflammatory cells, cytokine release, and fibroblast activation leading to chronic remodelling and matrix deposition (268). The recruitment of circulating fibrocytes to injured lung and their maturation to tissue fibroblasts may contribute to collagen deposition and fibrotic foci development.

A chimeric mouse model with GFP-expressing bone marrow cells was used in conjunction with bleomycin-induced lung injury, a reversible model of lung fibrosis, and animals were monitored for the migration of GFP-positive cells into the lungs following injury (269). More than a quarter of the resulting GFP⁺ lung cells expressed collagen-1, a significant increase over saline treated mice, and represented more than 80% of all collagen-expressing cells in the lung. In this study, bone marrow-derived fibroblasts were negative for αSMA expression, in the presence and absence of TGF-β1, a critical mediator of would repair and fibroblast differentiation. Increased levels of CXCL12 (SDF-1α) and CCL21 (SLC), the ligands for CXCR4 and CCR7 respectively, as well as increased mRNA expression of the receptors, were also found in the bleomycin treated lungs. This evidence supports the hypothesis that fibrocytes can respond chemotactically and migrate to sites of injury. In parallel studies, GFP⁺ bone marrow cells were found in fibrotic lung and skin, with approximately one quarter of GFP-positive cells also expressing αSMA (270). This observation is supported by in vitro work that showed increased fibrocyte differentiation and expression of αSMA following TGF-β1 stimulation, as well as the expression of CCR3, CCR5, CCR7, and CXCR4 mRNA (271). Additional murine studies of skin wounding identified a α-SMA⁺ myofibroblasts population derived from sex-mismatched bone marrow donors (272).

Several chemokines and their respective receptors have been implicated in fibrocyte recruitment, and investigated as therapeutic targets for fibrotic disease. Impaired fibrocyte recruitment has been reported in CCR2⁻/⁻ mice, which was corrected following transplantation of
CCR+ marrow. *In vitro*, CCL2 (MCP-1) could recruit fibrocytes and stimulate collagen-1 production (273). Mice deficient for CCL12 (MCP-5), the analog to human CCL2, were further shown to be protected from FITC-induced lung injury (274). CCL3 and the counterpart receptor CCR5 can also participate in fibrocyte recruitment following lung injury. Pulmonary fibrocyte numbers and collagen accumulation after bleomycin administration was found to be reduced in CCL3−/− and CCR5−/− mice (275).

The CXCR4-SDF-1 axis has also been studied as a key mediator of fibrocyte mobilization and recruitment. Several groups have reported CXCR4 expression by fibrocytes (271, 276, 277), as well as increased SDF-1 (CXCL12) levels following lung injury (278, 279). Classically, this pathway is essential for migration, survival, and development of multiple cell types, including human hematopoietic CD34+ and stromal STRO-1+ stem cells (280). SDF-1 is constitutively expressed in the bone marrow, as well as other tissues, and functions to regulate the trafficking and localization of immature and maturing leukocytes, including bone marrow stem cells, neutrophils, T cells, and monocytic cells via CXCR4 interactions (281). Therapeutically, inhibition of CXCR4 by the compound AMD3100 is being investigated to optimize bone marrow cells mobilization into the peripheral blood for use in autologous bone marrow transplant (282). Fibrocyte regulation by modulation of chemokine ligand-receptor interactions has been proposed as a novel avenue for therapy. Initial observations found that CD45+Collagen-1+CXCR4+ fibrocytes accumulate in the lungs of bleomycin treated mice and that neutralizing anti-SDF-1 antibody could prevent this infiltration. Neutralizing antibody therapy further reduced collagen deposition and attenuated fibrosis (277). Subsequent studies using chimeric mice with GFP+ bone marrow found that following bleomycin injury nearly all GFP+ lung infiltrating fibrocytes were CXCR4+, whereas 46% expressed CCR2 and 9% expressed CCR7 (283). As CXCR4 expression is known to be regulated by HIF-1α, it was further found that CXCR4 is upregulated on fibrocytes in hypoxic conditions and hypoxic preconditioning increased the migratory capacity of fibrocytes to SDF-1. This increase was found to be mediated via the PI3-kinase pathways downstream of the PDGF receptor, and that Rapamycin treatment could inhibit bleomycin-induced pulmonary fibrocyte accumulation. Taken together, these data present a strong argument that regulation of the CXCR4/SDF-1 axis could provide a novel and effective therapeutic target for fibrotic diseases.

### 1.6.3 Evidence for Circulating Fibrocytes in Human Disease

On the foundation of many animal based studies, the role of circulating fibrocytes in human
disease has been evaluated. CD45<sup>+</sup>Collagen-1<sup>-</sup>, CD34<sup>+</sup>Collagen-1<sup>-</sup>, and CXCR4<sup>+</sup>Collagen-1<sup>-</sup> cells have been indentified within lung tissue sections of pulmonary fibrosis patients, and a positive correlation between the number of fibroblastic foci and lung fibrocytes was found, while no fibrocytes were found in normal lungs (276). Studies of tissue sections from obliterative bronchiolitis patients following lung or bone marrow transplant also identified fibrocyte infiltration and a relationship between pathological remodelling and fibrocyte numbers counted (284). The quantification of circulating fibrocytes in human interstitial lung disease found increased CXCR4<sup>+</sup> fibrocytes in UIP/NSIP patients, in addition to increased plasma SDF-1 levels (285). Circulating fibrocytes were further shown to be increased in IPF patients compared to healthy and ARDS patients, with a greater increase observed during acute disease exacerbations (286). Circulating fibrocyte numbers were determined to be an independent early predictor of mortality, with the mean survival of patients with a fibrocyte level higher than 5% of total blood leukocytes being 7.5 months compared to 27 months for patients with less than 5%.

Fibrocytes have been cited as the precursor of bronchial myofibroblasts in asthmatic patients (287). Human bronchial mucosa cells expressing CD34 and collagen-1 were increased following allergen exposure and a subgroup also expressed αSMA. In vitro, TGF-β1 also increased fibrocyte proliferation and αSMA expression, and is known to be up-regulated in airways of asthmatic patients. Furthermore, an outgrowth of collagen-1 positive fibroblasts was found in cells cultured from the BALF of asthmatic patients, with a subgroup also expressing αSMA (288).

Extending from the observations linking fibrocytes to pulmonary diseases, fibrocytes have been similarly implicated in other pathologies. Cultured CD34<sup>+</sup> bone marrow cells from human rheumatoid arthritis patients can differentiate into fibroblast-like type B synoviocytes following stimulation with stem cell factor and GM-CSF (289), and circulating fibrocytes were similarly found to be increased in the peripheral blood of RA patients (290). A link between fibrocytes and systemic fibrotic disease has been reported.

The inflammatory response observed in the skin of systemic scleroderma patients is marked by the infiltration of immune cells, likely leading to scar formation (291), although an early report found a lack of CD34<sup>+</sup> spindle-shaped cells in the skin of scleroderma patients when compared to other connective tissue disorders (292). The recently identified disease Nephrogenic Fibrosing Dermopathy (NFD), first described in 1997, has been described as a mimicker of systemic
scleroderma that occurs exclusively in patients with renal dysfunction (293). Biopsies from patients show accumulation of spindle-shaped cells expressing CD34, CD45 and Collagen-1 in the dermis, suggesting that these cells originate from circulating fibrocytes that are recruited to the skin (294, 295). This disease is also noted to progress to pulmonary fibrosis in a disproportionate number of cases (293).

1.7 Summative Statement

This review of current literature in the field of lung disease and transplantation, and the progenitor cells associated with these conditions serve to provide the reader with context and preliminary knowledge with which to interpret the experimental data presented in this thesis. As an organ constantly exposed to the external environment, several mechanisms must be in place within the lung to combat infection and damage from inhaled toxins, yet as is demonstrated by this review of several common chronic lung diseases, these mechanisms often fail. One must question what specific changes or deficiencies are present in patients affected by these lung diseases and how alterations in the intrinsic repair processes can lead to the development of disease. Although much literature exists describing immunologic and biochemical methods to maintain organ homeostasis, the role and function of both endogenous and exogenous stem/progenitor populations is still widely unknown. Although many hypotheses and isolated pieces of data suggest mechanisms, the exact processes by which the respiratory epithelium repairs itself remain controversial. In addition, knowledge of the biological changes associated with aberrant tissue repair and/or the development of fibrosis is also incomplete. While the biological phenomena of repair and fibrosis are both influenced by the local tissue microenvironment, it is also speculated that cells and effector molecules from outside the lung may also contribute. Changes in important exogenous cell populations and their products acting within the lung may enhance or inhibit important actions required for tissue homeostasis or repair. Further research is required to determine the critical alterations in the natural repair processes that contribute to disease pathogenesis. Many studies have been reviewed to support the aim of quantification of cell populations or their products to further the understanding of these biological changes in disease states. Identification of biomarkers to serve as indicators of disease status or progression, or to simply identify important pathways or populations that are altered in disease, is critical to the fundamental understanding of many lung diseases and to the development of novel therapies. The following work aims to build on current knowledge and contribute important
further information to better understand the contribution of bone marrow-derived cell populations in lung disease, repair, and allograft rejection and to generate further testable hypotheses regarding changes in progenitor cells within these contexts.
1.8 HYPOTHESES

Taken as a whole, this study is initially based on the hypothesis that the adult bone marrow contains tissue-specific progenitor cell populations capable of exiting the marrow, existing in the peripheral blood, and being recruited to injured lung. It was further hypothesized that both chronic lung disease and acute injury following lung transplant can influence the number of progenitor cells, namely CCSP$^+$ epithelial-like progenitors and CD45$^+$Collagen-1$^+$ fibrocytes. It was also hypothesized that protein mediators of cell chemotaxis are altered in response to lung disease or injury and that these changes are important in controlling the number of progenitor cells. Lastly, it was hypothesized that the number of progenitor cells measured would be predictive of important clinical measures of injury or disease including spirometric lung function measurements and oxygenation.

Detailed hypotheses are outlined at the beginning of each chapter.

1.9 STUDY RATIONALE

This study was designed to investigate a wide spectrum of clinical lung disease and injury to gain important information regarding bone marrow-derived cell populations in terms of numbers and response. A heterogeneous patient population was studied in a variety of clinical situations in order to generate an initial understanding of the corresponding progenitor cell profiles.

1.10 STUDY AIMS

This study aimed to quantify the number of CCSP$^+$ epithelial-like progenitor cells and CD45$^+$Collagen-1$^+$ fibrocytes in 3 main clinical situations:

1. End-stage lung disease
2. Lung transplantation
3. Chronic lung allograft dysfunction following transplantation

This study further aimed to determine key protein mediators of lung progenitor cell mobilization and/or trafficking in the 3 scenarios defined above.

Lastly, the study aimed to identify the relationships between progenitor cell number and clinically relevant outcomes to investigate the function of these measurements as a biomarker for disease status or progression.

Specific aims are outlined at the beginning of each chapter.
Chapter 2

Materials and Methods
2.1 Patient acquisition, Informed consent and Data storage

All patients included in the study were recruited from the Toronto Multi Organ Transplant Program between Nov 2007 and January 2011. Initial Research Ethics Board (REB) approval was obtained on November 22 2007 and subsequently renewed each following year. Three consent forms were utilized for Lung Transplant, Liver Transplant, and Post-Transplant Patients respectively ( Appendix 1-3).

End-stage lung disease patients were approached at the time of lung transplant listing by the relevant surgeon. Liver transplant patients were consented following living donor approval, approximately 1 week prior to transplantation. Post-transplant patients between 1-10 years following transplant were consented upon return for regularly scheduled clinic visits or annual updates. In all cases, informed consent was obtained by encouraging open dialogue regarding all expectations and potential risks involved in study participation, as well as clear explanation of expected benefits of participation. All components of the experiments described herein were designed and executed following the guidelines outlined in the Nuremberg Code of research ethics for experimentation with human subjects (296).

All patients and corresponding samples were given a unique alphanumeric code to remove all personal identifiers. A password secured file was maintained to store and link all personal data to the matching coded samples for all experimental analysis or subsequent biobanking.

2.2 Sample Processing

2.2.1 Bone Marrow

Bone marrow was collected from the exposed sternal bone at the time of lung transplant. A blunt end of a surgical scalpel was utilized to remove the sample from the bone and then transferred to Telfa absorbent cotton dressing. The dressing containing the bone marrow sample was then placed into a 50ml conical tube containing PBS (-Mg/-Ca) + 0.1% FBS+100 Units Heparin storage solution. The sample was kept at room temperature until processing. Samples were analyzed within 24 hrs of collection.

The Telfa pad was removed from storage solution and remaining cells were strained through a large gauge filter back into original solution. Total cellular matter was centrifuged at 400 xg for 5min at 4°C. Resulting pellet was resuspended in 2ml of analysis buffer containing PBS (-Mg/-Ca)
and 0.1% FBS. Sample was then layered over 1ml of Ficoll Pacque Plus (Stem Cell Technologies) and centrifuged at 400xg for 30 minutes at 25°C with the brake off. The resulting interface layer was removed by transfer pipette and collected in a 15ml conical tube (BD) and repelleted. Total cell isolate was counted using a haemocytometer and resuspended to a final concentration of 1x10^6 cells per ml of buffer.

2.2.2 Peripheral Blood

Peripheral blood samples (10ml) were collected into heparanized vaccutainers (BD) from the systemic circulation at the time of lung transplant, following anaesthetic but prior to reperfusion of the first lung. Samples were mixed and then stored on a rocking platform until processing. Samples were analyzed within 24 hrs of collection.

Half of the peripheral blood sample (4-5ml) was transferred to a 15ml Falcon tube and centrifuged at 3000 RPM for 10 minutes at 4°C with the brake off. Resulting plasma layer was removed and maintained at 4°C prior to ultimate storage at -80°C. Buffy coat white cell layer was then removed and transferred to 4ml of red cell lysis buffer and incubated for 15min. 10ml of analysis buffer was then added to wash the lysed cells and entire sample was centrifuged at 1500RPM for 5 minutes at 4°C. Total buffy coat cell isolate was counted using a haemocytometer and resuspended to a final concentration of 1x10^6 cells per ml of buffer.

The remaining peripheral blood sample (4-5ml) was diluted 1:1 with analysis buffer and layer over Ficoll Pacque Plus (Stem Cell Technologies) and centrifuged at 400xg for 30 minutes at 25°C with the brake off. The resulting interface layer was removed by transfer pipette and collected in a 15ml conical tube, then re-spun into a pellet. Total resulting peripheral blood mononuclear cell (PBMC) isolate was counted using a haemocytometer and resuspended to a final concentration of 1x10^6 cells per ml of buffer.

2.3 Flow cytometry

2.3.1 CCSP^+ Cells

Cells were analyzed at a concentration of 1x10^6 per tube. Samples were initially blocked for 30min with 10% goat serum at 4°C. Following washing (addition of 1ml analysis buffer and centrifugation at 1500RM for 5min), if applicable, samples were first incubated with surface antibody for receptors/lineage markers at manufacturer recommended concentrations. After two
washes samples were resuspended in 100ul of FAB containing 10% goat serum, 10% Fc Block (Miltenyi) and 0.5ul of rabbit-anti-mouse/human CCSP primary antibody (Upstate Laboratories) or Rabbit IgG (R&D Systems) isotype control antibody, and incubated for 30min at 4°C. Two subsequent washes were applied and then samples were resuspended in analysis buffer containing 0.5ul goat-anti-rabbit AlexaFluor 488 secondary antibody (Invitrogen) for 30min at 4°C. Two final washes were applied prior to ultimate sample storage in 4% paraformaldehyde solution. Samples were stored for up to 48 hrs at 4°C in the dark.

2.3.2 Fibrocytes

Buffy coat samples were analyzed at a concentration of 1x10^6 per tube. Following washing (addition of 1ml analysis buffer and centrifugation at 1500RM for 5min), samples were first incubated with surface antibody for CD45 (BD Pharmingen) at manufacturer recommended concentration (5ul). Following two washes, cells were incubated with 250ul of Cytoperm solution (BD) for 20 minutes at 4°C. All subsequent washes were performed using a 1X Cytoperm/Cytowash (P/W) solution. Following two washes samples were resuspended in 100ul of P/W solution containing 10% goat serum and 1ul of Rabbit-anti-Human Collagen Type 1 primary antibody (Rockland) or Rabbit IgG isotype control antibody (R&D Systems), and incubated for 30 min at 4°C. Two additional washes were applied prior to incubation with Goat-anti-Rabbit AlexaFluor 488 secondary antibody (Invitrogen) at a 1:1000 dilution for 30 min at 4°C. Two final washes were applied prior to ultimate sample storage in 4% paraformaldehyde solution. Samples were stored for up to 48 hrs at 4°C in the dark.

2.3.3 Data Analysis

All samples were analyzed using a Coulter Cytomics FC500 (2-laser, 5-color) analyzer. Data were analyzed using FlowJo software package for Windows.

CCSP⁺ cells were initially gated based on the forward scatter-vs-side scatter (FSC/SSC) plot to define the cell regions region. An initial gate was defined for FSC/SSC low cells. A negative staining gated was subsequently set on this population, as defined by the isotype control sample using a frequency-vs-FL1 dot plot with a 1% threshold for detection. Positive expression for CCSP was defined by subtracting the total positive cells with set gate minus the initial 1% threshold (described in Chapter 3, Figure 3.1.1). Double positive cell population were defined by first gating on surface receptor/lineage marker positivity followed by CCSP expression analysis.
Fibrocytes were first gated based on the forward scatter-vs-side scatter (FSC/SSC) plot to define the entire leukocyte region. Fibrocytes were then analysed in the same fashion as CCSP\(^+\) cells, setting the receptor/lineage marker gate first and subsequently analyzing Collagen-1 positivity on a frequency-vs-FL1 dot plot (described in Chapter 3, Figure 3.1.2).

2.4 Quantitative PCR

2.4.1 SYBR Green

Real-time quantitative PCR detection was performed using SYBR Green detection method (Applied Biosystems). In brief, total RNA was prepared from isolated human PBMCs or BMCs using the RNeasy Kit (Qiagen). For RNA, reverse transcription for first-strand cDNA was generated using Superscript II (Sigma) according to manufacturer’s protocol. Real-time PCR (SYBR green detection method; Applied Biosystems) was performed for amplification of the CCSP or Collagen-1 gene products. Positive control lung tissue was collected from explanted recipient lungs, subject to dispase digestion (Stem Cell Technologies) to isolate single cell suspensions, and RNA was isolated in parallel. Control RNA samples not subjected to reverse transcription were used to determine cDNA contamination. Specificity of the product was determined by analysis of the dissociation curve for control and experimental reactions. Intron-spanning primers were also designed to increase specificity of the product and exclude genomic DNA products.

2.5 Bio-Plex Plasma Protein Array (BioRad)

Plasma samples were collected from peripheral blood by high speed centrifugation as described in section 2.2.2. Plasma samples were stored at -80°C for a period of 2 days up to 3 years.

Prior to assay, 1 volume of plasma sample was diluted with 3 volumes of sample diluents. Coupled magnetic beads were not premixed and a master mix was prepared from the stocks provided, immediately prior to use. The Bio-Plex Pro II wash station with preloaded magnetic programs for the Bio-Plex Pro flat bottom filter plates was used. Assay was performed as described in product literature with no alternations and the assay was analyzed immediately following preparation. Bio-Plex Manager software was used for data acquisition.
The following targets were analyzed:

Table 2.1A: 17-plex Assay Targets

<table>
<thead>
<tr>
<th>Group 1 Cytokines</th>
<th>Group 2 Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>IL-3</td>
</tr>
<tr>
<td>IL-7</td>
<td>IL-16</td>
</tr>
<tr>
<td>IL-8</td>
<td>MIF</td>
</tr>
<tr>
<td>IL-10</td>
<td>MIG</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>SCF</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>SCGF-β</td>
</tr>
<tr>
<td>IP-10</td>
<td>SDF-1</td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1B: 14-plex assay Targets

<table>
<thead>
<tr>
<th>Group 1 Cytokines</th>
<th>Group 2 Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>HGF</td>
</tr>
<tr>
<td>bFGF</td>
<td>MIF</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>MIG</td>
</tr>
<tr>
<td>IP-10</td>
<td>SCF</td>
</tr>
<tr>
<td>MCP-1</td>
<td>SCGF-β</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>SDF-1</td>
</tr>
<tr>
<td>RANTES</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
</tr>
</tbody>
</table>

2.6 In vitro Cell Assays

2.6.1 Cell culture

All human bone marrow samples were cultured on standard tissue culture plastic and maintained in low glucose DMEM (prepared in-house) supplemented with 10% FBS. Cultures were passaged at 80% confluency using trypsin and re-plated between 1:2 and 1:4 dilutions.

