ALTERNATIVELY ACTIVATED MACROPHAGE MARKERS ANDIDIOPATHIC PULMONARY FIBROSIS
INVESTIGATING THE ROLE OF DECTIN-1 AS A MARKER OF PROFIBROTIC MACROPHAGES IN THE PROGRESSION OF PULMONARY FIBROSIS

BY

HEMISHA PATEL, B.Sc. (HONOURS)

Thesis Submitted in Partial Fulfillment of the Requirements for the Degree Master of Science

McMaster University © Copyright by Hemisha Patel, June 2018
MASTER OF SCIENCE (2018)  McMaster University
(Medical Sciences)  Hamilton, Ontario

TITLE: Investigating the Role of Dectin-1 as a Marker of Profibrotic Macrophages in the
Progression of Pulmonary Fibrosis.

AUTHOR: Hemisha Patel, B.Sc. (Honours)

SUPERVISOR: Dr. Kjetil Ask, Ph.D.

NUMBER OF PAGES: 56;
ABSTRACT

An estimated 45% of all deaths can be attributed to various chronic fibroproliferative diseases. Idiopathic pulmonary fibrosis (IPF) is the most common form of interstitial lung disease which is characterized by progressive decline in lung function. While the pathogenesis of IPF is not fully understood, alternatively activated macrophages (M2) have been implicated as a key contributor to the fibrotic process. The plasticity of macrophages in vivo challenges the ability to specifically target the M2 macrophage phenotype across species. Previous bioinformatic analysis from our lab identified Dectin-1/Clec7a as a unique marker of M2 macrophages in both human and murine model systems. The expression of the transmembrane receptor Dectin-1 has not been elucidated in the context of pulmonary fibrosis. To prevent the progression of fibrosis by targeting alternatively activated macrophages, we investigated the expression of Dectin-1 in IPF and an experimental model of fibrotic lung disease. Our data demonstrated that while protein expression of Dectin-1 was increased in archived lung tissues of patients with IPF, mRNA expression of this receptor was downregulated in the tissues of these IPF patients. Gene expression of Dectin-1 was shown to be increased in monocyte-derived macrophages, further suggesting a circulatory component contributing to lung fibrosis. As expected, we confirmed that Dectin-1 was highly expressed past the injury phase of the bleomycin-model of induced pulmonary fibrosis which aligns with the increased immune infiltrates at this time point. Preliminary work into the time dependency of the resolution phase of the bleomycin-induced model of lung fibrosis was shown. All in all, our data suggests that Dectin-1 may be a useful marker in
characterizing and differentiating phenotypes of macrophages implicated in the fibrotic process. Future efforts aim to gain insight into the functional requirement of Dectin-1 in the alternative activation of profibrotic macrophages to identify novel therapeutic targets for fibrotic lung disease.
ACKNOWLEDGMENTS

A myriad of people whose unconditional love and support have made the completion of my Master’s a reality.

First and foremost, I would like to extend my biggest thank you to my supervisor and friend, Dr. Kjetil Ask. To someone who has supported me fully in all my endeavors, especially through my moments of panic and excitement – thank you. Your creativity and optimism towards research are unmatched, and this blurb alone cannot even begin to express my gratitude. Whether it is through Bluetooth-powered lab meetings or finishing up diamond quality documents, you have always had my back and that is an aspect of these last two years that I will never forget. Thank you for everything.

Thank you to my committee members, Dr. Jeremy Hirota, Dr. Todd Hoare and Dr. Kim Jones, for their encouragement and constructive feedback throughout this process. It has been a journey and I appreciate that they were able to guide me along the way.

I would like to take the opportunity to thank all the members of the Ask lab, whose friendships I value greatly. From the crazy timepoint experiments and group ventures leading up to the completion of this thesis, it is the support from my colleagues that has kept me driven. The lab was my second home due to their company and support.

Last but not least, a special thank you to my family. In times of uncertainty and big changes, my parents and younger brother have consistently provided me with constant love and encouragement.

I am extremely fortunate for all the guidance I have received from all my friends, family, colleagues and supervisors, without whom none of this would have been possible.
TABLE OF CONTENTS

Introduction .......................................................................................................................... 1
    Interstitial Lung Diseases (ILD) .......................................................... 1
    Idiopathic Pulmonary Fibrosis (IPF) ......................................... 1
    Current Treatments of IPF ......................................................... 3
    Role of Macrophages in Fibrosis .................................................. 4
    Bioinformatic Identification of M2 Macrophage Biomarkers ......... 6
    Dectin-1/Clec7a and Spleen Tyrosine Kinase (Syk) ......... 8

Hypothesis ...................................................................................................................... 10

Objectives & Rationale ..................................................................................................... 10
    Objective 1: To examine Dectin-1 expression in FFPE tissues of IPF patients ........ 10
    Objective 2: To characterize the expression of Dectin-1 expression in circulating cells
                  of IPF patients ........................................................................ 12
    Objective 3: To examine Dectin-1 expression in an experimental model of pulmonary
                  fibrotic disease ..................................................................... 13

Materials and Methods ................................................................................................... 15
    Development of Tissue Microarray (TMA) ........................................ 15
    Monocyte Isolation and Macrophage Differentiation ................. 15
    Extraction of RNA and RNA Quality Assessment .................. 16
    nanoString® Gene Expression Assessment ................................... 17
    Bleomycin-Induced Experimental Model of Lung Fibrosis .......... 18
    Harvesting of Lungs from Mouse Specimens .............................. 18
    Lung Histology .............................................................................. 18
    Immunohistochemistry (IHC) .......................................................... 19
    Image Acquisition and Halo® Quantification .............................. 19
    Single Cell Isolation of Murine Lung Tissues ......................... 19
    Flow Cytometry .............................................................................. 20
    Statistical Analysis ....................................................................... 20