2.6.2 Trans-well migration Assays

All BMCs and PBMCs used for migration assays were isolated using standard protocols described above (section 2.2). Initially $1 \times 10^6$ cells were layered on top of a 5μm pore membrane...
insert and placed in contact with standard DMEM media + 10% FBS + cytokine of interest in a 24-well tissue culture plate (Costar). Cytokines used included IP-10, RANTES, SDF-1, SCGF-β (100ng/ml, Peprotech). Cells were left to migrate for 2 hours and all cells recovered in the lower chamber were collected and counted for subsequent flow cytometric analysis for CCSP expression. The percentage of CCSP+ cells of the total migrated cells was determined and used to calculate absolute CCSP+ cells migrated, and correct for variable CCSP+ cell content in the initial cell mixture. The calculations applied were as follows:

(1) Total cells migrated x % CCSP+ = Absolute CCSP+ cells migrated

(2) Absolute CCSP+ in starting sample/ Absolute CCSP+ cells in migrated sample

= corrected CCSP+ migrated

2.7 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM) for repeated individual experiments or standard deviation (SD) for pooled results from a single experiment. For comparison of 2 groups a two-tailed students T-test was applied with a statistically significant p-value set at 0.05. For comparison of greater than 2 groups a 1-way ANOVA was used with a Dunnett’s multiple comparison correction post-test applied to a p-value of 0.05, which compares all groups versus a control group. Paired T-tests or repeated measures ANOVAs were used when appropriate. Correlational analysis of 2 variables was performed with correlation coefficient and p-values determined based on linear regression. Multiple correlation analyses were modified using a Bonferroni correction of the p-value and adjusting the required significance level using the formula α2 = α1/n, where α1 is the original p-value (0.05), α2 = the corrected p-value, and n= the number of comparisons made. This correction is used to increase the stringency of the null hypothesis and reduce the likelihood of a type-1 error, where the null hypothesis is rejected when no difference truly exists. Normality was tested using the D’agostino and Pearson omnibus test, which first computes the skewness and kurtosis to quantify how far from Gaussian the distribution is in terms of asymmetry and shape, and then calculates how far each of these values differs from the value expected with a Gaussian distribution. Further discussion of non-parametric analysis is discussed in section 6.1.2.
Chapter 3

Bone Marrow-Derived Progenitor Cells in End-Stage Lung Diseases
HYPOTHESIS

It was hypothesized that an altered progenitor cell profile would be found in end-stage lung disease patients. Specifically, it was hypothesized that a loss of CCSP\(^+\) epithelial-like progenitor cells would be measured in end-stage patients, while an increase in CD45\(^-\)Collagen-1\(^+\) fibrocytes would be associated with fibrotic lung disease. It was also hypothesized that these changes in progenitor cell number or phenotype may be specific to the underlying disease of origin, i.e. progenitor cell profiles may be associated with a specific disease pathology. Furthermore, these changes in progenitor cell numbers may be predictive of disease status as defined by lung function measurements. It was further hypothesized the populations of bone marrow-derived cells in question have the ability to migrate in response to chemokines produced by the chronically injured lung, and that the level of specific protein mediators in the plasma may be predictive of disease or of progenitor cell numbers.

AIMS

This study aimed to (1) quantify CCSP\(^+\) cells in the bone marrow and peripheral blood of end-stage lung disease patients, (2) quantify CD45\(^-\)Collagen-1\(^+\) fibrocytes in the peripheral blood of end-stage lung disease patients, (3) determine if progenitor cell profiles are predictive of underlying disease or lung function, (4) to elucidate important circulating protein mediators of progenitor cell recruitment.

RESULTS

3.1 Progenitor Cells Quantification by Flow Cytometry

Bone marrow cells (BMCs) were isolated from the exposed sternum of lung transplant recipients by scraping technique, then deposited onto an absorbent dressing and stored in a saline-based solution. Peripheral blood samples were also collected in a heparinized vaccutainer prior to reperfusion of the first lung. Bone marrow and peripheral blood mononuclear cells (PBMCs) were analyzed for cell surface CCSP expression by indirectly labelled antibody staining. Following flow cytometric analysis, the resulting data were first gated based on forward and side-scatter parameters to exclude debris and any contaminating populations, and to enrich for cells within the lymphocyte fraction (Figure 3.1.1A). Samples stained with isotype-matched control antibodies were used to set a threshold of 1% false-positive staining, and all reported percentages are based on this strategy (Figure 3.1.1B).
Fibrocytes were identified by cell-surface CD45$^+$ staining, followed by permeabilization and intracellular Collagen-1 staining. Following flow cytometric analysis, the resulting data were first gated to select for the peripheral blood leukocyte (PBL) fraction based on forward and side scatter plotting (Figure 3.2.1A). Subsequent gating first selected the CD45$^+$ population, defined as an increase above the 1% isotype threshold, and then ultimately selected the Collagen-1 dual-positive population (Figure 3.1.2B).
Figure 3.1.2 - Representative CD45<sup>+</sup>Collagen-1<sup>+</sup> Fibrocyte Flow Gating Strategy.

(A) Leukocyte gate on SS/FS

(B) CD45/Collagen-1<sup>+</sup> gating

**Figure 3.1.2** – Representative CD45<sup>+</sup>Collagen-1<sup>+</sup> Fibrocyte Flow Gating Strategy. (A) Initial cell gate to exclude cell debris, based on forward and side scatter plot. (B) Gating strategy for CD45 and Collagen-1<sup>+</sup> cell populations, based on IgG isotype staining set as 1%.

All data reported in this thesis were generated based on this analytic strategy and the highest level of consistency was maintained in order to reliably compare results between patients. Further experimental details can be found in the material and methods section (Chapters 2.2-2.3).

### 3.2 Identification of CCSP<sup>+</sup> and Collagen-1<sup>+</sup> Cells by PCR

Isolated bone marrow and peripheral blood cells were analyzed for population-specific mRNA expression by SYBR Green real-time quantitative PCR methodology. Amplification of CCSP mRNA in BMCs and PBMCS (Figure 3.2.1) and Collagen-1 mRNA expression in PBLs (Figure 3.2.2)
was observed for a representative number of patients. Expression levels were lower for both genes when compared to control cells isolated from lung tissue, but the dissociation curves of each product matched that of the control tissue. No amplification was observed in non-reverse transcribed control samples (No RT) (Figure 3.2.1B and 3.2.2B).

**Figure 3.2.1 - SYBR Green PCR Analysis of CCSP⁺ Cells**

(A)

![Amplification curve of human lung (positive control), bone marrow (BM) and peripheral blood cells (BC) analyzed for CCSP⁺ mRNA expression by SYBR Green PCR, including the corresponding dissociation curves.](image)

(B) No RT controls

![Aliquots of the same samples not subjected to reverse transcription (RT) used as a negative control, including the corresponding dissociation curves.](image)
Figure 3.2.2 – Representative SYBR Green PCR Analysis of Collagen-1⁺ Cells. (A) Amplification curve of human lung (positive control), bone marrow (BM) and peripheral blood cells (BC) analyzed for Collagen-1⁺ mRNA expression by SYBR Green PCR, including the corresponding dissociation curves. (B) Aliquots of the same samples not subjected to reverse transcription (RT) used as a negative control, including the corresponding dissociation curves.

3.3 Cell Phenotyping

Flow cytometric cell-surface analysis was used to determine the level of expression of lineage markers CD34 and CD45 on CCSP⁺ or Collagen-1⁺ cells (Figure 3.3A-C). Both CD34 and CD45 surface expression was measured for CCSP⁺ bone marrow and peripheral blood mononuclear cells, as well as on Collagen-1⁺ cells. Approximately half of the CCSP⁺ bone marrow cells were also positive for CD34 or CD45 (Fig 3.3.A). Nearly all peripheral blood cells expressed CD45, conferring this lineage to a majority of CCSP⁺ PBMCS, while only a small subset of peripheral blood cells co-
expressed CD34 and CCSP (Fig 3.3B). Additionally, a majority of peripheral blood leukocytes co-express CD45 and Collagen-1, as this is used as the basis for fibrocyte identification, and a smaller proportion co-express both CD34 and Collagen-1 (Fig 3.3C). Some variability in these proportions was noted between patients. A representative result is shown.

**Figure 3.3 - Surface Phenotyping of Progenitor Cell Populations**

(A) CD45 or CD34 dual-staining in conjunction with on CCSP on human bone marrow cells. (B) CD45 or CD34 dual-staining in conjunction with on CCSP on human peripheral blood mononuclear cells. (C) CD45 or CD34 dual-staining in conjunction with on Collagen-1 on human peripheral blood leukocytes.
3.4 Study Patient Demographics

A total of 156 lung transplant recipients were included in this study. The mean age was 50.9 ± 14.9 yrs and the mean body mass index (BMI) was 23.7 ± 4.6. A slightly higher number of males than females were included (57.7% vs. 42.3%), which did not reach statistical significance. Of the total samples patients, 27.6% of the patients were diagnosed with COPD/Emphysema, 33.3% with pulmonary fibrosis, 21.8 with CF, 9% with PH, 5.1% with BO(S), 1.3% with BAC, and 1.9% indicated as other, including sarcoidosis and pneumonia. A majority did not have diabetes (73.1%), while non-insulin dependent and insulin dependent diabetics accounted for 10.9% and 12.8% of patients respectively. Retransplant patients accounted for 5.1% of all included (Table 3.1A).

Samples were collected between November 2007 and January 2011 for analysis of progenitor cell populations. During this period, an additional 114 lung transplants were performed at the Toronto General Hospital where samples were not obtained during to logistical considerations and need for urgent flow cytometry, which was not always available. Comparing the demographics of the patients included in the study to the demographics of all patients transplanted in the same time period, age, gender distribution, indication for transplant, diabetes status, BMI, and graft number are all similar between the two groups (Table 3.1A).

A total of 36 lung donors were also sampled for bone marrow and peripheral blood. The characteristics of the sampled lung donors versus the total donor population for all recipients sampled and the total donor population for all transplants performed between November 2007 and January 2011 were compared. No statistically significant differences in terms of age, BMI, cause of death, or mechanism of death were found. Of note, fewer diabetic donors were included in the sampled set compared to the other 2 groups (Table 3.1B).
Table 3.1A – Lung Recipient Characteristics in All vs. Included Patients

<table>
<thead>
<tr>
<th></th>
<th>All Transplant Recipients (Nov 07 – Jan 11)</th>
<th>All Included Transplant Recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at transplantation (SD), yrs</td>
<td>51.8 ± 14.1</td>
<td>50.9 ± 14.9</td>
</tr>
<tr>
<td>Gender, total (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>157 (58.1)</td>
<td>90 (57.7)</td>
</tr>
<tr>
<td>Female</td>
<td>113 (41.9)</td>
<td>66 (42.3)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD / Emphysema + Alpha-1 Antitrypsin Deficiency</td>
<td>72 (26.7)</td>
<td>43 (27.6)</td>
</tr>
<tr>
<td>Pulmonary Fibrosis + Scleroderma</td>
<td>99 (36.7)</td>
<td>52 (33.3)</td>
</tr>
<tr>
<td>Cystic Fibrosis + Bronchiectasis</td>
<td>54 (20.0)</td>
<td>34 (21.8)</td>
</tr>
<tr>
<td>PPH + Eisenmenger’s + Congenital Abnormalities</td>
<td>19 (7.0)</td>
<td>14 (9.0)</td>
</tr>
<tr>
<td>Retransplant + BO</td>
<td>15 (5.6)</td>
<td>8 (5.1)</td>
</tr>
<tr>
<td>BAC</td>
<td>2 (0.0)</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>Other</td>
<td>9 (3.3)</td>
<td>3 (1.9)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Insulin Dependent</td>
<td>31 (11.5)</td>
<td>17 (10.9)</td>
</tr>
<tr>
<td>Insulin Dependent</td>
<td>35 (13.0)</td>
<td>20 (12.8)</td>
</tr>
<tr>
<td>No Diabetes</td>
<td>198 (73.3)</td>
<td>114 (73.1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (2.22)</td>
<td>5 (3.2)</td>
</tr>
<tr>
<td>BMI, mean (SD), kg/m²</td>
<td>23.8 ± 4.6</td>
<td>23.7 ± 4.6</td>
</tr>
<tr>
<td>Graft Number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>260 (96.3)</td>
<td>148 (94.9)</td>
</tr>
<tr>
<td>Second</td>
<td>10 (3.7)</td>
<td>8 (5.1)</td>
</tr>
<tr>
<td></td>
<td>All Donors (Nov 07 - Jan 11)</td>
<td>All Donors for Recipients Included</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td><strong>Gender, n (%)</strong></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>121 (44.8)</td>
<td>66 (42.3)</td>
</tr>
<tr>
<td>Female</td>
<td>147 (55.2)</td>
<td>88 (56.4)</td>
</tr>
<tr>
<td><strong>Cause of Death</strong></td>
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<td></td>
</tr>
<tr>
<td>Cerebrovascular/Stroke</td>
<td>148 (54.8)</td>
<td>82 (52.6)</td>
</tr>
<tr>
<td>Anoxia/Hypoxia</td>
<td>28 (10.4)</td>
<td>17 (10.9)</td>
</tr>
<tr>
<td>Head Trauma</td>
<td>61 (22.6)</td>
<td>37 (23.7)</td>
</tr>
<tr>
<td>Overdose</td>
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</tr>
<tr>
<td>Motor Vehicle Accident</td>
<td>3 (1.1)</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>Spontaneous Intracranial Hemorrhage</td>
<td>4 (1.5)</td>
<td>4 (2.6)</td>
</tr>
<tr>
<td>Primary CNS Tumor</td>
<td>1 (0.4)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Gunshot</td>
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<td>2 (1.3)</td>
</tr>
<tr>
<td>Cardiac Arrest/MI</td>
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</tr>
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<td>Unknown</td>
<td>2 (0.7)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Other</td>
<td>15 (5.6)</td>
<td>6 (3.8)</td>
</tr>
<tr>
<td><strong>Mechanism of Death</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracranial Hemorrhage/Stroke</td>
<td>150 (55.6)</td>
<td>87 (55.8)</td>
</tr>
<tr>
<td>MVA</td>
<td>31 (11.5)</td>
<td>18 (11.5)</td>
</tr>
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<tr>
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<td>5 (1.9)</td>
<td>1 (0.6)</td>
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<tr>
<td>Blunt Injury</td>
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<td>8 (5.1)</td>
</tr>
<tr>
<td>Seizure</td>
<td>13 (4.8)</td>
<td>8 (5.1)</td>
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<tr>
<td>Gunshot Wound</td>
<td>7 (2.6)</td>
<td>5 (3.2)</td>
</tr>
<tr>
<td>Natural Causes</td>
<td>1 (0.4)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Other</td>
<td>15 (5.6)</td>
<td>8 (5.1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>4 (11.1)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI, mean (SD), kg/m²</strong></td>
<td></td>
<td>28.0 ± 23.5</td>
</tr>
</tbody>
</table>
Of the 156 lung transplant recipients sampled for progenitor cell analysis and the additional 36 samples collected from lung donors, 183 were analyzed for CCSP\textsuperscript{+} PBMCs, 151 were analyzed for CCSP\textsuperscript{+} BMCs, and 92 were analyzed for CD45\textsuperscript{+}Collagen-1\textsuperscript{-} PBLs (Table 3.2). No differences in the distribution of analyzed samples based on indication for transplant was noted when compared to the total sampled patient dataset.

Table 3.2 – End-Stage Lung Disease Patient Samples Analyzed.

<table>
<thead>
<tr>
<th>Indication for Tx</th>
<th>BM samples Analyzed for CCSP\textsuperscript{+}</th>
<th>PBMC samples Analyzed for CCSP\textsuperscript{+}</th>
<th>PBL samples Analyzed for fibrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>CF</td>
<td>27</td>
<td>33</td>
<td>14</td>
</tr>
<tr>
<td>COPD/Emphysema</td>
<td>33</td>
<td>41</td>
<td>22</td>
</tr>
<tr>
<td>IPF</td>
<td>41</td>
<td>53</td>
<td>26</td>
</tr>
<tr>
<td>PH</td>
<td>11</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>BAC</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>SARCOIDOSIS</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Donors</td>
<td>31</td>
<td>32</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>151</td>
<td>183</td>
<td>92</td>
</tr>
</tbody>
</table>

3.5 Progenitor Cells Numbers in End-stage Lung Diseases

Quantification of the two progenitor cell populations identified variability among disease groups (Figure 3.4 A-C). On average, a larger percentage of CCSP\textsuperscript{+} cells were measured in the peripheral blood compared to the bone marrow. An increased percentage of CCSP\textsuperscript{+} cells was found in the BMC (mean = 2.21 ± 0.38%, p < 0.01) and PBMC (mean = 3.35 ± 0.67%, p < 0.001) of CF patients, compared to lung donors (BMC mean = 1.06 ± 0.17%, PBMC mean = 1.87 ± 0.22%). A decrease in CCSP\textsuperscript{+} PBMCs was also noted for BOS patients compared to donors (mean = 0.98 ± 0.30%, p=0.042). The percentage of peripheral blood fibrocytes was increased in patients with IPF (mean = 2.49 ± 0.52%, p < 0.05) and BOS (mean = 4.78 ± 1.17%, p < 0.001) compared with donors (mean = 0.87 ± 0.13%).

The ratio of CD45\textsuperscript{+}Collagen-1\textsuperscript{-} fibrocytes to peripheral blood CCSP\textsuperscript{+} cells was calculated to further highlight the relative changes in progenitor cell populations (Figure 3.4D).
Figure 3.4 - Progenitor Cell Measurements Separated by Disease.

(A) Percentage of bone marrow cells (BMCs) positive for CCSP in each disease group, compared to lung donors. (B) Percentage of peripheral blood mononuclear cells (PBMCs) positive for CCSP in each disease group, compared to lung donors. (C) Percentage of peripheral blood leukocytes (PBLs) positive for CD45 and Collagen-1 in each disease group, compared to lung donors. (D) Ratio of CCSP$^+$ PBMCs to CD45$^-$Collagen-1$^+$ fibrocytes in each disease group, compared to lung donors. 1-way ANOVA with Dunnett’s post-hoc test.

A significant relationship was identified between bone marrow and peripheral blood CCSP$^+$ cell percentage ($p < 0.0001$, $r^2 = 0.23$). No correlation was found for the number of CD45$^-$Collagen-1$^+$ fibrocytes and BMC or PBMC CCSP$^+$ cells (Figure 3.5)
Figure 3.5 - Relationships Between Progenitor Cell Populations.

(A) Correlation between the percentage of CCSP\(^+\) PBMC and CCSP\(^+\) BMCs. (B) Correlation between the percentage of CD45\(^-\)Collagen-1\(^+\) fibrocytes and CCSP\(^+\) BMC. (C) Correlation between the percentage of CD45\(^-\)Collagen-1\(^+\) fibrocytes and CCSP\(^+\) PBMC.

Separating and comparing progenitor populations based on gender did not identify any significant differences (Figure 3.6).

Figure 3.6 - Progenitor Cell Populations by Gender

(A) Percentage of CCSP\(^+\) bone marrow cells (BMCs) in male vs. female lung recipients. (B) Percentage of CCSP\(^+\) peripheral blood mononuclear cells (PBMCs) in male vs. female lung recipients. (C) Percentage of CD45\(^-\)Collagen-1\(^+\) fibrocytes in peripheral blood of males vs. female lung recipients.