Results .............................................................................................................................. 22
    Objective 1: To examine Dectin-1 expression in FFPE tissues of IPF patients .......... 22
                  Trichrome expression increases in fibrotic regions of IPF patients ........ 22
                  Expression of CD206+ cells increases in fibrotic regions of patients with IPF . 22
                  Dectin-1 expression increases in fibrotic regions of patients with IPF ......... 23
                  Dectin-1 transcript is downregulated in FFPE lung tissues from IPF patients .. 24
    Objective 2: To characterize the expression of Dectin-1 expression in circulating cells
                  of IPF patients ........................................................................ 25
                  Dectin-1 gene expression from human monocyte-derived macrophages ....... 25
Objective 3: To examine Dectin-1 expression in an experimental model of pulmonary fibrotic disease
Flow cytometric expression of Dectin-1 in bleomycin-induced fibrotic model
Expression of Dectin-1\(^+\) macrophages in a murine model of lung fibrosis

Discussion

References

Figures & Tables
LIST OF TABLES AND FIGURES

Table 1: Diagnostic Criteria for AE-IPF.................................................................2

Figure 1: IL-6 addition increased CLEC7A expression in in vitro stimulated BMDMs and THP-1s........................................................................................................7

Figure 2. Area quantification of Trichrome stained TMA........................................47

Figure 3. Cytonuclear quantification of CD206 stained TMA..................................48

Figure 4. Area quantification of Clec7a stained TMA..............................................49

Figure 5. Gene Expression in Typical Fibrotic Response........................................50

Figure 6. Dectin-1 Associated Gene Expression....................................................51

Figure 7. CCL18 transcript levels in cytokine-stimulated human primary macrophages derived from patients diagnosed with IPF.........................................................52

Figure 8. CLEC7A and SYK transcript levels in cytokine-stimulated human primary macrophages derived from patients diagnosed with IPF........................................53

Table 2: Myeloid Lineage Flow Panel....................................................................54

Figure 9. Dectin-1 expression in macrophages phenotyped in the bleomycin-induced fibrotic model via flow cytometry analysis.........................................................55

Figure 10. Masson’s Trichrome Assessment in Time Course of Bleomycin-Induced Model of Lung Fibrosis..............................................................56

Figure 11. α-SMA Assessment in Time Course of Bleomycin-Induced Model of Lung Fibrosis...........................................................................................................57
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>Alpha Smooth Muscle Actin</td>
</tr>
<tr>
<td>AAM</td>
<td>Alternatively Activated Macrophage</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone-Marrow Derived Precursor Murine Cells</td>
</tr>
<tr>
<td>CCL18</td>
<td>Chemokine (C-C motif) Ligand 18</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix Proteins</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin Fixed Paraffin Embedded Tissue</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>GEO</td>
<td>Gene Expression Omnibus</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic Pulmonary Fibrosis</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILD</td>
<td>Intestinal Lung Disease</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral-Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Tumor Growth Factor Beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
</tbody>
</table>
DECLARATION OF ACADEMIC ACHIEVEMENT

This is a declaration that all the work entailed in this Master’s thesis was completed by Hemisha Patel under the supervision of Dr. Kjetil Ask. I conducted the majority of the work involving the design, data collection, and analysis. Background analysis involving bioinformatics was completed by Dr. Anna Dvorkin-Gheva.
INTRODUCTION

Interstitial Lung Disease (ILD)

Interstitial lung diseases (ILDs) refers to a group of diffuse parenchymal lung disorders associated with scarring and inflammation of the lung interstitium (Antoniou et al., 2014). Fibrosis, or scarring of tissues, resulting from an excessive deposition of extracellular matrix (ECM) components is the key pathological process contributing to the development and mortality of a large group of ILDs (Wynn & Ramalingam, 2012). Depending on the nature of the disease, the accumulation of ECM proteins in the lung interstitium, including fibronectin and collagen, can lead to disrupted gas exchange, increased difficulty in breathing, decreased lung compliance and eventual respiratory failure and death (Luca Richeldi, Collard, & Jones, 2017). While ILDs often have no distinguishable underlying causes, they are frequently associated with specific environmental exposures, connective tissue diseases, genetics or drugs (Fischer & du Bois, 2012). Although current research revolving around ILDs aims to better define and characterize the cellular and molecular mechanisms behind the progressive nature of ILDs, their clinical diagnoses are currently classified based on their specific radiological, histopathological, and clinical features (Fischer & du Bois, 2012).

Idiopathic Pulmonary Fibrosis (IPF)

The most common form of ILD is idiopathic pulmonary fibrosis (IPF). IPF is a chronic progressive lung disease which follows an unpredictable clinical course of progression. Upon diagnosis, the median survival of patients with IPF is within 2-3 years (Wynn, 2004). Approximately 15,000 of Canadians are affected with IPF today, while a
projected 6,000 new diagnoses are made yearly. While IPF is usually slowly progressive in nature, some affected patients experience an acute exacerbation of IPF (AE-IPF), which is an accelerated deterioration of respiratory condition alongside fibrosis (D. S. Kim et al., 2006). This rapid decline is often associated with poor outcomes in affected patients and may be triggered by mechanical stress, infections, drug toxicity or other unidentified potentiators (Hyzy, Huang, Myers, Flaherty, & Martinez, 2007). Patients are clinically diagnosed with AE-IPF based on the diagnostic criteria outlined in Table 1 (Lipsi et al., 2018).

Table 1: Diagnostic Criteria for AE-IPF (Lipsi et al., 2018). A list outlining the clinical criteria for the diagnosis of conclusive AE-IPF. Adapted from Lipsi et al. (2018).

<table>
<thead>
<tr>
<th>Diagnostic Criteria</th>
<th>Definition criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous or concurrent diagnosis of IPF</td>
<td>The onset of lung injury within 1 week of a known clinical insult or new or worsening respiratory symptoms</td>
</tr>
<tr>
<td>Acute worsening or development of dyspnea typically &lt; 1 month in duration</td>
<td>Bilateral opacities—not fully explained by effusions, lobar/lung collapse, or nodules</td>
</tr>
<tr>
<td>Computed tomography with new bilateral ground-glass opacity and/or consolidation superimposed on a background pattern consistent with usual interstitial pneumonia pattern</td>
<td>Respiratory failure not fully explained by cardiac failure (left heart failure or pulmonary embolism) or fluid overload (bronchoalveolar lavage)</td>
</tr>
<tr>
<td>Deterioration not fully explained by cardiac failure or fluid overload</td>
<td></td>
</tr>
<tr>
<td>Acute worsening of hypoxemia from baseline arterial blood gas</td>
<td></td>
</tr>
</tbody>
</table>

After the onset of symptoms, a clinical diagnosis of IPF begins by attempting to identify underlying etiologies of the interstitial lung disease (Cottin et al., 2014). More specifically, this requires exclusion of any other known causes of ILD. In the case that no detectable etiology can be identified, a pulmonary function test is performed. An IPF diagnosis requires the presence of a usual interstitial pneumonia (UIP) pattern based on a
high-resolution CT (HR-CT) scan (Cottin et al., 2014). If possible a surgical lung biopsy may be combined with the HR-CT. There is growing evidence that multi-disciplinary discussion between pulmonologists, radiologists and pathologists are needed to examine all clinical evidence related to the ILD in order to accurately diagnose IPF in patients (Cottin et al., 2014).

**Current Treatments of IPF**

Currently, there are two FDA approved drugs for inhibiting the progression of fibrosis: pirfenidone and nintedanib (Karatzas, Bourdakou, & Kolios, 2017). Pirfenidone is a form of oral antifibrotic therapy. In a phase 3 study, IPF patients administered 2403 mg/day of pirfenidone were demonstrated experienced reduced disease progression reflected by improved progression-free survival and reduced decline in vital capacity (King et al., 2014). Moreover, treatment with pirfenidone was associated with fewer deaths as outcomes and patients administered the drug experienced an acceptable side-effect profile (King et al., 2014). The cellular and molecular mechanisms underlying pirfenidone have not been identified. The second approved drug is the tyrosine kinase inhibitor, nintedanib, which has similarly been shown to reduce the decline of lung function through a measure of forced vital capacity (L. Richeldi, 2014). Nintedanib functions by inhibiting multiple tyrosine kinases including PDGF, VEGF and FGF (Bonella, Stowasser, & Wollin, 2015). Despite the availability of these therapies, they have been shown to have insufficient levels of therapeutic benefit in comparison to the significant side effects experienced by patients receiving these treatments. The most
effective and viable treatment option to directly improve the respiratory capacity of IPF patients and those with end-stage chronic respiratory failure is lung transplantation (Thabut & Mal, 2017). Transplantation, however, is evidenced by a median survival rate of approximately 5.8 years. In addition, patients with lung transplants often experience subsequent complications. For instance, chronic lung allograft dysfunction (CLAD) develops in approximately 50% of lung transplant recipients five years after the lung transplant (Thabut & Mal, 2017).

**Role of Macrophages in Fibrosis**

Although the pathogenesis of IPF remains to be fully elucidated, it evidently includes a chronic inflammatory response in the lungs which triggers remodeling of healthy tissue through the accumulation of extracellular matrix components (Bringardner, Baran, Eubank, & Marsh, 2008; Gifford, Matsuoka, Ghoda, Homer, & Enelow, 2012). One of the hallmark features of fibrotic lung disease is the accumulation of myofibroblasts, which synthesize and deposit ECM as they are primary modulators in the wound healing process (Coward, Saini, & Jenkins, 2010). Fibroblasts differentiate into myofibroblasts upon receiving cytokine signals, such as transforming growth factor-beta (TGF-β) with endothelin-1 (Abraham, Eckes, Rajkumar, & Krieg, 2007). More recently, macrophages have become an active area of research, as these immune cells produce profibrotic cytokines involved in this fibroblast to myofibroblast differentiation process (Sindrilaru & Scharffetter-Kochanek, 2013).

Macrophages are phagocytic antigen-presenting cells vital in both innate and adaptive immunity. As myeloid immune cells, their receptors respond to pathogenic
ligands originating from bacteria, viruses, and parasites (Murray & Wynn, 2011). These cells play vital roles in the metabolism, development, and maintenance of homeostasis while contributing to a wide range of diseases (Byrne, Maher, & Lloyd, 2016).