In addition, no correlations were found when patients were separated by body mass index (BMI) (Figure 3.7).
Figure 3.7 - Progenitor Cell Populations by BMI

(A) Correlation between the percentage of CCSP\(^+\) bone marrow cells (BMCs) and body mass index (BMI). (B) Correlation between the percentage of CCSP\(^+\) peripheral blood mononuclear cells (PBMCs) and body mass index (BMI). (C) Correlation between the percentage of CD45\(^-\)Collagen-1\(^+\) fibrocytes and body mass index (BMI).

The relationship between progenitor cell numbers and age was next investigated. A significant relationship was found between increased age and fewer CCSP\(^+\) progenitor cells in both the bone marrow (p=0.021, \(r^2 = 0.037\)) and peripheral blood (p=0.015, \(r^2 = 0.034\)), but no relationship between age and circulating fibrocytes existed (Figure 3.8.1A-C). When CF patients are excluded from the analysis, this significant relationship no longer exists, while a relationship between increased age and fewer CD45\(^-\)Collagen-1\(^+\) fibrocytes emerges (p=0.029, \(r^2 = 0.067\)) (Figure 3.8.2 A-C).

Figure 3.8.1 - Progenitor Cell Populations by Age

(A) Correlation between the percentage of CCSP\(^+\) bone marrow cells (BMCs) and recipient age (yrs). (B) Correlation between the percentage of CCSP\(^+\) peripheral blood mononuclear cells (PBMCs) and age (yrs). (C) Correlation between the percentage of CD45\(^-\)Collagen-1\(^+\) fibrocytes and age (yrs).
To determine if the increase in progenitor cell populations noted in specific patient groups is a result of generalized inflammatory cell changes, the total blood leukocyte and lymphocytes were collected for each patient. No differences were found in total lymphocytes counts between diseases. The mean lymphocyte counts for each disease group fell within the normal reference of reference (1.5-3.4 bil/L) (Figure 3.9A). A decreased number of total leukocytes was noted in BOS, COPD, IPF (p < 0.001), and PH (p < 0.01) patients when compared to donors (Figure 3.9B).
Figure 3.9 - Reference Blood Cell Counts by Disease

(A) Total lymphocyte counts in recipients, compared by disease. (B) Total leukocyte counts in recipients separated by disease, compared to lung donors. Reference cells counts are presented as bil/L. Normal ranges are presented as determined by the Toronto General Hospital diagnostic testing laboratory. 1-way ANOVA with Dunnett’s post-hoc test.

Based on leukocyte counts and the percentage of CCSP⁺ PBMCs or CD45⁺Collagen-1⁺ PBLs, the absolute progenitor cell number was calculated for each population by dividing the total leukocyte counts (bil/L) by the percentage of progenitor cells measured and was then compared by disease groups. A significant increase in the number of CCSP⁺ PMBCs was also found for absolute cell values (mean = 48.99 ± 9.47, p < 0.01), while the decrease in CCSP⁺ PBMCs was no longer significant for BOS patients (Figure 3.10A) compared to donors (mean = 22.36 ± 3.47). The absolute number of CD45⁺Collagen-1⁺ PBLs was also significantly increased in BOS (mean = 71.2 ± 26.48, p < 0.001) and IPF (mean = 28.49 ± 7.56, p < 0.05) patients, when compared to absolute fibrocyte numbers in lung donors (mean = 9.25 ± 2.05) (Figure 3.10B).
Figure 3.10 - Absolute Progenitor Cell Numbers by Disease

(A) CCSP⁺ peripheral blood mononuclear cell (PBMCs) percentage normalized to reference total leukocyte counts. (B) CD45⁺Collagen-1⁺ fibrocyte percentage normalized to reference total leukocyte counts. 1-way ANOVA with Dunnett’s post-hoc test.

3.6 Progenitor Cell Numbers and Lung Function

In order to investigate the predictive value of progenitor cell quantification, lung function measurements were collected for each patient. Measures of the forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) were recorded as the percentage of the predicted value (% predicted). The ratio of the two absolute values was also used as a predictor of progenitor cell numbers. Taking the entire patient data set together, no correlations were found between CCSP⁺ BMCs (Figure 3.11A), CCCSP⁺ PBMCs (Figure 3.11B), or CD45⁺Collagen-1⁺ PBLs (Figure 3.11C) and lung function measurements.
Further subgroup analysis of lung function measurements and progenitor cell numbers grouped by underlying disease was performed. While some trends were identified, statistically significantly correlations were not found.

3.7 Chemokine Receptor Profiling

To investigate the hypothesis that bone marrow-derived progenitor populations can be recruited to damaged tissue via the peripheral circulation, the expression of key chemokine receptors was measured on a representative subset of patients. A subset of CCSP$^+$ BMCs and
PBMCs was found to express the CC receptors CCR2 (CD192) and CCR4 (CD194), as well CXC receptors CXCR3 (CD183) and CXCR4 (CD184) (Figure 3.12A-D).

**Figure 3.12 - CCSP* Cell Chemokine Receptor Expression**

(A) CCR2  (B) CCR4  (C) CXCR3  (D) CXCR4.

Peripheral blood fibrocytes have also been reported to express CXCR4 (277), an observation that was further confirmed in a subset of the lung transplant recipients included in this study. A majority of isolated leukocytes gated on Collagen-1 were found to be dual-positive for CXCR4 (Figure 3.13A-C).
Figure 3.13 – CXCR4 Expression by Peripheral Blood Fibrocytes. (A) Total peripheral blood leukocytes gated on CXCR4 expression based on initial isotype gating. (B) Isotype for Collagen-1 set at 1% of the CXCR4⁺ cells. (C) Percentage of CXCR4⁺ cells also positive for Collagen-1.

3.8 Plasma Protein Array Analysis

Quantification of protein mediators of cell trafficking in the plasma from lung transplant recipients and donors was performed to elucidate important factors. Targets were chosen from the total available assay product list and were selected based on biological activity and pre-existing evidence of these factors participating in chemotaxis and cell migration. A multiplex array was used to simultaneously determine the level of the 17 selected cytokines (Table 3.4). Comparing differences in protein levels between control individuals that include both lung donors and healthy volunteers (n=22) and patients with CF (n=17), COPD (n=13), or IPF (n=15) identified a number of interesting changes. An increased level of IP-10 (mean = 1657 ± 556pg/ml vs. 443.2 ± 64.7 pg/ml), MCP-1 (mean = 331.9 ± 86.98pg/ml vs. 134.9 ± 26.17 pg/ml), and MIG (mean = 9324 ± 4408pg/ml vs. 950 ± 176.1pg/ml) was measured in IPF patients compared to healthy controls (n=6 lung donors, n=4 liver donors, and n= 12 healthy volunteers) (Figure 3.14A-C). Additionally, the level of MIF was specifically increased in CF patients compared to controls (mean = 24473 ± 9880pg/ml vs. 6087 ± 2629pg/ml) (Figure 3.14D).
Table 3.3 – 17-Plex Plasma Cytokine Array Targets

<table>
<thead>
<tr>
<th>Group 1 Cytokines</th>
<th>Group 2 Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>IL-3</td>
</tr>
<tr>
<td>IL-7</td>
<td>IL-16</td>
</tr>
<tr>
<td>IL-8</td>
<td>MIF</td>
</tr>
<tr>
<td>IL-10</td>
<td>MIG</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>SCF</td>
</tr>
<tr>
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<td>SCGF-β</td>
</tr>
<tr>
<td>IP-10</td>
<td>SDF-1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>RANTES</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.14 - Plasma Cytokine Levels in End-Stage Lung Disease Patients vs. Healthy Controls.

Comparison of plasma cytokine concentrations between cystic fibrosis (CF) (n=19), chronic obstructive pulmonary disease (COPD) (n=16), and idiopathic pulmonary fibrosis patients (IPF) (n=17) compared to lung donor and healthy volunteer controls (n=10). Statistically significant differences were found for (A) IP-10, (B) MCP-1, (C) MIG, and (D) MIF levels. 1-way ANOVA with Dunnett’s post-hoc test.
Correlational analysis of plasma cytokines and progenitor cell populations identified some novel relationships. Taking all samples together, the level of Stem Cell Growth Factor-β (SCGF-β) was predictive of the number of CCSP⁺ BMCs (n = 44, p=0.034, $r^2 = 0.097$) and PBMCs (n=66, p=0.008, $r^2 = 0.11$) (Figure 3.15 A-B). Subgroup analysis by underlying disease did not identify any further relationships.

**Figure 3.15 - SCGF-β Plasma Concentrations Predict CCSP⁺ BMCs and PBMCs.**

Conversely, the concentration of Monocyte Chemotactic Protein-1 (MCP-1) was found to be predictive of the number of CD45⁺Collagen-1⁺ fibrocytes (n = 37, p=0.0052, $r^2 = 0.21$) (Figure 3.16).

**Figure 3.16 - MCP-1 Plasma Concentrations Predict CD45⁺Collagen-1⁺ Fibrocyte Numbers.**
As a panel of 17 cytokines was quantified in this experiment, statistical corrections must be made to address the issue of multiple comparisons. Of these targets, IL-1β concentrations consistently fell below the range of assay detection, and were therefore excluded from further analysis. A Bonferroni correction was applied to the significance level in the form of $\alpha_2 = \frac{\alpha_1}{n}$, where $\alpha_1$ = the originally set alpha (0.05), $\alpha_2$ = the newly defined alpha, and n=the number of comparisons (in this case, 16). From this equation, $\alpha_2 = 0.05/16 = 0.003$. When this correction is applied to the correlational analysis of progenitor cells and plasma proteins, the significance is lost for CCSP$^+$ BMCs and SCGF-β, but is maintained for CCSP$^+$ PBMCs and SCGF-β (Figure 3.15A-B). When the relationship between MCP-1 and CD45$^+$Collagen-1$^+$ fibrocytes is re-analyzed, the new p-value is calculated to be 0.083 (from $p = 0.0052*16$), which only reaches significance at the level of $p < 0.1$.

3.9 In Vitro Migration Studies

To determine the ability of CCSP$^+$ epithelial-like progenitor cells to migrate in response to cytokine stimuli, in vitro trans-well assays were used. Migration to the factors RANTES, IP-10, and SDF-1 was assessed. Comparing the migration potential of CCSP$^+$ PBMCs between healthy (H) volunteers and end-stage lung disease transplant recipients (R) found no significant differences between the two groups, although a trend toward increased migration was observed for R vs. H. Within group comparison by stimuli found that recipient PBMCs could migrate to a significantly greater degree in response to SDF-1 compared to no treatment (NT) ($p<0.05$) (Figure 3.18A). Migration of recipient BM was also compared by stimuli, and a significant response to SDF-1 was also found compared to no treatment ($p<0.05$) (Figure 3.18B).
Figure 3.17 – *In vitro* Migration of CCSP$^+$ BMCs and PBMCs

(A) Migration of isolated recipient bone marrow cells in response to chemotactic stimuli RANTES (n=7), IP-10 (n=7), SDF-1 (n=10), or SCGF-β (n=4), compared to untreated cells (n=12). (B) Comparison of isolated CCSP$^+$ peripheral blood mononuclear cells from control lung donors vs. lung recipients, in response to RANTES (n = 9D, n = 11R), IP-10 (n = 9D, n = 11R), SDF-1 (n = 9D, n = 13R), or SCGF-β (n = 4D/R), compared to untreated cells (n = 9D, n=14R). 1-way ANOVA with Dunnett’s post-hoc test.

3.10 Plasma CCSP Analysis

Additional patient plasma analysis was performed to assess the level of secreted CCSP protein. A commercially available ELISA was used to quantify CCSP in stored plasma from CF (n=8), COPD (n=9), and IPF (n=9) patients compared to Donors (n=6). A higher level of circulating CCSP was measured in IPF patients when compared to lung donors (p < 0.001) (Figure 3.18A). When comparing the relationship between CCSP plasma concentration and CCSP$^+$ BMC or PBMCs, no correlations were found (Figure 3.18B-C).
Figure 3.18 - CCSP Plasma Measurements by ELISA

(A) Plasma CCSP concentration in IPF (n=9), CF (n=9), COPD (n=8) patients compared to lung donors (n=6), as determined by Enzyme-Linked Immunosorbent Assay (ELISA). 1-way ANOVA with Dunnett’s post-hoc test. (B) Relationship between CCSP+ bone marrow cells (BMCs) and plasma CCSP concentration (n=25). (C) Relationship between CCSP+ peripheral blood mononuclear cells (PBMCs) and plasma CCSP concentration (n=37).
SUMMARY OF CHAPTER 3 RESULTS

The data presented in this chapter provide evidence of altered progenitor cell numbers in end-stage lung disease patients and to investigate mechanisms responsible for these changes. To this end, it can be stated that an increased percentage and absolute number of CCSP⁺ BMCs and PBMCs can be measured in patients with Cystic Fibrosis, while a decreased number of circulating CCSP⁺ cells is associated with Bronchiolitis Obliterans Syndrome. In addition, an increase in percentage and absolute number of CD45⁺Collagen-1⁺ fibrocytes is characteristic of pulmonary fibrosis and BOS patients. These changes were not found to be associated with age, gender, or BMI. In the complex set of end-stage lung disease patients studied, progenitor cell number was not predictive of lung function measurements. Investigation of chemokine receptor expression by CCSP⁺ cells identified CCR2, CCR4, CXCR3, and CXCR4 and their associated ligands as potential pathways involved in cell recruitment. Plasma protein quantification further identified alterations in inflammatory mediators between disease groups. Specifically, IP-10 and MCP-1 were increased in IPF patients, MIF was increased in CF patients, and MIG was increased in IPF, COPD, and CF patients. Comparison of plasma protein levels to cell numbers found a relationship between SCGF-β and CCSP⁺ BMCs and PBMCs, while CD45⁺Collagen-1⁺ cells were associated with MCP-1 plasma concentration. Interestingly, plasma levels of both SDF-1 and MCP-1 predicted better lung function measurements as indicated by % predicted FEV1 and the FEV1/FVC ratio. Functional investigation of CCSP⁺ cell migration identified a greater response to the chemotactic stimuli RANTES, IP-10, SDF-1, and SCGF-β in end-stage lung disease patients compared to healthy controls, observation that reached statistical significance for SDF-1. Additional plasma analysis for CCSP concentration found a significantly higher level of circulating CCSP in IPF patients, although no correlation between CCSP⁺ cell numbers and plasma CCSP concentration was found.
CHAPTER 3 DISCUSSION

The initial finding of a CCSP\(^+\) population within the mouse bone marrow and peripheral blood was subsequently reproduced in human samples. To further validate this observation, mRNA PCR analysis was also established for both mouse and human, supporting the hypothesis that a subset of bone marrow and peripheral blood cell possess the CCSP gene and express the mRNA encoding CCSP. The PCR data argue against the conclusion that secreted CCSP is simply non-specifically binding the surface of neighbouring cells, leading to a false identification of a truly CCSP positive population. Further protein expression validation was provided by Western blot experiments where CCSP protein was detected in isolated BMCs and PBMCs. The vast majority of cell measurements presented in this thesis were generated using antibody-based flow cytometric analysis. To initially validate these measurements, a number of optimization experiments were performed. Serial measurements of progenitor cell percentage were made on single blood samples stored over time, as well as serial blood samples taken over time from the sample person. For each of these conditions, the coefficient of variation (CV) was calculated based on the standard deviation and the mean of the measurement made. It was determined that the variation resulting from experimental differences and individual changes was small enough (5\%) to allow for comparison between patients and between samples processed at different times.

Standardized sample preparation and cell isolation techniques were also determined prior to the collection of study data, and these procedures were maintained throughout. This process included repeated analysis of a single sample over time and repeated analysis of multiple samples from a single individual, with the aim to determine reproducible staining procedures that produced consistent data with a low coefficient of variation between analyses. Fibrocyte staining procedures were based on previously published protocols. As the identification of a CCSP\(^+\) BMC and PBMC population was novel, it was important to define the most reliable staining methods for quantification. An initial Ficoll prep, blocking of Fc receptors, and lysis of contaminating red cells were all found to be important steps in isolating the relevant cell population and reducing non-specific staining. Following data acquisition, gating protocols were also predefined and consistently maintained for all data analysis.

Patient demographics were compared between those sampled for the study and all transplants performed within the same timeframe. This was done to ensure that no selection bias
had occurred. This comparison also suggests that our conclusions would not be different had another subset of patients been selected. No major differences were noted between the subset sampled and the total population in terms of age, gender distribution, BMI, indication for transplant, diabetes status, or graft number. Demographic comparisons were also made for lung donors. Again, there was no evidence that the sampled donor subset was significantly different from the total population. This fact supports the hypothesis that the sampled population described in by the observations presented in this thesis are representative of a total end-stage lung disease patient population.

Based on flow cytometric cell analysis and quantification, it was found that a greater percentage of CCSP$^+$ cells are present in the bone marrow and peripheral blood of Cystic Fibrosis patients. The mechanism responsible for this is not fully understood. It has previously been reported that bronchial epithelium from CF patients is more proliferative than in non-CF airways (297). In models of epithelial regeneration comparing humanized airway xenografts from CF and non- tissue, CF-derived cells portrayed a greater proliferative potential but were further characterized by remodelling, delayed differentiation, and altered pro-inflammatory and MMP responses (298). It is possible that CF patients experiences an impaired or sub-optimal epithelial repair response following tissue injury, resulting in prolonged and unresolved signals aimed to correct this situation. Sustained efforts to induce repair would lead to persistent inflammation without accomplishing the intended tissue regeneration, and could lead to accumulation of epithelial progenitor cells. Signals responsible for cellular recruitment are often redundant and may originate from several cells populations within the lung, including the damaged epithelial cells themselves. Conversely, BOS patients were found to have a decreased number of CCSP$^+$ epithelial-like progenitor cells in the peripheral blood. This may be due to alteration of recruitment signals or impaired trafficking ability in BOS patients. As many of these patients are taking immunosuppressive therapies including calcineurin inhibitors, anti-proliferatives, and corticosteroids, there is some possibility that these pharmaceuticals might inhibit progenitor cell recruitments. This suggestion may not fully explain the observed decrease, as many pulmonary fibrosis patients are also on some of these medications. A full comparison of progenitor cell profiles based on different pharmaceutical regimen would an important further direction for this work. This analysis could include comparison by dosing, length of time taking the medication, and effects of medication switching.
Circulating fibrocyte numbers were also altered in end-stage lung disease patients. As has been previously reported, an increase in peripheral blood CD45⁺Collagen-1⁺ cells was found in pulmonary fibrosis patients (286). Additionally, the novel observation of increase fibrocytes in BOS patients was also made. It is hypothesized that this increase is associated with and potentially contributes to the fibrotic pathology found in these patients. It is further hypothesized that these fibrocytes can localize within the lung and contribute to the activated fibroblast population, secreting matrix and perpetuating the development of fibroblastic foci. Again, a number of these patients may be taking immunosuppressive drugs, but this has been shown to be largely ineffective as therapy (52). It is possible that these drugs do not target the correct cell population, as it has been shown that inhibition of the SDF-1/CXCR4 axis is can attenuate experimental lung fibrosis (277). Taken together the ratio of CD45⁺Collagen-1⁺ fibrocytes to CCSP⁺ epithelial-like progenitor cells was shown to significantly distinguish fibrotic diseases (IPF, BOS) from other pathologies. This fact underlines the unique contributions of each cell type and presents a unique composite measurement with which lung disease patients can be profiled.

A significant relationship was found between CCSP⁺ cells measured in the bone marrow and in the peripheral blood. This observation may suggest that the intrinsic bone marrow pool of progenitor cells can determine the numbers that are released to the peripheral blood, although other mechanisms aside from total pool available likely contribute to peripheral blood mobilization. No relationship between CCSP⁺ BMCs or PBMCs was found with circulating fibrocytes. Based on the differences in cell ratios, one might expect an inverse relationship to be present. Lack of relationship may be due to variability between patients and within the data set. Furthermore, differences in progenitor cell numbers were not observed based on gender or BMI. Decreased age was found to predict higher CCSP⁺ cell numbers, although this correlation was lost when CF patients were exclude from the analysis, suggesting that age is likely a confounding variable and that the true relationship is between cell number and diagnosis. Interestingly, when CF data are excluded, a significant correlation between fibrocytes and decreased age is observed. This may be an important observation that is more specific to the target patients most affected by fibrocyte numbers (BOS, IPF). Alternatively, this may result from BOS patients having higher fibrocytes as well as generally being lower in age at re-transplant, again confounding the data.