Historically, macrophages have been classified as either acquiring a pro-inflammatory or M1 (classically activated) phenotype or a pro-fibrotic or M2 (alternatively activated) phenotype (Murray & Wynn, 2011). However, macrophages are extremely plastic cells capable of being polarized by the stimuli in the surrounding microenvironment, causing phenotypic shifts in these cells (Braga, Agudelo, & Camara, 2015). Current research using in vivo studies suggest that macrophage phenotypes lie on a wide spectrum, whereby macrophages can exhibit qualities of varying phenotypes concurrently to different degrees (Sica & Mantovani, 2012). For the purposes of this thesis, “M2” macrophages refer to alternatively activated macrophages in vitro as opposed to “M2-like” macrophages observed in vivo settings. These “M2-like” macrophages in vivo are exposed to a variety of external factors and fluctuating cytokine milieu that cannot be mimicked using in vitro settings, where select and known concentrations of recombinant proteins are exposed to homogenous cultures of macrophages over set time-periods. Specifically, through in vitro studies, undifferentiated macrophages can be polarized to the M1 phenotype through interferon-gamma (IFN-γ) and bacterial lipopolysaccharide (LPS) exposure, while the M2 macrophage can be polarized by stimulating naïve macrophages with the cytokines IL-4 and IL-13 (Weischenfeldt & Porse, 2008).

Typically after injury, during the early inflammatory response, M1 macrophages predominate and increase the phagocytic capacity of the cells while producing
proinflammatory cytokines such as TNF-α, IL-12, and IL-23 (Braga et al., 2015). After the inflammatory phase, M2 macrophages involved in the wound healing process release anti-inflammatory cytokines such as IL-10 to resolve inflammation and produce pro-fibrotic factors such as TGF-β which stimulate the differentiation of fibroblasts, epithelial cells, and progenitor cells to facilitate tissue repair for the restoration of healthy tissue architecture post-inflammation (Wynn & Vannella, 2016). If this wound-healing and resolution process become dysregulated, excessive profibrotic factors in the milieu can result in eventual fibrosis of the tissues (Wynn & Vannella, 2016). Therefore, specific targeting and manipulation of the profibrotic M2 macrophage phenotype would enable the prevention of fibrotic progression.

**Bioinformatic Identification of M2 Macrophage Biomarkers**

There currently exists a lack of knowledge surrounding M2 macrophage markers present in both human and murine model systems, which can be commonly measured for more direct translational studies (Roszer, 2015). With the efforts of Pavithra Parthasarathy, a previous Master’s student in our lab, and Dr. Anna Dvorkin-Gheva from McMaster’s Immunology Research Center in Hamilton, Ontario, this issue has been addressed using a bioinformatics approach. Gene Expression Omnibus (GEO)-deposited datasets of murine and human macrophage phenotype gene signatures were used to identify biomarkers for the M2 macrophage phenotype which were a) unique to the profibrotic phenotype, in comparison to M1 and naïve (M0) macrophages, and b) shared between both human and mouse model systems. Six genes were found to be significantly
upregulated for the M2 phenotype in both human and murine systems. *CLEC7A* was one of the identified genes which encodes the membrane receptor Clec7a or Dectin-1. Upon identification of Dectin-1 as a potential marker for profibrotic macrophages, we validated this finding using nanoString® assessment and characterized the expression of Dectin-1 *in vitro* in both murine (bone marrow-derived macrophages) and human (THP-1 monocytic cell line) systems to be specifically expressed on alternatively activated (M2) macrophages stimulated with IL-6 as shown in Figure 1 below.

**Figure 1.** IL-6 addition increased CLEC7A expression *in vitro* stimulated BMDMs and THP-1s. Bone marrow-derived macrophages and THP-1 macrophages were cultured *in vitro* under polarizing conditions. IL-4 and IL-13 stimulated M2 macrophages (middle column), M2+IL-6 stimulated macrophages (right column) or unstimulated macrophages (left column). Cells were stained for Clec7a and analyzed through flow cytometry. M2+IL-6 stimulated BMDMs and THP-1s showed increased Clec7a expression compared to M2 or control conditions.
This *in vitro* validation experiment in both THP-1 cells and BMDMs aligned with our *in vivo* findings where the addition of IL-6 in the bleomycin-induced model of fibrosis was associated also with increased alternatively activated macrophages in lung tissues (Ayaub et al., 2017; Ayaub et al., 2016). IL-6 is a pleiotropic inflammatory cytokine which has been demonstrated to enhance the M2 polarization process via increased expression of the IL-4 receptor (Mauer et al., 2014). In IPF, IL-6 has been shown to be associated with progressive fibrotic disease and acute exacerbations in IPF patients, while blocking IL-6 has been associated with reduced pulmonary fibrosis (Collard et al., 2010; Le et al., 2014).

**Dectin-1/Clec7a and Spleen Tyrosine Kinase (Syk)**

Dectin-1, encoded by *CLEC7A*, is a small transmembrane receptor that is a member of the C-type lectin family of pattern-recognition receptors (PRRs) (Goodridge et al., 2011). As this receptor specifically recognizes and binds to β-glucans polysaccharides derived from the cell walls of fungi and yeast, it has been characterized for its role in antifungal immunity (Brown et al., 2003). Dectin-1 is predominately expressed on macrophages, dendritic cells, and neutrophils (Taylor et al., 2002). Upon ligation of β-glucan, Dectin-1 recruits the adaptor protein CARD9 to phosphorylate spleen tyrosine kinase (Syk) which in turn initiates a signaling cascade which induces phagocytosis and leads to the activation of nuclear factor-κB (NF-κB) and MAPK (Kimura et al., 2014).

The Dectin-1 Syk mediated pathway has been demonstrated to be activated and involved in the alternative activation of macrophages as Dectin-1 was upregulated *in vitro* within 4 hours when stimulated with IL-4 and IL-13, cytokines which induce alternative
activation of macrophages (Willment et al., 2003). Moreover, the activated Syk pathway has also been implicated in renal interstitial fibrotic disease where it was demonstrated that suppressing Syk in an experimental mouse model of renal fibrosis inhibits the progression of kidney fibrosis, shown by a reduction in mRNA expression of Arginase-1 (murine M2 macrophage marker) (Chen et al., 2016). While the Dectin-1 pathway has been shown to be involved in the bleomycin-induced model of skin fibrosis, Dectin-1 signaling and receptor expression has yet to be investigated in the context of pulmonary fibrosis (Pamuk et al., 2015).

Combined, this information suggests that increased levels of IL-6 could potentiate and further activate M2-like, profibrotic macrophages and further contribute to the progression of fibrosis in patients. The overall hypothesis of our lab is that M2-like, profibrotic macrophages are required for the progression of pulmonary fibrosis and constitute a valid therapeutic target to prevent progression of fibrotic lung disease. However, testing this hypothesis is challenging due to the lack of consistent reliable markers between the two species. Here, I have attempted to contribute to increasing our understanding of M2-like profibrotic macrophages in fibrotic disease by characterizing Dectin-1 expression both in human and murine systems.
HYPOTHESIS

Based on the previous data and observations in the literature, I hypothesize specifically that Dectin-1 is expressed on alternatively activated macrophages (AAMs) both in patients with fibrotic lung disease and in experimental models of lung fibrosis. To address this hypothesis, I will examine the expression of Dectin-1 in the lungs of patients diagnosed with IPF and in an experimental model of lung fibrosis. Further, as pulmonary AAMs are derived from monocytes stemming from the bone marrow and through the circulation, I hypothesize that Dectin-1 will be expressed in primary monocytes exposed to macrophage stimulating factor and profibrotic cytokines. It is believed that the characterization of Dectin-1 expression in both human and murine systems of fibrotic lung disease may identify a potential biomarker for alternatively activated macrophages in fibrosis that could ultimately be used as a tool to target cells expressing this receptor to prevent the progression of pulmonary fibrosis. To address this specific hypothesis, I have designed three main objectives outlined below.

OBJECTIVES & RATIONALE

Objective 1: To examine Dectin-1 expression in FFPE tissues of IPF patients

In order to effectively determine whether Dectin-1 is a biomarker profibrotic (M2-like) macrophages, the bioinformatic analysis from which the receptor was derived must be verified. Since the bioinformatic analysis was conducted using in vitro data of macrophages stimulated with only IL-4 to produce an M2-like phenotype, it is necessary to confirm that Dectin-1 is clinically relevant. Here, a previously developed tissue
microarray (TMA) from our lab consisting of formalin fixed paraffin embedded (FFPE) lung tissue cores from human patients with varying fibrotic diseases was used as a tool to examine the protein expression of different biomarkers of interest. Positivity of fibrosis in the fibrotic cores of the TMA would first be confirmed using Masson’s Trichrome staining. This would be followed by examining the expression of the target cell of interest, profibrotic macrophages, using the well-characterized macrophage mannose receptor marker CD206 in the TMA (Yu et al., 2016). CD206 has been demonstrated to be highly selective towards profibrotic macrophages in both human and murine systems (Ploeger et al., 2013; Stifano & Christmann, 2016).

Assuming the TMA shows positive expression for both fibrosis and profibrotic macrophages in the fibrotic cores, Dectin-1 protein expression in FFPE lung tissues from patients with IPF and other fibrotic diseases would then be compared to those from control patients. This aim is especially important in that while Syk signaling pathways downstream of Dectin-1 have been shown to be implicated in murine fibrotic diseases such as renal interstitial fibrosis (Ma, Blease, & Nikolic-Paterson, 2016) and scleroderma (Pamuk et al., 2015), it has yet to be characterized in human patients with pulmonary fibrotic diseases.

As protein expression cannot be directly predicted by gene expression at the mRNA level due to highly variable post-transcriptional and translational regulations, it is important to differentiate the expression of Dectin-1 at both protein and transcript levels (Diaz-Munoz & Turner, 2018). Thus, sensitive mRNA quantification in human fibrotic diseases will be performed via nanoString ® assessment using extracted RNA from FFPE
human lung tissues of 10 control patients and 12 IPF tissues. As a positive control to ensure that the gene expression analysis is reliable, extracellular matrix and macrophage-related genes typically upregulated in the fibrotic process, including MMP7, CCL18, Col1A1, and Col3A1, will be assessed in FFPE tissue cores of both IPF and control patients. Using the same extracted samples of RNA, the transcript level of CLEC7A and SYK which are the genes of interest will then be assessed using the same nanoString® gene code set.

**Objective 2: To characterize the expression of Dectin-1 expression in circulating cells of IPF patients**

In line with Objective 1, the clinical relevance of Dectin-1 must be examined before establishing the receptor as a potential biomarker for profibrotic macrophages. Since there is increasing evidence that circulating monocytes contribute to the fibrotic process as the population of these immune cells were shown to be increased in AE-IPF (Mathai et al., 2010; Moeller et al., 2009), gene expression will also be assessed from cultured primary macrophages derived from the whole blood of human patients with fibrotic lung diseases. As a positive control for the method of gene expression assessment, the RNA extracted from this *in vitro* experiment will first be assessed for chemokine ligand 18 (CCL18) transcript expression in patients with IPF. The gene for CCL18 codes for cytokine CCL18 which is secreted by antigen presenting cells such as macrophages (Chang et al., 2010). CCL18 gene expression is expected to be higher in IPF patients since its production by naïve macrophages has been demonstrated to be highly upregulated in the presence of fibroblasts and collagen, often present in a profibrotic
milieu (Schraufstatter, Zhao, Khaledyanidi, & Discipio, 2012). Gene expression analysis of CLEC7A and SYK will then be assessed using the same nanoString® gene code set in patients with IPF.