It was suggested that changes in progenitor cell numbers may results from global changes in inflammatory cell state by disease. The total leukocyte and lymphocyte count was compared by
disease and no differences were noted for lymphocytes, while leukocytes were decreased in all patients groups excluding CF when compared to lung donors. This comparison may not be ideal, as lung donors suffer from many effects of injury or brain death which might increase their white cell counts. Of note, all groups excluding CF and donors cell within the normal range for leukocyte counts. Taking these data and determining absolute progenitor cell number (positive cells counts (%) x total leukocyte counts) confirmed the conclusions reached with percentage data, although the decrease in BOS CCSP⁺ PBMCs was no longer significant. These data suggests that the changes found in progenitor cell numbers between diseases are not simply a result of underlying changes in total inflammatory cell numbers.

When comparing progenitor cell numbers with lung function measurement (FEV₁, FVC, or FEV₁/FVC ratio) no significant relationships were observed. Subgroup analysis comparing these values within each disease group did not reveal any further correlations. The aim of this analysis was to determine if progenitor cell profiles could predict disease status. The data presented cannot determine if progenitor cell numbers can predict disease progression, and a longitudinal study following patients forward in time would be required to address this important question. It is possible that these spirometry variables are not the ideal markers of disease. There is much literature suggesting ideal measures and scores for each disease group (see Introduction). With a retrospective study design, much of the data required for composite scores are incomplete or not available. To properly define the ability of progenitor cell measurements to predict outcomes, a prospective study would be required where all pre-determined endpoints are consistently collected. Radiographic scores, 6-minute walk tests (distance and desaturation), diffusing capacity of carbon monoxide (DLCO) or dyspnea scores may all provide greater insight toward answering this question. Patients have also been largely grouped by disease type, but this does not account for variability within these diseases, which may reduce the specificity of even disease-specific outcome markers. Additionally, survival and mortality data are often the most robustly predictive endpoints, and the design of this study does not provide this type of data.

To address the potential mechanisms involved in progenitor cell recruitment from the bone marrow to the injured lung, several studies were performed to determine protein mediators potentially contributing to this process. Chemokine receptor analysis determined that CCSP⁺ cells express CCR2, CCR4, CXCR3, and CXCR4. CCR2 binds the ligand Monocyte Chemoattractant Protein-1 (MCP-1), suggesting that CCSP⁺ cells may share a common lineage with monocytes and
may be recruited in parallel. CCR4 interacts with several ligands including MCP-1, Macrophage Inflammatory Protein (MIP-1), RANTES, and TARC. Expression of this receptor by CCSP⁺ cells further suggests their potential to be recruited in states of inflammation. The CXCR3 receptor binds to the chemokines Monokine Induced by Gamma Interferon (MIG), Interferon Gamma-Induced Protein 10 kDa (IP-10), and Interferon-inducible T-cell Alpha Chemoattractant (I-TAC), all of which are important in the leukocyte response to pathogens and subsequent immune response. As mentioned previously, CXCR4 is important in stem cell recruitment, acting by binding the ligand Stromal-Derived Factor-1 (SDF-1), as well as binding the Macrophage Migration Inhibitory Factor (MIF) (299, 300). The largest subgroup of CCSP⁺ cells was those which co-expressed CXCR4. CXCR4 expression was also confirmed for circulating fibrocytes, as has been previously published (277), suggesting a large range of function for the ligand, SDF-1. It is possible that each double-positive cell subset is induced and recruited in different states. It is also possible that subsets exist that express multiple chemokine receptors, capable of responding to various stimuli, which could be tested with multiple flow-cytometric staining. The expression of a particular marker does not however confirm functional ability for a cell to bind or respond to a particular ligand.

A panel of 17 cytokines were quantified in the plasma from end-stage lung disease patients and lung donors. The targets selected were chosen based on previous evidence and literature indicating chemotactic ability and relevance to cell recruitment with the aim to test the hypothesis that these factors are involved in progenitor cell response to lung injury. Hypothesis generation was also intended, stemming from these observations, further functional experiments could be designed to directly test the response to any identified targets.

Comparing plasma protein concentration level between end-stage lung disease group, an increase in IP-10 and MCP-1 was found in IPF patients, suggesting that CCR2 and CCR4 positive CCSP⁺ cell may be specifically stimulated in this group. Fibrocytes also express CCR2 (273), suggesting this ligand may be responsible for the increased percentage observed in IPF patients. CCR4 is not expressed by fibrocytes (271, 301). The level of MIG was significantly higher in all end-stage patients compared to lung donors, implicating CXCR3⁺ cells and the immune response across pathologies. Alternatively, MIF was specifically increased in CF patients, suggesting that this pathway may be responsible for the increased CCSP⁺ cell numbers found in this group and further implicating CXCR4 expressing cells.
Investigating the direct relationship between progenitor cells and plasma cytokine concentrations identified a correlation between Stem cell growth factor-beta (SCGF-β) and both CCSP⁺ BMCs and PBMCs. Although this protein was not found to be increased in end-stage patients this relationship may imply a requirement for this factor by CCSP⁺ cells. SCGF-β is a reported growth factor for primitive hematopoietic progenitor cells (289). Serum concentrations of SCGF have further been used as an indicator of hematopoietic recovery following stem cell transplantation (302). It has also been reported that SCGF together with VEGF allows differentiation of endothelial cells from human progenitor cells (303). Taken together, this may suggest that SCGF-β can act as a growth or maintenance factor for CCSP⁺ cells. Conversely, MCP concentration was found to predict circulating fibrocyte number, further implying a role for CCR2⁺CD45⁺Collagen-1⁺ cells and the relationship between fibrocytes and monocytes.

In vitro migration studies were performed to investigate the ability of CCSP⁺ cell to directly respond to protein stimuli. A significant migration was noted for CCSP⁺ BMCs and PBMCs isolated from lung transplant recipients in response to SDF-1. It was also observed that cells isolated from end-stage lung disease patients had a great migratory response to all stimuli compared to cells from healthy individuals.

Final plasma analysis aimed to quantify the level of CCSP itself in end-stage patients. An increased level was measured in IPF patients by ELISA, which confirms previous results. CCSP has been used in a number of studies to quantify lung injury (see Introduction), but the aim of this analysis was to determine relationships between CCSP and CCSP⁺ cells. No correlations were found between plasma concentration and cell numbers. This may provide some evidence that leukocytes are not simply trapping CCSP on the cell surface, and that measured differences are not simply due to the variability in that mechanism, although this cannot be ruled out completely. This observation also suggests that CCSP⁺ cells are not actively secreting CCSP in the circulation.

From these data, the response of bone marrow-derived cells was next questioned in the context of acute lung damage following donor lung harvesting, preservation, and transplantation.
Chapter 4

The Effect of Lung Transplantation on Progenitor Cell Populations
HYPOTHESIS

It was hypothesised that CCSP⁺ or CD45⁺Collagen-1⁺ peripheral blood cells may respond to acute lung injury, such as seen following lung transplant. Specifically, based on animal models, it was hypothesized that the number of CCSP⁺ epithelial-like progenitor cells will increase within 48 hrs following injury. It was further hypothesized that changes in progenitor cell numbers may predict clinical outcome following transplant. Finally, it was believed that these changes are mediated by chemokine-based recruitment of cells and that important changes in plasma protein levels may predict cell number or outcome.

AIMS

These studies were designed to (1) quantify CCSP⁺ epithelial-like progenitors cells and CD45⁺Collagen-1⁺ fibrocytes at 24 and 48 hrs post-lung transplant, (2) determine if progenitor cell response is predictive of outcomes following transplant, (3) to elucidate important circulating protein mediators of progenitor cell recruitment in acute lung injury.

RESULTS

4.1 Patient Demographics

A subset of the end-stage lung disease patients undergoing lung transplant (described in Chapter 3) were followed post-transplant. A total of 48 patients were included post-transplant. Of these, all were analyzed for CCSP⁺ PBMCS at 24 hrs, and 31 of these were also analyzed at 48 hrs. In addition, 18 of these patients were analyzed for CD45⁺Collagen-1⁺ fibrocytes at 24 hrs and 15 of these were re-analyzed at 48 hrs (Table 4.1). The indications for transplant within this data set were representative of the total end-stage lung disease patient data set (Chapter 3), and included BO (n=3), CF (n=14), COPD (n=10), PF (n=16) and PH (n=4) and other (n=1).

Samples were collected between July 2008 and January 2011. In this time, 123 end-stage lung disease patients were sampled at the time of transplant (Chapter 3). Comparing the demographics of the patients included in the post-transplant subset to the demographics of all patients sampled in the same time period, no significant differences were noted in terms of age, gender distribution, indication for transplant, diabetes status, BMI, or graft number (Table 4.1A). Comparing lung donor characteristics further highlighted the representative nature of the subset and no differences were found between groups (Table 4.1B). Finally, factors specific to the
transplant operation were also compared between the total study population and the post-subset, including transplant type, ischemic time, CMV mismatch, and length of hospital or ICU stay. Within these parameters no differences were found between groups (Table 4.1C).

### Table 4.1A – Lung Recipient Characteristics in All Included vs. Post-Transplant Followed Patients

<table>
<thead>
<tr>
<th></th>
<th>Included End-Stage Patients</th>
<th>Subset of Included Post-Transplant Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 156</td>
<td>n = 48</td>
</tr>
<tr>
<td><strong>Recipient</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at transplantation</td>
<td>50.9 ± 14.9</td>
<td>49.8 ± 16.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>89 (57.1)</td>
<td>30 (62.5)</td>
</tr>
<tr>
<td>Female</td>
<td>65 (41.7)</td>
<td>18 (37.5)</td>
</tr>
<tr>
<td><strong>Diagnosis, Total (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD / Emphysema +</td>
<td>43 (27.6)</td>
<td>10 (20.8)</td>
</tr>
<tr>
<td>Alpha-1 Antitrypsin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scleroderma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystic Fibrosis +</td>
<td>34 (21.8)</td>
<td>14 (29.2)</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPH + Eisenmenger’s +</td>
<td>14 (9.0)</td>
<td>4 (6.5)</td>
</tr>
<tr>
<td>Congenital Abnormalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retransplant + BO</td>
<td>8 (5.1)</td>
<td>3 (6.3)</td>
</tr>
<tr>
<td>BAC</td>
<td>2 (1.3)</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>3 (1.9)</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Insulin Dependent</td>
<td>17 (10.9)</td>
<td>7 (14.6)</td>
</tr>
<tr>
<td>Insulin Dependent</td>
<td>20 (12.8)</td>
<td>11 (22.9)</td>
</tr>
<tr>
<td>No Diabetes</td>
<td>114 (73.1)</td>
<td>30 (62.5)</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI, mean (SD), kg/m²</strong></td>
<td>23.7 ± 4.6</td>
<td>22.8 ± 4.6</td>
</tr>
<tr>
<td><strong>Graft Number</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>148 (94.9)</td>
<td>46 (95.8)</td>
</tr>
<tr>
<td>Second</td>
<td>7 (4.5)</td>
<td>2 (4.2)</td>
</tr>
</tbody>
</table>
Table 4.1B – Lung Donor Characteristics in All Included vs. Post-Transplant Followed Patients

<table>
<thead>
<tr>
<th></th>
<th>All Donors for Recipients Included</th>
<th>Donors for Included Subset of Post-Transplant Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 156</td>
<td>n = 48</td>
</tr>
<tr>
<td><strong>DONORS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>44.5 ± 18.1</td>
<td>44.1 ± 15.9</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>66 (42.3)</td>
<td>19 (39.6)</td>
</tr>
<tr>
<td>Female</td>
<td>88 (56.4)</td>
<td>29 (60.4)</td>
</tr>
<tr>
<td><strong>Cause of Death</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrovascular/Stroke</td>
<td>82 (52.6)</td>
<td>23 (47.9)</td>
</tr>
<tr>
<td>Anoxia/Hypoxia</td>
<td>17 (10.9)</td>
<td>5 (10.4)</td>
</tr>
<tr>
<td>Head Trauma</td>
<td>37 (23.7)</td>
<td>12 (25.0)</td>
</tr>
<tr>
<td>Overdose</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Motor Vehicle Accident</td>
<td>2 (1.3)</td>
<td></td>
</tr>
<tr>
<td>Spontaneous Intracranial Hemorrhage</td>
<td>4 (2.6)</td>
<td>3 (6.3)</td>
</tr>
<tr>
<td>Primary CNS Tumor</td>
<td>1 (0.6)</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>Gunshot</td>
<td>2 (1.3)</td>
<td></td>
</tr>
<tr>
<td>Cardiac Arrest/MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>6 (3.8)</td>
<td>4 (8.3)</td>
</tr>
<tr>
<td><strong>Mechanism of Death</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracranial Hemorrhage/ Stroke</td>
<td>87 (55.8)</td>
<td>25 (52.1)</td>
</tr>
<tr>
<td>MVA</td>
<td>18 (11.5)</td>
<td>10 (20.8)</td>
</tr>
<tr>
<td>Asphyxiation</td>
<td>8 (5.1)</td>
<td>2 (4.2)</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>5 (3.2)</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>Drug Intoxication</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Blunt Injury</td>
<td>8 (5.1)</td>
<td></td>
</tr>
<tr>
<td>Seizure</td>
<td>8 (5.1)</td>
<td>2 (4.2)</td>
</tr>
<tr>
<td>Gunshot Wound</td>
<td>5 (3.2)</td>
<td></td>
</tr>
<tr>
<td>Natural Causes</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>8 (5.1)</td>
<td>6 (14.3)</td>
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<tr>
<td>Other</td>
<td>8 (5.1)</td>
<td>6 (14.3)</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>11 (7.1)</td>
<td>2 (4.2)</td>
</tr>
<tr>
<td>No Diabetes</td>
<td>132 (84.6)</td>
<td>44 (91.2)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8 (5.1)</td>
<td>2 (4.2)</td>
</tr>
<tr>
<td><strong>BMI, mean (SD), kg/m²</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.0 ± 23.5</td>
<td>25.0 ± 4.6</td>
</tr>
<tr>
<td>Transplants</td>
<td>Included End-Stage Patients</td>
<td>Subset of Included Post-Transplant Patients</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>-----------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>n = 156</td>
<td>n = 48</td>
<td></td>
</tr>
<tr>
<td><strong>Transplants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single Lung Transplant, mean (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left Lung Transplant</td>
<td>11 (7.1)</td>
<td>5 (10.4)</td>
</tr>
<tr>
<td>Right Lung Transplant</td>
<td>8 (5.1)</td>
<td>5 (10.4)</td>
</tr>
<tr>
<td>Double Lung Transplant</td>
<td>134 (85.9)</td>
<td>37 (77.1)</td>
</tr>
<tr>
<td>Lung / Heart Transplant</td>
<td>3 (1.9)</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td><strong>Left Cold Ischemic Time, median (IQR), min</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left Cold Ischemic Time</td>
<td>325 (256 – 411)</td>
<td>354 (287 – 425)</td>
</tr>
<tr>
<td>Right Cold Ischemic Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Cold Ischemic Time, median (IQR), min</td>
<td>68 (59 – 80)</td>
<td>70 (65 – 81)</td>
</tr>
<tr>
<td>Left Total Ischemic Time, median (IQR), min</td>
<td>418 (331 – 484.8)</td>
<td>429 (368 – 496)</td>
</tr>
<tr>
<td>Right Total Ischemic Time, median (IQR), min</td>
<td>319 (253 – 422.5)</td>
<td>324 (266 – 410)</td>
</tr>
<tr>
<td><strong>CMV Match, mean (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-R+</td>
<td>31 (19.9)</td>
<td>10 (21.7)</td>
</tr>
<tr>
<td>D-R-</td>
<td>44 (28.2)</td>
<td>11 (23.9)</td>
</tr>
<tr>
<td>D+R-</td>
<td>25 (16.0)</td>
<td>13 (28.3)</td>
</tr>
<tr>
<td>D-R+</td>
<td>46 (29.5)</td>
<td>12 (26.1)</td>
</tr>
<tr>
<td><strong>Hospital Length of Stay, mean (SD), days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital Length of Stay</td>
<td>38.2 (36.4)</td>
<td>34.4 (23.1)</td>
</tr>
<tr>
<td><strong>ICU Length of Stay, mean (SD), days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICU Length of Stay</td>
<td>12.0 (16.3)</td>
<td>10.0 (10.6)</td>
</tr>
</tbody>
</table>
Of the patients followed in the post-transplant subset 48 were analyzed for CCSP PBMCs at 24 hrs and 31 of these were also analyzed at 48 hrs. In addition, 18 were analyzed for CD45^+Collagen-1^+ PBLs and 15 of these patients were reanalyzed at 48 hrs.

Table 4.2 – Patient Samples Analyzed Post-Lung Transplant

<table>
<thead>
<tr>
<th>Indication for Tx</th>
<th>PBMC samples @ 24hrs</th>
<th>PBMC samples @ 48hrs</th>
<th>PBL samples @ 24hrs</th>
<th>PBL samples @ 48hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOS</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CF</td>
<td>13</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>COPD</td>
<td>11</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>IPF</td>
<td>17</td>
<td>11</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>PH</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>48</strong></td>
<td><strong>31</strong></td>
<td><strong>18</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

4.2 Progenitor Cell Response Following Lung Transplantation

At 24 hrs post-transplant there was an increase in the percentage of CCSP^+ PBMCs in lung transplant recipients. Generally, a distinctive shift was observed on flow cytometric dot plot analyses based on forward scatter and positive CCSP staining (Figure 4.1A). This was also observed as a long tail-like pattern on histogram plots (Figure 4.1B). Representative data are shown.
The percentage of CCSP$^+$ PBMCs was quantified and a significant increase was measured at 24 hrs post-transplant (mean = 3.14 ± 0.41%) compared to pre-transplant baseline (T=0) (mean = 1.96 ± 0.23%, paired T-test p=0.0002) (Figure 4.2A). Comparing the subset of samples at all 3 time-points, no increase from baseline was found at 48 hrs (mean = 1.99 ± 0.26%, repeated measures ANOVA p > 0.05). The increase in CCSP$^+$ PBMCs was also calculated as change from baseline, and a similar observation of significant increase at 24 hrs (mean = 1.46 ± 0.36) and resolution by 48 hrs (mean = -0.19 ± 0.33) was observed (Figure 4.2B). A significant difference in the change from baseline was measured for cells at 24 hrs vs. 48 hrs (p=0.0094).
Figure 4.2 – Increase in CCSP⁺ PBMCs at 24 hrs Post-Lung Transplant

(A) Percentage of CCSP⁺ PBMCs at 0 hrs and 24 hrs post-lung transplant (n=48). Paired t-test. (B) The change in the percentage of CCSP⁺ PBMCs at 24 hrs (n=48) and 48 hrs (n=31) from baseline (0 hrs), as assessed by t-test.

Changes in the percentage of CD45⁺Collagen-1⁺ PBLs were also measured for a subset of post-transplant patients at 24 hrs and 48 hrs. No changes in fibrocyte numbers compared to pre-transplant levels were measured in this patient group at either time-point or when expressed as a change from baseline (Figure 4.3)

Figure 4.3 – No Change in CD45⁺Collagen-1⁺ Fibrocyte Numbers Post-Lung Transplant

(A) Percentage of fibrocytes at 0 hrs and 24 hrs post-lung transplant (n=18). Paired t-test. (B) The change in the percentage of CCSP⁺ PBMCs at 24 hrs (n=18) and 48 hrs (n=15) from baseline (0 hrs), as assessed by t-test.

Comparing changes in progenitor cell numbers by disease found a significantly greater increase of CCSP⁺ PBMCs in IPF patients vs. CF patients based on change at 24 hrs from time of transplant (mean = 2.52 ± 0.77 vs. 0.17 ± 0.44, p < 0.05) (Figure 4.4A). No change in CD45⁺Collagen-1⁺ cells at 24 hrs was found when separated by disease (Figure 4.4B).
To determine if the increase in progenitor cell populations noted post-transplant is a result of generalized inflammatory cell changes, the total blood reference leukocyte (total white blood cells) and lymphocytes values were collected for each patient. On average, a decrease in peripheral blood lymphocytes was measured at 24 hrs and 48 hrs post-transplant (mean = 0.82 ± 0.11, mean = 0.74 ± 0.08 respectively) compared to baseline (mean = 1.35 ± 0.17) (Figure 4.5A). Conversely, an increase in peripheral blood leukocytes was found in at 24 hrs and 48 hrs (mean = 14.74 ± 1.13, mean = 15.82 ± 1.15 respectively) compared to baseline (mean = 10.59 ± 1.06) (both repeated measures ANOVA, $p < 0.001$) (Figure 4.5B). The lymphocyte levels fell below the normal range for all 3 time-points, while the leukocyte levels were measured above the normal range, as determined by the Toronto General Hospital diagnostic testing laboratory.
4.5 - Reference Blood Cell Counts Post-Lung Transplant

(A) Reference Blood Cell Counts Post-Lung Transplant. (A) Total blood lymphocyte counts of all lung transplant recipients followed post-transplant at 0 hrs, 24 hrs, and 48 hrs. (B) Total leukocytes counts of all lung transplant recipients followed post-transplant at 0 hrs, 24 hrs, and 48 hrs. Reference cells counts are presented as bil/L. Normal ranges are presented as determined by the Toronto General Hospital diagnostic testing laboratory. 1-way repeated measures ANOVA with Dunnett’s post-hoc test.

Based on leukocyte counts and the percentage of CCSP⁺ PBMCs or CD45⁺Collagen-1⁺ PBLs, the absolute progenitor cell number was calculated for each population by dividing the total leukocyte counts (bil/L) by the percentage of progenitor cells measured, and these values were again compared by time-point. A significant increase in the number of CCSP⁺ PMBCs was also found at 24 hrs for absolute cell values (mean = 42.81 ± 7.42, p < 0.01) (Figure 4.6A), and no significant changes were found in absolute cell numbers at 48 hrs (mean = 32.20 ± 5.04) compared to baseline (mean = 22.02 ± 3.99). A significant difference was found between the change in CCSP⁺ PBMCs at 24 hrs minus baseline (0 hrs) and the equivalent change at 48 hrs minus baseline (0 hrs) (mean = 27.52 ± 6.31 vs. 11.71 ± 4.88, p < 0.05) (Figure 4.6B).
Figure 4.6 - Changes in Absolute Progenitor Cell Values Post-Lung Transplant

(A) Absolute CCSP⁺ peripheral blood mononuclear cell (PBMCs) values as normalized to reference total leukocyte counts. Paired t-test. (B) Change in absolute CCSP⁺ PBMCs at 24 and 48 hrs minus baseline (0 hrs). T-test. (C) Absolute CD45⁺Collagen-1⁺ fibrocyte values as normalized to reference total leukocyte counts. Paired T-test. (D) Change in absolute fibrocytes at 24 and 48 hrs minus baseline (0 hrs) T-test.

The absolute number of CD45⁺Collagen-1⁺ PBLs was not significantly increased in post-lung transplant patients at 24 hrs or 48 hrs, when compared to baseline (p > 0.05) (Figure 4.6C) and the change in fibrocytes from 24 hrs minus baseline (0 hrs) was not significantly different from the change at 48 hrs minus baseline (Figure 4.6D).

4.3 Progenitor Cell Numbers Following Liver Transplantation

To determine if the changes in progenitor cell numbers following transplant was specific to lung injury or if the response was generalized to all surgical procedures or organ transplantation, a group of live liver donors and recipients were studied. A total of 10 donor/recipient pairs were sampled at 0 hrs, 24 hrs, and 48 hrs following donation or liver transplant. Of the recipients, the
indications for transplant included Primary Sclerosing Cholangitis (PSC) (n=3), Alcoholic Cirrhosis (n=2), Hepatitis-C Virus (HCV) (n=2), Cryptogenic Disease (n=2), and Nonalcoholic Steatohepatitis (NASH) (n=1). All donors were deemed healthy. No significant changes in CCSP$^+$ cell percentage was measured in liver donors or recipients at 24 hrs (Figure 4.7 A and C) or when analyzed as changes from baseline (Figure 4.7 B and D).

**Figure 4.7 - Changes in CCSP$^+$ PBMCs Post-Liver Transplant**

Similarly, no change was observed for the percentage of CD45$^+$ Collagen-1$^+$ fibrocytes in liver donors and recipients at 24 hrs (Figure 4.8 A and C) or when analyzed as changes from baseline (Figure 4.8 B and D). Of note, a higher than normal level of fibrocytes was measure in some liver recipients, particularly those with diagnoses of Alcoholic Cirrhosis and Cryptogenic Disease, and this increase was maintained post-transplant (Figure 4.8 C-D).
Figure 4.8 - Changes in CD45$^+$Collagen-1$^+$ Fibrocytes Post-Liver Transplant

As for the lung transplant group, peripheral blood lymphocyte and leukocyte numbers were collected total to be used as a reference for cellular changes. No significant changes were found lymphocyte or leukocytes count for liver donors post-transplant (Figure 4.9 A-B).
As was observed for lung recipients, a decrease in peripheral blood lymphocytes at 24 hrs (mean = 0.56 ± 0.065) and 48 hrs (mean = 0.53 ± 0.068) compared to baseline (mean = 0.95 ± 0.17) (both p < 0.05), and an increase peripheral blood leukocytes was found at 24 hrs (mean = 13.61 ± 2.4) and 48 hrs (mean = 13.46 ± 1.67) post-liver transplant, compared to baseline (4.51 ± 0.64) (both p < 0.01) (Figure 4.10 A-B)
Based on leukocyte counts and the percentage of CCSP\(^+\) PBMCs or CD45\(^+\)Collagen-1\(^+\) PBLs, the absolute progenitor cell number was calculated for each population and at each time-point, represented as the change from baselines (0 hrs) (Figure 4.11A-D). No significant changes were found.

**Figure 4.11 – Absolute Progenitor Cell Numbers Post-Liver Transplant**

(A) Liver Donors  
(B) Liver Recipients

(C)  
(D)

Figure 4.11 – Absolute Progenitor Cell Numbers Post-Liver Transplant. The change in absolute CCSP\(^+\) peripheral blood mononuclear cell (PBMCs) values as normalized to reference total leukocyte counts for (A) live liver donors and (B) liver recipients. Change in absolute CD45\(^+\)Collagen-1\(^+\) fibrocyte values as normalized to reference total leukocyte counts for (C) live liver donors and (D) liver recipients. Assessed by t-test.

### 4.4 Progenitor Cell Numbers and Outcomes Following Lung Transplant

In order to investigate the predictive value of progenitor cell quantification, arterial blood gas (ABG) measurements were collected at ICU arrival, 24 hrs, and 48 hrs post-lung transplant. The ratio of partial pressure of oxygen (PaO2) to fraction of inspired oxygen in a gas mixture (FiO2) was used as an indicator of oxygenation, and patients were grouped as either P/F <150, P/F 150-300,
P/F >300, or extubated. No predictive value was found for pre-transplant CCSP\(^+\) PBMCs (T=0) and oxygenation at 24 hrs (Figure 4.12A). A trend towards greater CCSP\(^+\) PBMC percentage (%) at 24 hrs being associated with better oxygenation (P/F > 300), but this was not significant (Figure 4.12B). Interestingly, this association was not found for patients who have been extubated by 24 hrs. No significant differences were found based on the change in CCSP\(^+\) cells (Figure 4.12C) at 24 hrs post-transplant and oxygenation at 24 hrs, although again, a trend toward improved oxygenation (P/F > 150) and higher CCSP\(^+\) PBMCs was noted. In addition, a further improvement in P/F (>300) was noted at 72 hrs for patients with a greater number of CCSP\(^+\) cells at 24 hrs post-lung transplant (Figure 4.12D).

**Figure 4.12 – Relationship between CCSP\(^+\) PBMCs and Post-Lung Transplant Oxygenation**

(A) The percentage of CCSP\(^+\) PMBCs pre-lung transplant grouped by P/F at 24 hrs post-lung transplant (P/F < 150 n= 2, P/F 150-300 n = 18, P/F > 300 n = 19, ext n = 2). (B) The percentage of CCSP\(^+\) PMBCs at 24 hrs post-lung transplant grouped by P/F at 24 hrs post-lung (C) The change in percentage of CCSP\(^+\) PBMCs at 24 hrs minus baseline at 0 hrs, grouped by P/F at 24 hrs post-lung transplant. (D) The percentage of CCSP\(^+\) PBMCs at 24 hrs post-lung transplant grouped by P/F at 72 hrs post-lung transplant. (A-C: P/F < 150 n= 2, P/F 150-300 n = 18, P/F > 300 n = 19, ext n = 2)(D: P/F < 150 n= 1, P/F 150-300 n = 14, P/F > 300 n = 8, ext n = 13)
To further study the predictive value of CCSP$^+$ PBMC quantification post-lung transplant, the surrogate marker of length of stay (LOS) in the intensive care unit (ICU), or the transplant unit (Tx) was compared to cell numbers. LOS was calculated as days free of either ICU or Tx-unit, using the longest patient stay as references (115 days for ICU and 314 for Tx-unit). Comparing all end-stage lung disease patients from Chapter 3 and comparing their pre-transplant (T=0) CCSP$^+$ BMCs or PBMCs to ICU or Tx free days, it was found that fewer CCSP$^+$ BMCs were associated with less days in either the ICU or the Tx-unit ($p < 0.1$) (n=120) (Figure 4.13A-B). A similar trend was found for pre-transplant CCSP$^+$ PBMCs, although this did not reach significance (n=150) (Figure 4.13C-D).

Figure 4.13 - Pre-transplant CCSP$^+$ Cell Numbers and Hospital Stays.

(A) Correlation between the number of days patients were not in the ICU and the percentage of CCSP$^+$ bone marrow cells (BMCs) pre-transplant. (B) Correlation between the number of days patients were not in the transplant unit (Tx) and the percentage of CCSP$^+$ bone marrow cells (BMCs) pre-transplant. (n=120) (C) Correlation between the number of days patients were not in the ICU and the percentage of CCSP$^+$ peripheral blood mononuclear cells (PBMCs) pre-transplant. (D) Correlation between the number of days patients were not in the transplant unit (Tx) and the percentage of CCSP$^+$ peripheral blood mononuclear cells (PBMCs) pre-transplant. (n=150)
To investigate whether the mobilization of CCSP$^+$ cells following lung transplant is predictive of the degree of lung damage, the percentage of CCSP$^+$ PBMCs at 24 hrs and the change in CCSP$^+$ PBMCs from baseline was compared to hospital length of stays (n=48). No significant relationships were found (Figure 4.14A-C).

Figure 4.14 - Post-Transplant CCSP$^+$ PBMC Numbers and Hospital Stays. (A) Correlation between the number of days patients were not in the ICU and the percentage of CCSP$^+$ PBMCs at 24 hrs post-transplant. (B) Correlation between the number of days patients were not in the transplant unit (Tx) and the percentage of CCSP$^+$ PBMCs at 24 hrs post-transplant. (C) Correlation between the number of days patients were not in the ICU and the change in percentage of CCSP$^+$ peripheral blood mononuclear cells (PBMCs) at 24 hrs minus baseline (0 hrs). (D) Correlation between the number of days patients were not in the transplant unit (Tx) and the percentage of CCSP$^+$ peripheral blood mononuclear cells (PBMCs) at 24 hrs minus baseline (0 hrs). (n=48)
Finally, the relationship between CCSP$^+$ cell mobilization and lung ischemia time was also investigated. No correlations between lung ischemia time and CCSP$^+$ PBMC mobilization (% or change) was found (Figure 4.15A-B)

**Figure 4.15 – Post-Transplant CCSP$^+$ PBMC Numbers and Lung Ischemia Time.**

(A) Correlation between lung ischemic time (left and right) and the percentage of CCSP$^+$ PBMCs at 24 hrs post-lung transplant. (B) Correlation between lung ischemic time (left and right) and the change in CCSP$^+$ PBMCs at 24 hrs minus baseline (0 hrs). (n=48)

### 4.5 CCSP$^+$ PBMC Proliferation Post-Transplant

To determine if the measured increase in CCSP$^+$ PBMCs at 24 hrs post-lung transplant was due to cell proliferation, intracellular Ki67 stain was performed and analyzed by flow cytometry. No proliferation, as measured by dual Ki67$^+$/CCSP$^+$ staining, was detected for CCSP$^+$ BMCs or PBMCs in healthy control or lung transplant recipients (Representative samples shown) (Figure 4.16 A-D). Control cell lines WRO (human thyroid carcinoma cells) and A549 (human adenocarcinoma cells) grown in culture both stained positively for intracellular Ki67 (Figure 4.17 A-B).
Figure 4.16 – Absence of Proliferation of CCSP⁺ PBMCs by Ki67 Staining

Dual intracellular Ki67 and surface CCSP⁺ staining on (A) PBMCs isolated from a healthy individual, (B) BMCs isolate from a lung transplant recipient at 0 hrs, (C) PBMCs isolate from a lung transplant recipient at 0 hrs, (D) PBMCs isolate from a lung transplant recipient at 24 hrs post-transplant.
4.6 Chemokine Receptor Expression Post-Transplant

In order to investigate the mechanisms contributing to increased CCSP⁺ PBMCs post-transplant, further cell phenotyping was performed to determine chemokine receptor expression. The chemokine receptors CCR2, CCR4, CXCR3, and CXCR4 were measured on CCSP⁺ PBMCs (Figure 4.18 A-C), but no statistically significant changes were found. A significant increase in CXCR4⁺ CCSP⁺ PBMCs was measured at 24 hrs compared to baseline (0 hrs) was identified (n=5 per group, paired t-test, p = 0.015).
4.7 Plasma Protein Array Analysis Post-Transplant

Quantification of key plasma cytokines at 24 hrs post-transplant was performed by multiplex array. The same targets were used as described in Chapter 3.8 (Table 3.4). Comparing the differences in plasma proteins before (T=0) and after (T=24 hrs) transplant demonstrated an increase in both IL-8 and SCF levels post-transplant (Figure 4.19A-B). No increase in circulating SDF-1 protein levels was found (Figure 4.19C). Cytokine concentrations in lung donor plasma are shown for reference.
Comparing the percentage of CCSP$^+$ PBMCs at 24 hrs to the concentration of plasma proteins found a relationship between GM-CSF levels and CCSP$^+$ PBMCs ($p = 0.0036$, $r^2 = 0.30$) (Figure 4.20A). A similar relationship was found between GM-CSF plasma concentration and the change in CCSP$^+$ PBMCs at 24 hrs minus 0 hrs ($p = 0.011$, $r^2 = 0.24$) (Figure 4.20B). Although no significant increase in total plasma GM-CSF was found at 24 hrs compared to baseline (paired t-test, $p = 0.13$), a significant difference in GM-CSF concentration was noted between lung recipients at 24 hrs and control lung donors ($p=0.048$) (Figure 4.20C). In addition, a relationship between SDF-1 plasma protein concentration at 24 hrs and change in CCSP$^+$ PBMCs from baseline (24 hrs-0 hrs) was found ($p = 0.004$, $r^2 = 0.28$) (Figure 4.20D). There were no correlations between post-transplant CD45$^+$Collagen-1$^+$ fibrocytes and plasma protein concentrations.
Figure 4.20 - Plasma Protein Concentrations and CCSP⁺ PBMCs Post-Transplant

Figure 4.20 - Plasma Protein Concentrations and CCSP⁺ PBMCs Post-Transplant. (A) Correlation between plasma GM-CSF concentration and (A) the percentage of CCSP⁺ PBMCs at 24 hrs post-lung transplant or (B) the change in percentage of CCSP⁺ PBMCs at 24 hrs minus baseline (0 hrs) (n=26). (C) GM-CSF plasma concentrations in lung donors (n=8), lung recipients at 0 hrs (n=22), and lung recipients at 24 hrs (n=22), paired t-test. (D) Correlation between plasma SDF-1 concentration and the change in percentage of CCSP⁺ PBMCs at 24 hrs minus baseline (0 hrs) (n=25).

4.8 Relationship between Plasma Cytokines and Clinical Outcomes.

Plasma protein concentrations were compared to the three clinical outcomes, lung oxygenation, hospital stays, or ischemia time. No relationships were found for protein levels at 24 hrs and lung oxygenation, although a trend toward increased SDF-1 and GM-CSF and worse lung function was observed at 24 hrs (Figure 4.21A-B). A significant relationship was noted between the level of plasma SDF-1 at 24 hrs post-transplant and fewer ICU free days. No relationships were found between ischemic times and plasma cytokines.
4.9 In Vitro Migration Studies

To determine if the migration potential of CCSP\(^+\) PBMCs was altered following transplant, \textit{in vitro} trans-well assays were performed. Migration in response to RANTES, IP-10, SDF-1, and SCGF-\(\beta\) was investigated. No significant differences in migration potential were found for CCSP\(^+\) PMBCs at 24 hrs compared to 0 hrs in response to any stimuli (\(p > 0.05\)) (Figure 4.22). As was found with pre-treatment samples, significant migration by CCSP\(^+\) PBMCs isolate 24 hrs post-transplant was found in response to SDF-1 when compared to untreated (NT) controls (\(p < 0.05\)) (Figure 4.22).
Figure 4.22 - In Vitro Migration of CCSP⁺ PBMCs at 0 hrs vs. 24 hrs Post-Transplant

Migration of isolated lung recipient peripheral blood mononuclear cells (PBMCs) at 0 hrs vs. 24 hrs post-lung transplant in response to RANTES, IP-10, SDF-1, or SCGF-β. Each time point (0 hrs or 24 hrs) is analyzed by 1-way ANOVA with Dunnett’s post-hoc test compare to no treatment (N.T.). Within treatment comparison (0 hrs vs. 24 hrs by cytokine) is analyzed by paired-test.
SUMMARY OF CHAPTER 4 RESULTS

The data presented in this chapter serve to provide evidence of the capacity for CCSP⁺ epithelial-like progenitor cells to respond to injury and epithelial damage following lung transplantation, resulting in an increased percentage of circulating cells in patients at 24 hrs. It was further shown that this response is specific to lung transplantation and it not likely due to a steroid-mediated mobilization, as determined by control subjects (liver transplant recipients) who also received high-dose intra-operative immunosuppression but did not demonstrate an increase in CCSP⁺ peripheral blood cells. Outcomes investigated in relation to the progenitor cell response did not definitely link oxygenation, ICU or hospital length of stay, or ischemia time to the level of cell response. Studies aimed to elucidate mechanism identified a specific increase in CCSP⁺CXCR4⁺ PBMC subset. Interestingly, the level of CCSP⁺ PBMCS (% and change) did correlate with plasma levels of GM-CSF and SDF-1. Further proteomic plasma analysis identified an increase in IL-8 and SCF post-transplant but not SDF-1 or GM-CSF. In vitro migration studied intended to assess functional cell response to chemotactic signals following transplant identified that CCSP⁺ PBMCs isolated from lung recipients at 24 hrs post-transplant could significantly migrate in response to SDF-1 when compared to untreated cells, but to a lesser degree than cells isolated pre-transplant. Taken together, these data suggest a complex mechanism of progenitor cell recruitment following lung injury, which may likely involve the SDF-1/CXCR4 axis but may also be sensitive to ligand and receptor fluctuations.
CHAPTER 4 DISCUSSION

The rationale for the work presented in this chapter was based on observations in models of mouse lung injury utilizing the toxin naphthalene to damage Clara cells. In this mode of acute epithelial injury it was found that an increase in the percentage of CCSP$^+$ cells could be measured in the peripheral blood and bone marrow at 2 days following injury, which resolved as epithelial repair occurred and cell numbers returned to baseline by day 20 (259). Moving from this finding it was hypothesized that a similar response may occur in acute human lung injury. The situation of lung transplantation provided a predictable and somewhat homogenous epithelial cell injury resulting from ischemia reperfusion for which to study.