**Objective 3: To examine Dectin-1 expression in an experimental model of pulmonary fibrotic disease**

Considering Dectin-1 was bioinformatically derived as a *shared* marker in both human and murine models, a similar comparison of Dectin-1 expression must be conducted in a relevant experimental murine model as was done with the human fibrotic disease samples in Aims 1 and 2. In this case, the well-characterized method of inducing fibrosis through the administration of bleomycin in C57BL/6 mice was employed as an experimental model for recapitulating key features of pulmonary fibrosis. Bleomycin administration leads to acute lung injury associated with inflammation, eventually resulting in a fibroproliferative phase characterized by excess ECM deposition (Redente et al., 2011). During the fibrotic phase of the bleomycin model, approximately 14 days post-administration, it has been demonstrated that the profibrotic CD206+ macrophage phenotype is elevated (Ji et al., 2014).

To conduct a similar assessment of Dectin-1 receptor expression through the progression of the bleomycin-induced experimental model of fibrosis, single digested mononuclear lung cells will be isolated from C57BL/6 mice to characterize Dectin-1+ cells in different populations of macrophages through flow cytometric analysis. Additionally, the progression of fibrosis in the bleomycin-induced model will be
examined through typical immunohistochemistry indictors of fibrosis, including Masson’s Trichrome staining depicting the level of extracellular matrix deposition in the murine lung tissues, and α-SMA staining which indicates myofibroblast infiltration in these tissues.
MATERIALS AND METHODS

Development of Tissue Microarray

A 304-core tissue microarray (TMA) containing 26 patients diagnosed with IPF and 17 control patients was developed in our lab by previous Master’s student Karun Tandon, alongside Dr. Anmar Ayoub, Dr. Nathan Hambly, Dr. Jean-Claude Cutz and Dr. Asghar Naqvi. Human lung tissues were obtained from a biobank of pulmonary diseases located at St. Joseph’s Hospital in Hamilton, Ontario. From these tissues, 0.6mm fibrotic and non-fibrotic cores were selected to create the TMA. Similarly, 1mm cores from both fibrotic and non-fibrotic regions were stored in RNA-free tubes for RNA extraction.

Monocyte Isolation and Macrophage Differentiation

Approximately 20-30mL of fresh whole blood was obtained from control and IPF patients into BD™ Vacutainer™ Plastic K2EDTA tubes (Thermo Fisher Scientific, REF# 12947666). Upon collection, patient blood was processed within 2-3 hours. CD14+ monocytes were isolated directly from the whole blood using the EasySep™ Direct Human Monocyte Isolation Kit (STEMCELL Technologies Inc., REF #19669). The solution of isolated CD14+ monocytes was centrifuged at 1200 rcf for 7 minutes at room temperature, with the brake switched on. The supernatant was then decanted, and the pellet of monocytes was resuspended in approximately 5mL of RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, 1% P/S and 25 µg/mL fungizone. The CD14+ monocytes were next counted using the Countess Automated Cell Counter, and the cell density was adjusted to 40 x 10^4 cells/mL for further monocyte enrichment using complete RPMI
1640. 50uL of monocytes were plated on flat-bottom 96-well plates at a concentration of 40,000 cells per well. The CD14+ isolated monocytes were treated with 50 ng/mL recombinant human M-CSF (PeproTech, REF #300-25) for 6 days, with a supplementation of complete RPMI 1640 medium with human M-CSF after 3 days. The differentiated macrophages were then primed with M-CSF (50ng/mL) with or without treatment of 10ng/mL of TGF-β (R&D Systems, REF #240-B-002) for 24 hours. The cultured macrophages were then stimulated for 24 additional hours with 10ng/mL of IL-4 (STEMCELL Technologies Inc., REF# 78045-1) or IL-6 (PeproTech, REF# 200-06). Cells are subsequently lysed with RNA lysis buffer and stored at -80°C for RNA extraction.

**Extraction of RNA and RNA Quality Assessment**

Total RNA from both cultured macrophages and FFPE tissue cores were extracted in preparation for nanoString gene expression analysis. Two or three one-millimeter FFPE lung tissue cores from the same patient are pooled into an Eppendorf tube where paraffin dissolver is used to deparaffinize the tissue core overnight. The tube was centrifuged, and the supernatant was decanted to wash the pelleted tissue with 100% ethanol. This step was repeated before allowing the open tube to air dry at room temperature. The lysate was processed by isolating its RNA using the NucleoSpin® total RNA FFPE kit (Macherey-Nagel, REF# 740982). Similarly, total RNA was isolated from the lysate of 100000 cultured cells in one Eppendorf using the NucleoSpin® RNA Plus kit (Macherey-Nagel, REF# 740984). The concentration of extracted RNA from both cultured macrophages and
FFPPE tissue cores was measured using Nanovue Plus spectrophotometer (GE Healthcare Life Sciences). The quality of extracted RNA was examined using the Bioanalyser 2100 (Agilent Technologies) where 400-500ng/mL of isolated RNA with an RNA integrity number (RIN) of 2 for FFPE tissue samples and 100ng/mL of isolated RNA with a RIN of 9 for freshly cultured cells was sufficient for nanoString assessment.