A subset of lung transplant recipients was followed and progenitor cell numbers were reanalyzed at 24 and 48 hrs following transplant. Comparing both donor and recipient demographics in the total population versus those included post-transplant found no significant differences. Further comparison of lung transplant parameters between groups also suggests that this subset was representative of the total population. This finding would suggest that conclusions derived from the subset analysis can be applied to the total study population and beyond this study to the population at large. Statistical analysis was based on a repeated measures approach, utilizing both a paired t-test for the 0 hr vs. 24 hrs comparison and repeated measures ANOVA to compare all 3 time points.

On average, an increase in CCSP$^+$ cells was measured in the peripheral blood at 24 hrs, which resolved by 48 hrs. The population appeared as a clear increase on flow cytometric dot plot analysis and could be visualized as a long tail extending from the main peak when represented by histogram. This suggests that as with the previous finding, the population is not strongly positive for the CCSP surface antigen and the mean shift in total fluorescence was low. Interim analysis of the findings indicated that data from the 48 hrs time point provided lesser value and subsequently only 24 hr samples were collected and analyzed.

Although an increase in CCSP$^+$ PBMCs is found for the population mean, it should be stated that some patients did not demonstrate this increase. It was also noted that the degree of increase was variable by patients. The underlying reasons for this are not yet clear. It can be stated that based on this analysis that the level of increase may be associated with underlying disease. A significant difference in the magnitude of CCSP$^+$ PBMC change was found between CF
and IPF patients, with CF patients increasing to a lesser degree. This observation may be due to the higher baseline levels measured for CF patients, implying an exhaustion or maximization of the cell population during chronic injury, preventing as large of an increase in response to acute injury. In contrast, no increase in circulating fibrocytes was measured at 24 or 48 hrs post-transplant. This observation suggests that these two marrow-derived populations are activated and recruited via separate mechanisms and may respond to unique signals. It is possible that fibrocyte numbers increase beyond 48 hrs, which may reflect changing cytokine patterns in the lung and peripheral blood. A complex process occurs within the lung following reperfusion injury, involving both inflammation and ultimately resolution and repair. It is possible that CCSP+ cells are recruited more robustly in the initial inflammatory phase and that CD45+Collagen-1+ fibrocytes participate in remodelling and tissue regeneration. The action of CCSP+ cells once recruited to the lung is not yet understood. Additional animal model utilizing CCSP+ conditional knock-out mice and naphthalene injury have indicated that a lack of bone marrow-derived CCSP+ cells confers a reduced survival, indicating that response of CCSP+ progenitor following injury is beneficial to repair (Bustos and Waddell, unpublished observations).

One clear limitation to these findings is the lack of evidence indicating the source and the ultimate destination of these cells. The data presented provide only a snap-shot of the true biological process. Animal models can be useful to address these questions. Lineage-tagged mice or sex-mismatch bone marrow transplants could be a useful addition to these observations, but would not fully answer the question in human transplantation. Sex-mismatched lung transplant recipients could be studied, and lung tissue analysed for recipient-derived CCSP+ cells within the transplanted lungs. Detailed genotyping of CCSP+ PBMCs could also be used to determine the origin of these cells. Work being done in collaboration with this project has used the bronchial brushing technique to study endogenous lung cells in a fairly non-invasive manner. This process has provided evidence of a CD45+CCSP+ cell isolated from the lungs of recipients at several time points following transplant. This evidence further supports the existence of a circulating bone marrow-derived population that is recruited into the lung.

To address the specificity of this observation, living liver donors and their matched recipients were included for peripheral blood analysis that the same time points. This was done to investigate the effects of (1) surgical injury, (2) anaesthetic, (3) organ transplantation, and (4) immunosuppression on progenitor cell response. It could be hypothesized that the observed
response in lung transplantation may be due to any of the above factors and not due specifically to lung injury. If was found that no significant CCSP\(^+\) PBMC or CD45\(^+\)Collagen-1\(^+\) cell increase was measured in liver donors or recipients, although there was some variability in response between patients. A group of 10 matched liver donors and recipients were studied to investigate this patient population, which may not accurately represent the variability present in a clinical population. A larger cohort of patients may be required to fully understand the progenitor cell response following surgical injury or high-dose immunosuppression. As these 10 patients had a spectrum of underlying disease indicating them for transplant, it may be useful to study a more homogenous group of liver recipients, as previous disease mechanisms such as the inflammatory or fibrotic milieu may influence progenitor cell response following transplantation. It is possible that the damaged tissue produces or releases specific signals capable of inducing a lung epithelial progenitor cell response. This may be a previously defined cytokine or factor, or alternatively may indeed be a novel stimulus capable of orchestrating a specific and unique reaction.

As in the previous chapter, white blood cell counts were collected and used to normalize the measured percentages to an absolute cell number, with the aim to ensure that observed changes in progenitor cell numbers were not due to an overall increase in the peripheral blood leukocyte or lymphocyte population. As with the end-stage data, all observations were confirmed with absolute cell numbers, further validating the observed increase. The lack of increase was also true for absolute cell number in the liver transplant subset. Of note, total blood leukocytes were found to be significantly increased at 24 hr and 48 hrs post-lung transplant, while lymphocyte counts were significantly decreased. This was also true for liver transplant recipients but not liver donors. The observation is believed to be the result of a combination of an inflammatory response to organ injury and the immunosuppressive action of the Solu-Medrol administration.

In order to determine if there was a relationship between CCSP\(^+\) PBMCs response to transplant and degree of lung injury, 3 variables were examined. First, oxygenation measurement in the form of PaO2/FiO2 ratio was used to infer the health and function of the transplanted lung, and this was compared to CCSP cell numbers pre-transplant and at 24 hrs. No significant trends were found linking cell mobilization and post-transplant oxygenation. When grouped by P/F ratio at 24 hrs post-transplant no difference was found in mean pre-transplant (T=0 hrs) cell percentage, suggesting that the baseline cell level does not impact outcome. When the same grouping were analyzed for cell percentage at 24 hrs a trend was noted where better oxygenation (P/F >300) was
associated with greater CCSP⁺ PBMC numbers, although this did not reach significance. This observation would suggest that the more CCSP⁺ PBMCs present in the peripheral blood may lead to greater cell numbers being recruited to the injured lung where they exhibit pro-regenerative or anti-inflammatory effects, leading to improved functioning of the transplanted lung. This trend was further observed when cell numbers were grouped based on P/F ratio at 72 hrs, suggesting that the positive effects can extend or increase over time. The second variable examined was length of stay values in either ICU or total hospital length of stay. These data are presented as days free of either ICU or hospital to accurately reflect that patients with shorter stays could have either recovered more quickly or perhaps have died without being discharged. Regardless, no correlations were found between either measurement and the number of CCSP⁺ PBMCs. These variables were examined to investigate CCSP⁺ PBMC mobilization as a predictor of outcome. It should be stated that multiple factors influence ICU and hospital stays, not simply lung health, which may include infections and other co-morbidities that CCSP⁺ cells would likely have no influence on. The final factor studied was the transplant-specific ischemia time. This was done to determine if the extent of ischemic injury was able to influence the progenitor cell response. No significant correlations were found between left or right lung ischemic times and CCSP⁺ PBMC percentage at 24 hrs or the change in cell numbers from 0-24 hrs.

As stated previously, it is not be clear if the increase in CCSP⁺ PBMCs is truly bone marrow-derived, or perhaps resulting from donor lung sloughing. It was also possible that the measured increase was due to some form of activation and proliferation of currently circulating CCSP⁺ cells. To address this point, dual flow cytometric analysis was performed to identify Ki67-positive proliferating cells. Based on a representative subset of patients, no proliferating cells could be identified in the peripheral blood CCSP⁺ fraction. This indicates that the measured response is not due to CCSP⁺ PBMC proliferation and that the changes in percentage are due to movement of cells from either the bone marrow or the lungs into the peripheral circulation.

In an effort to gain insight into the mechanisms responsible CCSP⁺ migration in response to lung transplant, surface chemokine receptors were measured on CCSP⁺ PBMCs at 0 hrs and 24 hrs. Dual positive staining was performed for CCR2, CCR4, CXCR3, and CXCR4 in conjunction with CCSP. Comparing changes in these 4 subsets at 0 vs. 24 hrs found a significant increase CXCR4⁺ CCSP⁺ PBMCs at 24 hrs, and not in any other subset. This observation implicates the actions of SDF-1 or potentially MIF, acting through CXCR4, as important mechanisms following transplant.
Moving forward to investigate the changes in cytokines concentrations following transplant, the same 17-plex plasma protein array (Table 3.4) was applied to 24 hr samples. A significant increase in both IL-8 and SCF was found at 24 hrs, although no significant increase in SDF-1 was identified. IL-8 is a cytokine produced by macrophages and epithelial cells, and is also stored in endothelial cells (304). It has been shown to be a potent chemoattractant for neutrophils, leading to their degranulation and contributing to inflammation (305). IL-8 has been shown to signal through both CXCR1 and CXCR2 (306), suggesting that measurement of these two receptors on CCSP⁺ cells may be informative. The observed increase in SCF following transplant implicates c-kit expressing cells in the response to this injury. Preliminary investigation on a limited number of samples has suggested that human CCSP⁺ BMCs and PBMCs express c-kit (data not shown), this would be another interesting marker to investigate. Changes in a double-positive subset of c-kit⁺CCSP⁺ PBMCs may also be an important population to study following transplant. A lack of increase in SDF-1 may be due to high level of cell expressing CXCR4 being present in the peripheral blood at this time, ultimately providing abundant receptor binding opportunities for this ligand and reducing the free protein concentration in the plasma. This observation may also implicate alternate ligands for CXCR4, including MIF, in progenitor cell homing.

Correlative analysis was performed to identify relationships between protein concentration and cell numbers. Interestingly, the level of GM-CSF, a known mobilization factor, was associated with the number of CCSP⁺ PBMCs at 24 hrs, as well as with the change in CCSP PMBCs from 0-24 hrs. Of note, the total GM-CSF level did not increase from 0 hrs to 24 hrs, but was still significantly higher than measured in healthy lung donor control plasma. Additionally, the level of SDF-1 did correlate with the change in CCSP⁺ PBMC percentage from 0-24 hrs, further associating with pathways with progenitor cell numbers.

Lastly, the functional ability of cytokines to induce CCSP⁺ PBMC migration was investigated utilizing in vitro trans-well assays. The response to IP-10, RANTES, SDF-1, and SCGF-β was compared at 0 hrs vs. 24 hrs. As may be expected, CCSP⁺ cells isolated at 24 hrs following lung transplant were capable of migrating to SDF-1, but not to other stimuli, although this ability was reduced in comparison to cells isolated from pre-transplant patients. This may be due to a reduced susceptibility to SDF-1 following transplant resulting from saturation of CXCR4 or over-stimulating in vivo prior to isolation.
Taken together the data presented in this chapter demonstrate a role for circulating epithelial-like progenitor cell population in the body’s response to injury following lung ischemic injury and transplantation. It also suggests that this response is specific to lung injury, is not due to steroid mobilization of leukocytes, and is not found for all progenitor populations. Protein mediators of this response likely include SDF-1 and GM-CSF, which may ultimately be useful in the design of novel reparative therapies and in the enhancement of endogenous tissue regeneration.
Chapter 5

Progenitors Cells in
Chronic Lung Allograft Dysfunction and BOS
HYPOTHESIS

As detailed in chapter 3, the analysis of progenitor cell populations in end-stage lung disease showed that patients with BOS at the time of re-transplant could be characterized by an increase in circulating fibrocytes and a decrease in peripheral blood CCSP$^+$ cells. This observation drove the hypothesis that an altered profile of bone marrow-derived cells could be predictive of BOS development following lung transplant, which may predate changes in clinically used spirometric testing. In conjunction, the secondary hypothesis is that a loss of CCSP$^+$ cells activity or recruitment and a parallel increase in fibrocyte activity may functionally contribute to the biology of BOS development and associated pathophysiology. Specifically, it was hypothesized that loss of CCSP$^+$ epithelial progenitors contributes to impaired lung regeneration and increased tissue remodelling, which is further augmented by greater fibrocyte recruitment and maturation to matrix-producing tissue fibroblasts. Variability in circulating cell numbers may be due to altered expression of chemotactic factors responsible for progenitor cell recruitment to damaged lung tissue.

AIMS

This study aimed to (1) apply a cross-sectional study design to quantify the level of circulating CCSP$^+$ cells and CD45$^+$Collagen-1$^+$ fibrocytes in patients 1-10 yrs following lung transplantation, (2) to determine possible associations between progenitor cell profiles and BOS status or other clinically relevant demographic factors, and (3) to identify protein mediators associated with progenitor cell levels or BOS status, with the hope of further hypothesis generation regarding the mechanism of recruitment or maintenance of progenitor cells following lung transplantation and chronic immunosuppression.

RESULTS

Peripheral blood samples were collected from patients at the time of re-transplant and from those returning for regularly scheduled clinic visits post-transplant. For the clinic outpatients, inclusion criteria included lung transplant recipients greater than 1-year and less than 10 years post-transplant who were free from acute infections. Patients were informed of the study objectives and requirements and participation was voluntary. A 10ml heparinised sample blood sample was subsequently collected by the clinical diagnostics lab. Blood samples were maintained at room temperature with contestant mixing until analysis within 24 hrs of collection.
5.1 Patient Demographics

The data collected from the group of end-stage BOS patients at time of re-transplant (n=7, from Chapter 3) were also included in this study of progenitor cells in chronic rejection following transplant. Of the 8 patients categorized as BOS in Chapter 3, one was not a re-transplant case and this patient was excluded from the dataset presented in this chapter. For the end-stage re-transplant BOS patients, the mean age at re-transplant was 27.86 ± 9.42yrs, the gender distribution was roughly equal, and the mean time from original transplant was 3.83 ± 0.76yrs. Cystic fibrosis was the most common indication for original transplant, but COPD, PF, and BO were also represented. A greater number of transplants were donor sex-match (n=5 M-M or F-F, vs. n= 2 F-M) and the mean donor age was 43.14 ± 17.20 (Table 1).

A total of 47 patients were included in the cross-sectional analysis of clinic out-patients. Of those, 14 were characterized as having BOS based on the ISHLT guidelines of ≥20% decrease in FEV₁ from previous baseline measurements (130), while 33 patients were defined as having stable lung function (Table 5.1). No significant differences in age, gender, original indication for transplant, donor age, or time from transplant to sample was noted between chronic BOS and stable patient groups. An equal proportion of chronic BOS patients had received sex-matched (M-M or F-F) transplants as has received sex-mismatched transplants (M-F or F-M), while a greater proportion of patients in the stable group had received sex-matched transplants. The stable patient group included 3 heart-lung transplant recipients, while the chronic BOS group did not include any.
Table 5.1 – Patients Demographics

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<tr>
<td><strong>Time Post Transplant</strong></td>
<td>Mean = 3.83 ± 0.76yrs</td>
<td>Mean = 4.48 ± 1.90yrs</td>
<td>Mean = 4.23±3.43yrs</td>
</tr>
</tbody>
</table>

5.2 Progenitors Cell in Patients Greater than 1-Year from Transplant

Peripheral blood samples were separated for CCSP⁺ mononuclear cell analysis and CD45⁺Collagen-1⁺ leukocyte analysis by Ficoll prep and high speed centrifugation respectively. Upon flow cytometric analysis of progenitor cell populations it was found that significantly fewer circulating CCSP⁺ PBMCs were measured in BOS patients (chronic and end-stage combined) (mean = 0.89 ± 0.14%) compared to patients categorized as stable (mean=1.59 ± 0.20%, p=0.0252) (Figure 5.1A). Conversely, an increase in circulating CD45⁺Collagen-1⁺ fibrocytes was found in BOS patients (mean = 1.92 ± 0.49%) compared to stable (mean = 0.82 ± 0.19%, p = 0.0253) (Figure 5.1B). Taking the ratio of these two measurements as a summative factor to further describe this trend also illustrated the altered levels of progenitor cells in BOS (mean = 1.68 ± 0.32 BOS vs. 0.92 ± 0.11 Stable, p = 0.0063) (Figure 5.1C)
Figure 5.1 - Circulating Progenitor Cell Profiles are Altered in BOS Patients.

(A) The percentage of CCSP$^+$ PBMCs in Bronchiolitis Obliterans Syndrome (BOS) vs. Stable patients. (B) The percentage of CD45$^+$Collagen-1$^+$ fibrocytes in BOS vs. Stable patients. (C) The ratio of CCSP$^+$ cells to CD45$^+$Collagen-1$^+$ fibrocytes in BOS vs. Stable patients. BOS n=21, Stable n=33). T-test.

5.3 Progenitor Cells Association to Time Following Transplant

To determine if the changes in progenitor cell numbers observed in post-transplant patients was a progressive process, the time from transplant to sample collection was compared to cell measurements. It was found that the level of CD45$^+$Collagen-1$^+$ cells significantly correlated to the number of days following transplant in clinic out-patients (p=0.044, $r^2 = 0.087$), while the number of CCSP$^+$ cells did not (Figure 5.2A-B). The computed ratio of CD45$^+$Collagen-1$^+$ cells was also found to correlate with increasing time post-transplant in this group (p=0.05, $r^2 = 0.082$) (Figure 5.2C). This correlation was no longer significant when end-stage BOS patients were included in the dataset.
Figure 5.2 Circulating CD45⁺Collagen-1⁺ Fibrocytes Increase with Time Post-transplant. (A) Correlation of CD45⁺Collagen-1⁺ fibrocyte numbers and days post-transplant. (B) Correlation between CCSP⁺ PBMCs and days post-transplant. (C) Correlation between the ratio of CD45⁺Collagen-1⁺/CCSP⁺ peripheral blood cells and time post-transplant.

5.4 Changes in Fibrocyte Surface Marker Expression in Chronic BOS vs. Stable

Further characterization of the circulating fibrocyte phenotype in chronic BOS (not end-stage) was addressed by measuring the levels of CD34⁺Collagen-1⁺ and CXCR4⁺Collagen-1⁺ double-positive cells in parallel to the standard CD45⁺Collagen-1⁺ population. No differences were found in single surface marker expression for CD34 or CXCR4 in chronic BOS vs. stable patients (Figure 5.3A). A significant difference was not found for CD34⁺Collagen-1⁺ and CXCR4⁺Collagen-1⁺ double-positive cells in chronic BOS vs. stable patients (Figure 5.3B).
Figure 5.3 - CD45, CD34, and CXCR4 Surface Expression in Chronic BOS

(A) CD45, CD34, and CXCR4 Surface Expression in Chronic BOS. Single surface expression of cells positive for (A) CD34 or (B) CXCR4 in patients greater than 1 year post-lung transplant. Dual-Collagen-1 and (C) CD34 or (D) CXCR4 patients greater than 1 year post-lung transplant. T-test.

5.5 - Progenitor Cells Numbers and Lung Function

The relationship between progenitor cell profiles and spirometric lung function measurements was also investigated. The ratio of forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC) was used as measure of obstruction in post-transplant patients. A weak trend toward better FEV1/FVC being associated with higher circulating CCSP$^+$ PBMCs was noted ($p=0.19$) (Figure 5.4A), while a significant association between higher FEV1/FVC and lower CD45$^+$Collagen-1$^+$ fibrocytes was found ($p=0.0011$, $r^2 = 0.13$) (Figure 5.4B). A similar relationship was found between the ratio of CD45$^+$Collagen-1$^+$ fibrocytes to CCSP$^+$ PBMCs and FEV1/FVC ($p=0.0013$, $r^2 = 0.12$) (Figure 5.4C)
**Figure 5.4 - Progenitor Cell Numbers and Lung Function**

(A) Correlation between FEV₁/FVC ratio and CCSP⁺ PBMCs. **(B)** Correlation between FEV₁/FVC ratio and CD45⁺Collagen-1⁺ fibrocytes. **(C)** Correlation between FEV₁/FVC ratio and CD45⁺Collagen-1⁺/CCSP⁺ cell ratio.

**5.6 Multiplex Protein Arrays Analysis of BOS vs. Stable Patient Plasma**

Further multiplex cytokine analysis was performed on plasma from BOS and Stable patients, and a total of 14 selected cytokines were analyzed (Table 5.2).
When plasma cytokine concentrations were compared in BOS (n= 10) or Stable (n=24) patients vs. controls (n = 8 healthy volunteers, n = 4 lung donors), a significant increase in Stem Cell Factor (SCF) was found in both Stable (mean = 473.6 ± 41.87) and BOS (mean = 475.3 ± 32.21) post-transplant patients compared to controls (mean = 282.9 ± 38.4, p<0.05) (Figure 5.5A). In addition, SDF-1 was significantly increased specifically in BOS patients (mean = 95.32 ± 14.06) but not in stable patients (mean = 85.87 ± 5.56) when compared to controls (mean = 60.66 ± 5.20, p < 0.05) (Figure 5.5B).

**Figure 5.5 - Stem Cell-Specific Factors are Variable in BOS vs. Stable Patients.**

(A)  
(B)

In addition, BOS patients had significantly increased plasma concentrations of VEGF (mean = 169.0 ± 27.51), MIG (mean = 3848 ± 844.3), and MIF (mean = 95241 ± 37092) compared to **Table 5.2 – 14-Plex Plasma Cytokine Array Targets**

<table>
<thead>
<tr>
<th>Group 1 Cytokines</th>
<th>Group 2 Cytokines</th>
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<tr>
<td>IL-10</td>
<td>HGF</td>
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<tr>
<td>bFGF</td>
<td>MIF</td>
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<td>GM-CSF</td>
<td>MIG</td>
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<td>IP-10</td>
<td>SCF</td>
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<td>MCP-1</td>
<td>SCGF-β</td>
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<tr>
<td>MIP-1α</td>
<td>SDF-1</td>
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<tr>
<td>RANTES</td>
<td></td>
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<tr>
<td>VEGF</td>
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</tbody>
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healthy controls (mean = 88.48 ± 34.78, 686.4 ± 142.0, and mean = 9782 ± 4627 respectively) (Figure 5.6 A-C)

**Figure 5.6 - Angiogenic and Inflammatory Factors are Increased in BOS Patients**

(A) (B) (C)

**Figure 5.6 Angiogenic and Inflammatory Factors are Increased in BOS Patients.** Plasma cytokine concentrations in control (n=12) vs. stable (n= 16) or BOS patients (n= 23) for (A) VEGF and (B) MIG and (C) MIF. 1-way ANOVA with Dunnett’s post-hoc test.

Comparison of plasma cytokine levels and progenitor cell numbers found a relationship between Monocyte Chemotactic Protein (MCP-1) and CCSP⁺ PBMCs (p=0.032, $r^2=0.14$), while a relationship between Stromal Derived Factor-1 (SDF) and CD45⁺Collagen-1⁺ fibrocytes (p=0.0049, $r^2$ =0.20) was also found (Figure 5.7A-B).
Figure 5.7 – Plasma Protein Concentrations and Progenitor Cell Numbers. (A) Correlation between plasma concentration of MCP-1 and the percentage of CCSP$^+$ PBMCs in stable (n=23), chronic BOS (n=10) and end-stage BOS patients (n=6). (B) Correlation between plasma concentration of SDF-1 and the percentage of CD45$^+$Collagen-1$^+$ fibrocytes in stable (n=23), chronic BOS (n=10) and end-stage BOS (n=6) patients.

As with the previous chapters, a panel of cytokines was quantified, in this case 14, and therefore statistical corrections for multiple comparisons must be made. A Bonferroni correction was applied to the significance level in the form of $\alpha_2 = \frac{\alpha_1}{n}$, where $\alpha_1$ = the originally set alpha (0.05), $\alpha_2$ = the newly defined alpha, and n=the number of comparisons (in this case, 14). From this equation, $\alpha_2 = \frac{0.05}{14} = 0.0036$. When this correction is applied to the correlational analysis of progenitor cells and plasma proteins, the significance is lost for CCSP$^+$ PBMCs and MCP-1, but is maintained for CD45$^+$Collagen-1$^+$ fibrocytes and SDF-1 (Figure 5.7A-B).
SUMMARY OF CHAPTER 5 RESULTS

Data presented in this chapter serve to provide evidence that progenitor cell profiles are skewed towards a fibrotic phenotype in bronchiolitis obliterans patients. Specifically, measurements of an increase in circulating CD45⁺Collagen-1⁺ fibrocytes in conjunction with a loss of CCSP⁺ PBMCs can distinguish BOS patients from stable lung function patients following lung transplant. Suggesting that this is a progressive process, an increased level of fibrocytes was found to be associated with increasing days post-transplant, although this was not true for all patients and an inverse relationship was not found for CCSP⁺ PBMCs and fewer days post-transplant. An important correlation between increased fibrocytes and greater lung obstruction, as measured by FEV1/FVC ratio, was also identified. A similar trend toward less lung obstruction and more CCSP⁺ PBMCs was also noted, which together resulted in a significant relationship between increased lung obstruction and increased CD45⁺Collagen-1⁺/CCSP⁺ cell ratio. These results emphasize the association between bone marrow-derived progenitor cells and the development of lung disease.

As in previous chapters, plasma cytokine levels were analyzed with the aim to determine important protein mediators of cell recruitment. Increases in stem cell-related factors SCF and SDF-1 were measured in post-transplant patients when compared to control subjects, with SDF-1 being specifically increased in BOS patients. Higher levels of the cytokines VEGF, MIF, and MIG were also specifically associated with the diagnosis of BOS. Examination of the relationship between plasma cytokine concentrations and progenitor cell numbers determined unique correlations between MCP-1 and CCSP⁺ PBMCs, while CD45⁺Collagen-1⁺ fibrocytes correlated with the plasma level of SDF-1. Taken together, this evidence is in line with previous conclusions and supports the hypothesis that alteration in progenitor cell number and recruitment can contribute to the development of BOS following lung transplantation.

CHAPTER 5 DISCUSSION

Chapter 3 of this thesis first examined the progenitor cell profiles in end-stage lung disease patients. As presented in these results, it was found that patients diagnosed with BOS who were analyzed at the time of re-transplant had an increased level of CD45⁺Collagen-1⁺ fibrocytes and a loss of CCSP⁺ PBMCs, which resulted in an altered ratio of these two progenitor cell populations when compared to control lung donor samples. Moving forward from this observation, a cross-sectional study design was employed to ask a similar question of patients at various time points following transplant, and to compare patients with stable lung function to those who has
developed BOS. The results of this study, as presented in this chapter, further confirm these initial findings and further suggest that BOS patients can be identified based on an altered progenitor cell profile that is skewed towards a fibrotic phenotype.

For the patients included in the cross-sectional analysis, only those greater than 1-year from transplant were approached for consent into the study and patients with diagnosed infections were also excluded. This was done with the goal of excluding patients who were returning to clinic with exacerbation of other conditions and to study patients who were otherwise healthy. This approach aimed to eliminate patients who may have had altered lung function not due to BOS or altered white blood cell counts that could be attributed a specific cause such as infection.

Along the lines of previous hypotheses, the increased fibrocytes measured in the peripheral blood may contribute to the fibrotic pathology associated with BOS. This may be associated with or aggravated by a parallel loss of CCSP⁺ cell recruitment to damaged lungs. Loss of CCSP⁺ cells in the peripheral blood may be partially attributed to the effects of immunosuppressive drugs that post-transplant patients take routinely. Effects of corticosteroids, including prednisone, are typically anti-inflammatory, through the inhibition of cell adhesion molecule expression, reduction of the inflammatory cytokine cascade, decreasing cell extravasation, and facilitating the apoptosis of activated cells. All of these mechanisms could contribute to a decrease in CCSP⁺ cells measured in the peripheral blood and loss recruitment to injured lung epithelium (307). It has also been shown that the mTOR inhibitor Rapamycin is cytotoxic to circulating endothelial progenitor cells (EPCs) and patients taking Rapamycin post-heart transplant had a reduced EPC colony count (308), although this would like represent only a small number of our patients.

A greater investigation into the effect of immune suppression on lung progenitor cell number and phenotype is an important extension of these initial findings. This comparison could include the individual regimen and dosing data will be collected from both cross-sectional and longitudinal study patients. This approach would aim to determine whether certain immunosuppressive drug regimens alter the progenitor cell profile. Progenitor cell profiles and phenotype will be compared between drug regimen groups to determine associations. The standard triple immunosuppressive protocol of corticosteroid (prednisone), calcineurin inhibitor (cyclosporin), cell cycle inhibitor (azathioprine) could be compared to the use of alternative agents
- tacrolimus or mycophenolate mofeil, in functionally declining patients. Patients receiving mTOR inhibitors, typically rapamycin, could also be compared to patient receiving standard immunosuppression. Dose and length of time receiving each drug should be considered.

Additionally, a multivariable analysis should be performed using known predictors of BOS, such as number of acute rejections, CMV mismatch and infection, HLA mismatch, PGD at the time of transplant, and anti-HLA antibody status to determine if progenitor populations are independently associated with BOS grade.

An important finding presented in this chapter is the relationship between lung function measurements and circulating progenitor cell numbers, as it was found that increased lung obstruction (reduced FEV1/FVC ratio) is associated with increased CD45⁺Collagen-1⁺ fibrocytes. An important extension of this observation would be a longitudinal study to determine predictive value of fibrocyte quantification. This study would require a cohort design where patients begin the study immediately post-transplant (T=0) and are followed serially in time with consistent lung function and progenitor cell measurements. Additional measures of function could include diffusing limit of carbon monoxide (DLCO) and 6-minute walk tests for distance and desaturation. It is hypothesized that changes in cell numbers may pre-date functional changes and may be an important predictor of BOS onset. If this is proven true, progenitor cell profiling could lead to improved patient monitoring and personalization of care.

Measurements of plasma proteins provide further information about the mechanism of progenitor cell recruitment and the complexity of this process. When comparing post-transplant patients versus healthy controls, an increased concentration of SCF was found in both stable and BOS patients. SCF acts through c-kit receptor (CD117), is an important cytokine in hematopoiesis and is highly expressed in the bone marrow (309). Given these properties, SCF has been shown to be an important component of the stem cell niche and a chemotactic factor driving stem cell migration (310). An increase in this factor post-lung transplant indicates an important role for stem and progenitor cells in the chronic rejection process. Although the primary effect of increased SCF may be on hematopoietic cells, stimulation and mobilization of tissue specific stem cell population is also possible. Determining the baseline and changes in expression of c-kit by CCSP⁺ cells and fibrocytes would be an important step in understanding the role of SCF post-transplant. The specific increase in SDF-1 is another important observation that supports previous
observations implicating SDF-1/CXCR4 in fibrocyte recruitment. An increased systemic SDF-1 level can contribute to the mobilization and localization of circulating fibrocytes to the injured lung. Increased level of VEGF and the inflammatory cytokines MIG and MIF were also found. VEGF acts as the key angiogenic factor, stimulating blood vessel formation. Interestingly, expression of VEGFR2 is an important identifier of endothelial progenitor cells. It is probable that both EPCs and CCSP+ epithelial progenitor cell populations are derived from a common precursor and that lineage determination is determined by a number of external and internal signals. Mixed phenotype progenitor cell population expression markers of both endothelial and epithelial lineages may exist and may respond to similar stimuli. Determining VEGFR expression on CCSP+ cells may be an important step in understanding this process and these relationships. Increased MIG was also noted in end-stage lung disease patients and MIF was specifically increased in CF patients (Chapter 3). It should be realised that within the means presented, a range of concentrations exist, representing the complexity of human disease and pathobiology. It is possible that more subtle differences define patients and simply grouping them as BOS or stable may mask important subgroups with unique cytokine expression patterns.

Investigating the relationships between plasma proteins concentrations and progenitor cell numbers again highlights the redundancy of these factors and pathways. As might be expected, circulating fibrocyte numbers strongly correlated with SDF-1 protein levels. Conversely, CCSP+ PBMCs correlated with plasma MCP-1 concentrations. This observation is opposite to the results presented in Chapter 3, where MCP-1 correlated with CCSP+ PBMCs. Of note, MCP-1 was also found to be increased in IPF patients, further suggesting a role for this factor or the cell with which it interacts in the process of fibrosis. It is again possible to suggest that CCSP+ epithelial-like progenitors and CD45+Collagen-1+ fibrocytes are derived from a common precursor, which may have a monocytes phenotype at some stage of development. Response to monocyte chemotactic protein may be variable depending on the microenvironment and level of activation/differentiation for each progenitor population in different physiological contexts.
Chapter 6

Final Discussion

and Future Directions
6.1 DISCUSSION

6.1.1 Synthesis of Results

When this study was first conceived, nothing was known about CCSP$^+$ cells in humans, other than that we were able to measure them in a handful of initial test experiments. With a completely blank slate, we aimed to create an understanding of how these cells participate and change in the context of lung injury. The preliminary hypothesis was that there would be some relationship between underlying epithelial or mesenchymal injury and the progenitor cells believed to contribute to these lineages. Moving from this it was further hypothesized that bone marrow-derived CCSP$^+$ cells would be more associated with epithelial injury and respond to perform a regenerative function, while circulating fibrocytes would become increased or activated in the context of tissue fibrosis and remodelling. When over 150 end-stage lung disease patients were profiled and their progenitor cells quantified in both the bone marrow and peripheral blood, a general agreement with these hypotheses was found. In cystic fibrosis patients, defects in epithelial cells leads to lung damage and further epithelial damage including ciliary collapse and a resulting loss of mucociliary clearance. In these patients an increase in CCSP$^+$ cells was found in both the bone marrow and peripheral blood, suggesting that bacterial infection or epithelial damage can lead to signalling mechanisms to act on exogenous progenitor populations. This is an interesting observation and may provide unique avenues of therapy for these patients. In theory, if CF patients received a bone marrow transplant from healthy individuals, the CCSP$^+$ population may be preferentially recruited to the lungs and could provide regenerative or anti-inflammatory functions. Importantly, this observation was not lost when measured cell percentages were normalized to total leukocyte counts, suggesting that the increase is not simply due to a global inflammatory state in these patients but is specific to CCSP$^+$ cells. In contrast, an increase in fibrocytes was found in IPF and BOS patients, which also supports the predetermined hypothesis. In these patients it is believed that circulating CD45$^+$Collagen-1$^+$ cell can be incorporated into fibrotic tissue where they mature to fibroblast or myofibroblasts, contributing to matrix deposition and remodelling.

In the situation of ischemic reperfusion injury widespread epithelial damage is also observed. In this context and based on animal models of lung injury, it was hypothesized that a measureable increase would be found in epithelial progenitor cells as they respond to this damage. It was indeed found that on average a significant increase in CCSP$^+$ cells could be found in the
peripheral blood of patients 24 hrs post-transplant. This was not accompanied by a parallel increase in fibrocytes and was significant even when normalized to leukocytes. Specificity of this response was also confirmed, as a lack of response was found for live liver donors and recipients. It could then be further hypothesized that CCSP$^+$ cell arriving in the lungs can contribute to tissue repair through paracrine mechanism, either to induce growth and differentiation of local Clara cells or by exerting an anti-inflammatory effect via CCSP itself.

Lastly, it was a natural direction to investigate the function and status of these progenitor cell populations in patients following lung transplant in the context of chronic rejection and development of Bronchiolitis Obliterans Syndrome. Previous evidence has suggested the loss of epithelial regeneration and increase in fibroproliferation may be augmented by an imbalance in the relevant progenitor cell profile. In a cross-sectional analysis of patients greater than 1 year from transplant a skewing of the profile was identified toward increased fibrocytes and a loss of CCSP$^+$ bone marrow-derived cells. This was accompanied by the observed correlation between CD45$^-$Collagen-1$^+$ fibrocytes numbers and time post-transplant. Indeed, the level of lung obstruction (FEV1/FVC) also correlated with increased CCSP$^+$/CD45$^-$Collagen-1$^+$ cells.

In an effort to understand the mechanisms of cell mobilization and recruitment and to generate further testable hypotheses, plasma protein analysis was used to identify change and relationships in pre-selected targets. Important observations in end-stage lung disease patients include an increase in MIF levels in CF patients. It was also found that CCSP$^+$ cells express CXCR4, as do fibrocytes. This increase may implicate MIF signalling through CXCR4, as opposed to the typical SDF-1 pathway, perhaps beginning to explain how 2 populations expressing the same receptor can respond in different fashions. In addition, IP-10 and MCP-1 was found to be increased in IPF patients, while MCP-1 levels correlate with CD45$^-$Collagen-1$^+$ cells in end-stage patients. It was also found that SCGF$^\beta$ levels could predict CCSP$^+$ PBMC and BMCs, suggesting that this factor may have maintenance or chemotactic effect on bone marrow-derived epithelial progenitors.

Following transplant, fast mobilization of bone marrow populations would be required to explain the increase in CCSP$^+$ cells at 24 hrs. A role for GM-CSF was identified in at this time point, a known and clinically applicable mobilizing agent. A relationship between both the absolute percentage of CCSP$^+$ cells and the change from 0-24 hrs was found with the level of plasma GM-CSF. Interestingly, the level SDF-1 also predicted the number of CCSP$^+$ cells at this time.
Lastly, the mechanisms of progenitor cell increase or loss in chronically rejecting and immunosuppressed patients was also investigated with plasma protein array analysis. Of note, in these patients, SDF-1 levels were specifically increased in BOS patients vs. stable lung function patients. In opposition to the end-stage lung disease patients, it was found the MCP-1 levels were predictive of CCSP$^+$ cell numbers and SDF-1 was predictive of CD45$^+$Collagen-1$^+$ fibrocytes.

Taken together, these data may indeed create more questions than they answers. It appears that redundant signals can act on both populations in different contexts. Indeed, the microenvironments and the patient characteristics are quite different in each of the 3 situations studied. A summary of this data suggests that response of progenitor cells can be highly influenced by the microenvironment and previous history, and not only by a specific ligand-receptor interaction.

6.1.2 Potential Pitfalls

It must first be stated that much of this work is observational, which makes absolute conclusions difficult. That being said, when phenomena are first identified much work is necessary to generate further hypotheses and elucidate avenues of more detailed investigation. With the study of human subjects, one must always begin with observational studies before interventional designs can be applied. The aim to do no harm must always be at the forefront of all research. This motivation explains the lack of some important elements of this research, including bone marrow samples following transplant and lung biopsy samples. When first developing concepts and hypotheses, as was the major aim of this work, further invasive sampling could not be justified in this patient population.

A majority of this work relies on antibody-based experimental techniques including flow cytometry and conjugated bead arrays. These techniques are subject to the possibility of non-specific binding and false-positives. We addressed this problem by utilizing various blocking and control procedures, but this problem cannot be fully eliminated. A threshold of non-specific binding was set for all experiments and positive readings were determined above this level.

Variability between underlying disease pathologies must also be considered when interpreting the results and the differences in progenitor cell numbers. Each disease, and in fact each person, represents a unique niche in which the progenitor cells exists. This niche is influenced
by the inflammatory milieu and the resulting pattern of cytokine expression. This can result in
greater cell proliferation and therefore greater cells numbers. The niche can also can also
influence gene transcription and contribute to both lineage determination and protein production.
Fewer progenitor cells may also result from an increase in cells death, lack of cell proliferation, lack
of cell mobilization and/or migration, or increased differentiation to a more mature phenotype.
Measured differences in progenitor cell numbers likely result from a combination of many factors
and it is possible that simply grouping by disease or as pre/post-transplant may mask important
subgroups that are more biologically relevant.