**nanoString® Gene Expression Assessment**

All isolated RNA was analyzed for gene expression using NanoString Technologies. The transcript expression levels of 48 human genes in the mRNA derived from cultured cells and FFPE tissue cores was quantified via NanoString’s nCounter technology. At least 10μL of 100ng/μL of total sample RNA was used for successful hybridization with the reporter and capture probes. Upon 21 hours of hybridization at 65°C, excess probes are removed, and the hybridized samples are immobilized for data collection where the expression of each individual target molecule is counted by the nCounter platform (Nanostring Technologies, Seattle, WA, USA). The gene expression data collected through this procedure are further analyzed using the nSolver Analysis Software v. 1.1, where the transcript counts were normalized to the positive controls and seven housekeeping reference genes, ACTB, B2M, PGK1, POLR2A, PPBI, RPLP2, UBC. All nanoString data are expressed as a fold change relative to a selected control and graphed as relative nanoString counts.
Bleomycin-Induced Experimental Model of Lung Fibrosis

10 to 12-week-old wild-type female mice (C57BL6/J background) were obtained from Jackson Laboratory and were housed at McMaster University in Hamilton, Ontario. Experimental pulmonary fibrosis was induced by intubating mice with 0.04U of bleomycin solubilized in 50uL of sterile saline, under isoflurane anesthesia (Hospira Healthcare Corporation). Control groups were intubated with sterile saline. Weights of all mice were recorded weekly and animals will be sacrificed at day 0 (baseline), day 14 (inflammatory and injury phase), day 21 (fibrotic stage) and day 35 (resolution phase).

Harvesting of Lungs from Mouse Specimens

Upon measurement of lung function as described above, anesthetized mice were sacrificed by severing the descending aorta. After exsanguination, the lungs were excised and washed with 600uL of phosphate-buffered saline to collect bronchoalveolar lavage fluid (BALF) for cell differential analysis. The four lobes of the right lung were rinsed with PBS and isolated with surgical suture before being excised and snap-frozen in liquid nitrogen. The frozen right lobes were transferred into a –80°C freezer for lung homogenization and RNA isolation assays. The left lung attached to the cannula was excised for histological analysis as described below.

Lung Histology

10% formalin was used to inflate the left lobe of each lung at a pressure of 30 cmH2O for 3-5 minutes. The lungs were then stored in 10% formalin for 48 hours before being
transferred into 70% ethanol. Each lobe was dissected into superior, middle and inferior sections. The sections were cut into 4 µm sections after being embedded in paraffin wax. The tissue sections were then stained with hematoxylin and eosin (H&E), alpha-smooth muscle actin (α-SMA) and Masson's Trichrome green by the John Mayberry Histology facility within the McMaster Immunology Research Centre.

**Immunohistochemistry (IHC)**

All histological processing and staining, including cutting and immunohistochemistry staining of FFPE tissues, was conducted by the John Mayberry Histology facility within the McMaster Immunology Research Centre. On a Bond RX (Leica), tissues were stained with CD206 (Abcam REF# AB64693) at a dilution of 1:8000 in PBS with 1% BSA. Dectin-1/CLEC7A antibody was ordered from Biolegend (REF #355402).

**Image Acquisition and Halo® Quantification**

The stained tissue slides were captured using an Olympus VSI 120 Slide Scanner microscope system (Olympus) at 200x resolution. Quantification of specific stains was performed by HALO™ Image Analysis Software through cytonuclear and area quantification modules (Halo Plus 3 WS, Indica Labs).

**Single Cell Isolation of Murine Lung Tissues**

Upon isolation of right lung from each mouse, the four lobes were finely minced using sharp surgical scissors on a piece of foil before being transferred into falcon tube with
5mL of digestion media made up of RPMI 1640 medium with 0.3mg/mL collagenase D and 2uL of DNase I for 2 hours at 37°C with agitation. The digested lungs were then crushed through a 100 µm filter (BD Falcon, Franklin Lakes, NJ, USA), centrifuged at 15000 rpm for 7 minutes before resuspension in FACS buffer (0.1% BSA in PBS) for flow cytometry analysis.

**Flow Cytometry**

Macrophages from single lung cells isolated from mice exposed to bleomycin were phenotyped by staining for cell surface markers. At least one million lung mononuclear cells from each mouse were resuspended in an antibody master mix containing the antibodies listed in Table 2. Table 2 indicates the myeloid panel of antibodies used to characterize the phenotypes of macrophages in the digested lung cells through flow cytometric analysis. Flow cytometric data were collected using LSRII and FACSDiva software (BD Biosciences) and the analysis was performed using the FlowJo software (version 10.4.2).

**Statistical Analysis**

All results are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 7.03 (GraphPad Software Inc.). Where two groups were compared, a normality test was conducted before using the appropriate two-tailed parametric or non-parametric t-test. Two or more groups and/or conditions were compared using a one-way analysis of variance (ANOVA), followed by Tukey’s multiple
comparison test. All preliminary mouse experiments were designed to use at least 5 mice per group. All cell culture experiments were performed in a 96-well plate with 4 or more biological replicates per condition. A p-value < 0.05 was considered as statistically significant.
RESULTS

Objective 1: To examine Dectin-1 expression in FFPE tissues of IPF patients

Trichrome expression increases in fibrotic regions of IPF patients

Extracellular matrix content was quantified in the TMA through Masson’s Trichrome staining to first confirm the level of fibrosis in the fibrotic cores of the TMA before using it as a tool to quantify the expression of other biomarkers (Figure 2A). The green stained collagen was selected for using the Halo® analysis software (Figure 2B). Trichrome quantification was grouped and quantified according to control, IPF (non-fibrotic regions) and IPF (fibrotic) patients. As expected, the percent of positively stained collagen area out of the total area of each individual core is significantly increased in the fibrotic regions of patients with IPF compared to both non-fibrotic regions of these IPF patients and control patients (Figure 2C). There is approximately a two-fold increase of positively stained Trichrome areas in fibrotic regions of IPF patients compared to the control patients (Figure 2C).