Another potential pitfall of this work is the issue of multiple statistical comparisons.
Corrections based on the Bonferroni method were made and conclusions adjusted accordingly. Yet,
when many variables are simultaneously compared an increased possibility of false-positives is
inherent. Pre-determined hypotheses and analytical approach were used to avoid ‘data-mining’
and to test a specific question as best as possible. It must also be stated that although the data set
as a whole includes a large number of patients, subgroup analysis by disease or time-point can
result in smaller groups being analyzed and therefore reducing the power. This would best be
overcome by using the subgroup analysis to generate hypotheses and then designing new studies
to test specific questions with proper power. In addition, the distribution of the data may also
effect the statistical conclusions. Normality tests based on the D’Agostino-Pearson omnibus test as
outlined in the material and methods (Section 2.7). The results of these analyses were quite
varied. The null hypothesis in these tests assumes that the data is samples from a Gaussian
distribution and determines this by first analysing the skewness and kurtosis of the data set and
then calculates how far each of these experimental values differs from the expected value in a
Gaussian distribution, computing a single p-value from the sum of these discrepancies. On
average, when large data sets were analyzed, such as cell numbers in all end-stage patents or
cytokine measurements across all patients the results were normally distributed but when smaller
subsets analyzes were considered these were often not Gaussian. Based on the recommendations
outlined in the literature provided with of the statistical software used, decisions about when to
use parametric vs. nonparametric tests should be made for an entire data set and not on a set by
set basis. It is also stated that small deviations from a normal distribution will not affect the results
of a t-test or ANOVA (311). Based on these recommendations, parametric statistical tests were
used and reported throughout this thesis.
6.2 CONCEPTS

6.2.1 The Bone Marrow and Peripheral Blood as a Lung Stem Cell Niche

All cells exist in the context of their surrounding microenvironment, including interactions with matrix, other cell populations, and circulating factors. In the context of circulating cell populations that exist mainly in suspension, the relative influence of protein factors in relation to other structural elements is greater. It is also true that this environment can change quickly as cytokine and chemokine levels fluctuate and can be rapidly cleared. It is commonly believed that cells in the circulation can respond to even small fluctuations in their environment or niche, and these fine changes can have large consequences on cell homing and on fate decisions. The investigation of the correct combination of factors required to support self-renewal or directed differentiation is a greater area of research, relevant to many area of basic and translational medicine. In the case of hematopoietic stem cells, similar to the populations described here as they both initially exist in the bone marrow and also in the peripheral circulation, it has been shown that variable cytokine combinations can influence cell function. It was further shown that even small changes in concentration (20ng/ml vs. 300ng/ml) could distinguish between a stimulatory effect versus an inhibitory effect on cell expansion (312). This concept is relevant to the results presented in this thesis, as it was found that the same cytokine could be associated with different cell populations depending on context. It must also be remembered that a ligand binding to its receptor results in downstream signalling and changes in gene expression. These signalling pathways are complex and not fully understood for all cytokines; as such differing effects on cell functions are likely due to variable activation of the receptor or downstream modifications in the resulting singling pathways and relevant transcription factors (313).

The effect of pre-treatment may also be relevant to these results. When comparing cell populations in end-stage patients versus the response to lung transplant, is may be important to consider the effect of long-term exposure to relevant cytokines versus newly induced factors. A differing response to a given factor may be influenced by what pathways have already been activated or perhaps over-activated. In clinical stem cell mobilization, pre-treatment strategies with IL-3 followed by GM-CSF were shown to augment mobilization of circulating progenitors (314). Enhancement has also been shown using antibodies against the β2 integrins LFA-1 and Mac-1 by blocking attachment of cell to bone marrow stroma (315). Similar mechanism may be
involved in lung progenitor cell mobilization and homing and may help explain varying responses between patients based on their history.

Some of these questions may be answered using micro-fluidic culture models where cells are exposed to different factors or different concentrations and then tested for functional response, including differentiation or migration.

6.2.2 Lung Progenitor Cells as a Biomarker

The value of biomarkers is essentially based in generating deeper understanding of a biological process through quantifying an easily accessible surrogate factor. This paradigm has been applied to numerous disease states and relationships identified between cells or soluble factors and relevant outcomes. There are two main aims of this type of work, first to gain greater biological understanding through observing relevant changes and second to develop diagnostic or monitoring tools to better personalise therapy.

The first of these was the major objective of this study. By taking a snapshot of the progenitor cells status across patients and scenarios an understanding of how these changes reflect the cell and molecular biology associated with a disease or condition. In the context of lung progenitor cells, it was also important to simply provide further evidence of their existence and relevance in human disease. Much controversy exists regarding their value and function, and if nothing more, this work supports a role for bone marrow-derived cells in lung disease and repair. The complexity of human disease often makes clear associations difficult to find. In this study, clinical outcomes such as lung function did not reliably predict cell percentages, except for in the BOS study. This may be due to the multiple confounding factors present in these patients that are more powerful predictors of outcome. This does not mean that there is not a biological function underlying the changes observed. Further studies are required to determine the specific function of these cells, which may promote their value as a biomarker.

Secondary to this aim, is the hope of finding important targets that can be modulated for therapeutic benefit. Once reliable relationships between a biomarker and disease outcome have been determine, it is plausible to design targeted therapy to modify the outcome. This approach has already been applied to a mouse model of pulmonary fibrosis, targeting circulating fibrocytes by inhibiting the SDF-1-CXCR4 axis. In this model, the experimental fibrosis was attenuated and outcomes were improved (283).
6.3 FUTURE DIRECTIONS

6.2.1 Longitudinal Studies

The main study design utilized throughout this work is based on a cross-sectional analysis. Typically, this approach is used first to test initial hypotheses and inform power calculations for larger, longitudinal studies. In order to provide greater causative evidence that progenitor cell populations contribute to lung disease or repair, longitudinally designed studies would be a useful further direction.

For the end-stage disease patients, samples were collected at the time of transplant. One advantage of this approach is that there would be less variability within each disease group, as all should have advanced pathology. The drawback is that comparing clinical parameters such as lung function or histology is less informative because the range of outcomes is smaller and most patients will have similar characteristics. By analyzing progenitor cell numbers as disease advances it will be easier to define how the two parameters changes together over time. This type of design would be easiest in disease such as Cystic Fibrosis, where disease onset and diagnosis occur nearly simultaneously. For examples, blood samples and potential cord blood samples could be collected from infants diagnosed with CF and then followed serially in time as disease progresses. This could be designed to coincide with routine clinical evaluation and progenitor cell analysis could be compared to validated markers of disease such as rate of infection, lung function, or hospital stays. With more complex disease like COPD or IPF, diagnosis and disease onset are never simultaneous. Pathophysiological changes occur well in advance of clinical disease symptoms and level of progress is hard to define. In this study design, initially diagnosed patients can be graded based on lung biopsy histology or spirometry and then followed serially in time to determine progression. Again, blood samples could be timed with routine care and measurements. An additional advantage of longitudinal studies is that survival can be used as an important outcome measure. An obvious disadvantage of a longitudinal design is patient loss, as it is always difficult to maintain patient compliance, particularly when many chronic lung diseases progress over several years. A larger demand on resources and personnel could also be considered a drawback.

For the immediate post-transplant studies, a mini-longitudinal approach was used, as samples were collect from the same patient at 0, 24, and 48 hrs. Again, this design was used to test initial hypotheses and generate further ones. A more comprehensive timeline would be useful
to better encompass the changes occurring within the lung following transplant and range of progenitor cell response. A proposed timeline of 0, 2, 6, 18, 24, 36, 48, and 72 hrs would better match spectrum of potential changes. This study has the benefit of \( t=0 \) being exactly defined and all patients can therefore be more easily compared.

Finally, the study of progression to Bronchiolitis Obliterans Syndrome (BOS) would continue from the early post-transplant observations. A cohort study could be designed where all patients begin at 1-year from transplant and are followed at 6-month intervals. Peripheral blood progenitor cell measurements could then be matched to declines in lung function, with time to BOS onset and survival being important outcomes.

A value inherent to longitudinal studies is that changes in care, including prescription medication and pulmonary rehabilitation can be compared to changes in the predicted marker. This will serve to provide greater understanding into the relationships between disease status and progenitor cell profiles. If validated, these measurements may be useful as indicators of response or progression and may be more sensitive than changes in lung function or survival.

A clear disadvantage to both cross-sectional and longitudinal designs is that they are both examples of observational studies and do not directly address questions of cause and effect. Clearly, experimental and interventional studies provide greater power but are often not possible or not justified by sufficient preliminary data, which is why observational studies are first required. Changes in parameters not directly manipulated by the study, such as drug changes or rehab that are prescribed as part of routine care, can be used to study the effect of these interventions on progenitor cell profiles.

6.2.2 Transcriptional Analysis of Progenitor Populations

It was found that significant differences in progenitor cell populations can be measured in patients with different diseases, and that CCSP\(^+\) epithelial-like progenitor cells can respond following lung transplant while CD45\(^-\)Collagen-1\(^+\) fibrocytes are increased in pulmonary fibrosis and BOS. Yet, the true phenotype and lineage of these cells is not fully understood. Changes were measured by flow cytometry and are based on surface marker expression, while changes in signal transduction pathways and gene expression were not addressed. Moving forward from these observations, it can be asked if the cell populations measured are truly the same between patient groups or if genetic or molecular changes may define cellular sub-groups. Simply, what is the
difference between CCSP⁺ cells in CF patients and CCSP⁺ cells in IPF patients? What properties contribute to the changes in total numbers that were quantified in this study?

Microarray genetic analysis could provide a useful and powerful approach to begin answering these questions. A number of cDNA array platforms are available to detect changes in expression levels between samples, including Affymetrix, Agilent, and Illumina technologies. A study could be designed to compare expression patterns between CCSP⁺ BMC or PBMCs and CCSP⁻ cells, which our laboratory has previously done with mouse cells but not human. The total peripheral blood or bone marrow cell population would first require sorting by MACs or FACs to separate the two populations. Some pitfalls of this approach would be cross-contamination of cell populations resulting in a less than pure sample after sorting, as well as the possible alterations that cell sorting and the associated stress or damage might have on the cells and their gene expression.

Once optimized sorting strategies were determined and a baseline expression pattern was defined for CCSP⁺ cells in healthy individuals, comparison arrays could be run to study differences in various end-stage lung diseases and following transplant. Comparisons could include (1) CF vs. IPF vs. COPD vs. Healthy patients, (2) 0 hrs vs. 18 hrs vs. 24 vs. 48 hrs post lung transplant, which could be done or a group of patients with good outcome and a group with poor outcomes, and (3) long-term post-transplant patients who develop BOS vs. those who do not. Of course, a similar set of experiments could be done to examine CD45⁺Collagen-1⁺ fibrocytes in the same conditions. This has also been previously done in mouse samples to compare fibrocytes cultured in serum vs. serum-free media (316) and to investigate changes in gene expression as human fibrocytes differentiate into adipocytes (266).

These experiments would be useful to further define subsets of progenitor cells that may be specifically induced or altered in the context of lung injury or repair. Arrays can also be performed to compare DNA methylation status and patterns between groups or to investigate microRNA (miRNA) expression, many of which are important in controlling lung cell fate and differentiation (317-319). These questions could be applied to either cell population or compared between the two.
6.2.3 Proteomic Analysis of Secreted Products

As much of the work in this thesis was based on the hypothesis that bone marrow-derived cells can be recruited to the lung following injury, it was also believed that protein mediators could be produced by injured tissue and released to the peripheral circulation, acting as signals and guides to migrating cells. Measurements of specifically chosen targets were performed in plasma samples, but this approach was far from comprehensive. The panels of 18 and 14 cytokines chosen for these studies were included based on previous literature or strong biological plausibility that they might be important in the specific processes studied. The advantage of this approach is the specificity and directness of the hypotheses tested, potentially identifying important changes or relationships. The drawback is that novel or unexpected targets would not be identified using a pre-selected approach. Larger-scale proteomics would be a useful step forward in identifying differences between progenitor cell populations, as well as the factors responsible for their maintenance or migration. The value of genomic studies outlined above is great, but these methods do not always provide information about what happening at the protein level.

In vitro culture of CCSP$^+$ vs. CCSP$^-$ BMCs or PBMCs could be used to determine the protein secretome of these cells. Air-liquid interface culture methods could also be used ask the same questions in a more physiologically relevant model compared to adherent cultures. Alternatively, cultured CD45$^-$Collagen-1$^+$ fibrocytes could be compared to cultured lung fibroblasts and secreted factors similarly compared. Identification of factors secreted by these cells would be helpful in understanding the mechanisms these populations use to communicate with resident cells and how aid in tissue repair or damage.

More detailed profiling of patient plasma would be an important step forward when designing patient-specific therapies. If unique protein mediators were identified in specific patient subsets, defined by either disease or outcomes, one could devise pharmacologic interventions that specifically enhance or inhibit cell recruitment depending on desired outcome. More simply, if a protein mediator was identified that specifically acted to direct CCSP$^+$ or CD45$^-$Collagen-1$^+$ cells to the lung, then small molecule activators/inhibitors or antibody based targeting could be used to alter this process.
Chapter 7

REFERENCES
REFERENCES


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APPENDIX 1 –

Consent form: End-Stage Patients
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Title of Study: Identification and characterization of bone marrow-derived lung stem cells

Investigator: Dr. Tom Waddell

Sponsor: Canadian Institute for Health Research

You are being asked to take part in a research study.

Before deciding whether or not you want to be a part of this research study, it is important that you read and understand the following explanation of the proposed study procedures. The following information describes the purpose, procedures, benefits, discomforts, risks and precautions associated with this study. It also describes your right to refuse to participate or withdraw from the study at any time. In order to decide whether you wish to participate in this research study, you should understand enough about its risks and benefits to be able to make an informed decision. This is known as the informed consent process. Please ask the study doctor or study staff to explain any words you don’t understand before signing this consent form. Please take your time to make your decision and make sure all your questions have been answered to your satisfaction before signing this document. Feel free to discuss it with your friends and family.

BACKGROUND

Lung donor organ shortage can cause long waiting times and sometimes people die while waiting for a lung transplant. To help this problem, we would like to better understand the ways in which lung diseases lead to the need for transplant. The adult bone marrow contains cells that can grow and mature into specialized types of cells in the body, such as different lung cells, each with different functions. Some of these cells can travel from the bone marrow or blood into the lungs, suggesting that bone marrow cells may be involved in lung repair or damage. We would like to find out if these cells may be used to treat lung disease. Some of these cells may be useful in repairing damaged lung, while others may contribute directly to the disease process.

PURPOSE

You have been asked to take part in this study because you have a lung disease that requires a lung transplant. This study is designed to try to find out if some rare cells in bone marrow and blood affect lung diseases.

We will look at bone marrow and blood cells from both lung transplant donor and recipients, and also compare the characteristics and number of these cells between different lung diseases. Our primary objective is to learn more about the role of bone marrow cells in relation to lung repair and damage.

Our second question is about how the cells respond following lung transplantation. To answer this question we will look at cells in your blood at two time points following the transplant.
PROCEDURE

During your lung transplant, a small amount (1-2 teaspoons) of your blood will be drawn from a catheter used in every lung transplant, placed by your anaesthetist. While you are under general anesthetic during your lung transplant procedure, a small amount of your bone marrow will be taken from your sternum (breastbone). Taking this sample will add less than 30 seconds to your surgery.

For second part of the study, 2 additional blood draws will be taken while you are in the hospital recovering. These samples will also be 1-2 teaspoons and will be taken within the 2 days following your surgery, at approximately 24 and 48 hours. You will be in either ICU or step down and no additional needles will be required.

RISKS/DISCOMFORT

The blood sampling and bone marrow sampling will pose no additional risk to your health, nor will it affect the function of the transplanted lung or your recovery.

BENEFITS

There are no direct benefits to you by taking part in this study. Information learned from this study may benefit other patients in the future with your disease.

CONFIDENTIALITY

All information obtained during the study will be held in strict confidence. You will be identified with a study number only. No names or identifying information will be used in any publication or presentations. No information identifying you will be transferred outside the investigators in this study or this hospital.

PARTICIPATION

Your participation in this study is voluntary. You can choose not to participate or you may withdraw at any time without affecting your medical care.

COMPENSATION

You will receive no compensation for taking part in this study. If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. The reasonable costs of such treatment will be covered by your health insurance for any injury or illness that is directly a result of participation in this trial. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities.
QUESTIONS

If you suffer any side effects or other injuries during the study, or if you have any general questions about the study, please call the doctor in charge of this study, Dr. Tom Waddell at 416-340-3432.

If you have any questions about your rights as a research participant, please call Dr. R. Heslegrave, Chair of the University Health Network Research Ethics Board at (416) 340-4557. This person is not involved with the research project in any way and contacting him will not affect your participation in the study.

CONSENT STATEMENT

I have had the opportunity to discuss this study and my questions have been answered.

I consent to take part in the study with the understanding I may withdraw at any time without affecting my medical care. I have received a signed copy of this consent form. I voluntarily consent to participate in this study.

_________________________     __________
Study Subject's Name  (Please Print)    Signature   Date

I confirm that I have explained the nature and purpose of the study to the subject named above. I have answered all questions.

Lung Transplant Surgeon _________________________ Signature  _________________________ Date

IF THIS CONSENT HAS BEEN VERBALLY TRANSLATED:

I confirm that I have verbally translated this consent form for the study subject noted above, and in my opinion the study subject has understood what I have explained to them.

_________________________     __________
Name of Translator   Signature   Date

_____________________
Language of Translation   Relationship to Subject (if applicable)
APPENDIX 2 –

Consent form: Post-Transplant Patients
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Title of Study: Identification and characterization of bone marrow-derived lung stem cells

Investigator: Dr. Tom Waddell

Sponsor: Canadian Institute for Health Research

You are being asked to take part in a research study.

Before deciding whether or not you want to be a part of this research study, it is important that you read and understand the following explanation of the proposed study procedures. The following information describes the purpose, procedures, benefits, discomforts, risks and precautions associated with this study. It also describes your right to refuse to participate or withdraw from the study at any time. In order to decide whether you wish to participate in this research study, you should understand enough about its risks and benefits to be able to make an informed decision. This is known as the informed consent process. Please ask the study doctor or study staff to explain any words you don’t understand before signing this consent form. Please take your time to make your decision and make sure all your questions have been answered to your satisfaction before signing this document. Feel free to discuss it with your friends and family.

BACKGROUND

Following lung transplantation, the process of organ damage begins to occur as a result of rejection. To help this problem, we would like to better understand the ways in which cells in your blood respond following your transplant and how they contribute to the rejection process. The adult blood and bone marrow contain cells that can grow and mature into specialized types of cells in the body, each with different functions. Some of these cells can travel from the bone marrow or blood into the transplanted organ, suggesting that circulating cells may be involved in organ repair or perhaps damage. We would like to find how these cells contribute to organ damage in order to better treat disease and maintain organ function.

PURPOSE

You have been asked to take part in this study because you have had a lung transplant. This study is designed to try to find out if some rare cells in your blood affect your outcome after the transplant operation.

We will look at blood cells from both lung transplant recipients and healthy volunteers, to compare the characteristics and number of these cells between them. Our primary objective is to learn more about the role of blood cells in relation to organ repair and damage.
PROCEDURE

One blood sample will be requested from you by ventipuncture. The volume of blood will be approximately 10ml.

RISKS/DISCOMFORT

The blood sampling will pose no additional risk to your health.

BENEFITS

There are no direct benefits to you by taking part in this study. Information learned from this study may benefit other patients in the future with your disease.

CONFIDENTIALITY

All information obtained during the study will be held in strict confidence. You will be identified with a study number only. No names or identifying information will be used in any publication or presentations. No information identifying you will be transferred outside the investigators in this study or this hospital.

PARTICIPATION

Your participation in this study is voluntary. You can choose not to participate or you may withdraw at any time without affecting your medical care.

COMPENSATION

You will receive no compensation for taking part in this study. If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. The reasonable costs of such treatment will be covered by your health insurance for any injury or illness that is directly a result of participation in this trial. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities.
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I consent to take part in the study with the understanding I may withdraw at any time without affecting my medical care. I have received a signed copy of this consent form. I voluntarily consent to participate in this study.

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Lung Transplant Surgeon  Signature  Date

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