Expression of CD206+ cells increases in fibrotic regions of patients with IPF

To determine if there is increased expression of alternatively activated (M2) macrophages in the fibrotic regions of our TMA, individual cells stained positively for CD206, a well-characterized profibrotic macrophage marker, were quantified (Yu et al., 2016). A cytonuclear quantification method was used which prompts the Halo® analysis
program to differentiate the degrees of staining in each individual core. A representative core stained with CD206 in brown is shown before cytonuclear markup using the Halo® software (Figure 3A). The markup for each stained core differentiates blue cells (negative stain), yellow cells (low positive stain), orange cells (medium positive stain) and red cells (high positive staining) for quantification (Figure 3B). As expected, the percent of CD206 positive stained cells or the percent of alternatively activated macrophages out of the total number of cells per core was significantly increased in patients with IPF when compared to control patients (Figure 3C). CD206 expression was also quantified in nine IPF patients based on fibrotic and non-fibrotic cores. In line with our expectations, the percent of CD206+ cells in each core was increased in the fibrotic regions of these nine patients affected with IPF when compared to their non-fibrotic cores (Figure 3D).

**Dectin-1 expression increases in fibrotic regions of patients with IPF**

In order to examine Dectin-1 expression in fibrotic tissues of patients with IPF, the area of positively stained Dectin-1 areas were quantified relative to the area of each individual core in the TMA. Variability of Dectin-1 staining in brown is shown when comparing a TMA core taken from a control patient and a fibrotic core from an IPF patient (Figure 3A). Consistent with my hypothesis, the dectin-1 or Clec7A positive areas were significantly increased in the fibrotic regions of patients with IPF compared to the non-fibrotic regions of these patients and control patients (Figure 4B). There is high
variability in both non-fibrotic and fibrotic cores from IPF patients in terms of area quantification of Dectin-1 (Figure 4B).

Dectin-1 transcript is downregulated in FFPE lung tissues from IPF patients

To assess the expression of Dectin-1 at the transcript level in human fibrotic lung disease, isolated RNA from FFPE lung tissues of 10 control patients and 12 IPF patients was examined using a 48 gene code set through nanoString® gene analysis technology. The reliability of this gene expression analysis was confirmed by examining the gene expression of extracellular matrix and macrophage-related genes including MMP7, CCL18, Col1A1 and Col3A1 in our human lung tissues. MMP7, which is a profibrotic mediator highly expressed in IPF lung tissues, was found to be significantly increased in our IPF tissue samples compared to the control patients as expected (Figure 5A) (Pardo, Cabrera, Maldonado, & Selman, 2016). Similarly, CCL18 which is a cytokine that is constitutively expressed by antigen-presenting cells such as macrophages in profibrotic environments was also significantly increased in IPF patients compared to control patients (Figure 5B) (Chang et al., 2010). Both Col1a1 and Col3a1 are genes coding for extracellular matrix components type 1 and type 3 collagen respectively which are upregulated in the fibrotic process (Gimenez et al., 2017). As expected, Col1a1 and Col3a1 were significantly increased in patients with IPF compared to control patients (Figure C and D).

Upon confirmation of this gene analysis method via nanoString® Technologies through our positive control genes, the genes of interest CLEC7A/Dectin-1 and SYK
were both examined for their expression using the same tissue samples. Against our hypothesis, it was found that the CLEC7A transcript was significantly downregulated in patients with IPF compared to the tested control patients (Figure 6A). Additionally, the gene for SYK coding for the Syk protein downstream of the Dectin-1 receptor was unchanged in both IPF and control patients (Figure 6B).

**Objective 2: To characterize the expression of Dectin-1 expression in circulating cells of IPF patients**

**Dectin-1 gene expression from human monocyte-derived macrophages**

To determine whether Dectin-1 is increased in macrophages derived from patients with IPF, we isolated CD14+ from the whole blood of four IPF patients before culturing them *in vitro* under profibrotic conditions. The isolated monocytes were differentiated into macrophages by exposing the cells with human recombinant M-CSF for 6 days and then stimulated with TGF-β for 24 hours to prime the cells in a profibrotic milieu. The naïve macrophages were then polarized into profibrotic macrophages via stimulation of either IL-4 alone, IL-6 alone or using a combination of IL-4 and IL-6 for 24 additional hours. As a positive control, the transcript levels of CCL18 were first examined in these circulating monocyte-derived macrophages. Figure 7 shows that CCL18 transcripts were significantly increased with the both IL-4 and IL-6 stimulation combined compared to naïve macrophages at baseline and compared to IL-4 or IL-6 stimulated macrophages.
The addition of IL-4 or IL-6 alone did not significantly increase CCL18 transcript levels compared to naïve macrophages with or without TGF-β (Figure 7).

Upon confirming that this in vitro set up allows for a reliable method in examining gene expression in cultured macrophages from the circulation based on the gene expression of CCL18, the transcript levels of our genes of interest CLEC7A and SYK were assessed. Transcripts of CLEC7A were found to be present slightly higher in naïve macrophages primed with TGF-β compared to those at baseline which were cultured without TGF-β but this was an insignificant increase (Figure 8A). While there was no significant change in CLEC7A transcript expression with IL-6 stimulation alone compared to naïve macrophages with or without TGF-β, stimulation with IL-4 alone significantly increased the expression of CLEC7A in macrophages cultured without TGF-β (Figure 8A). There was no significant change in transcript levels of SYK in monocyte-derived macrophages with or without TGF-β and when polarized with IL4 or IL-6 cytokines (Figure 8B).

Objective 3: To examine Dectin-1 expression in an experimental model of pulmonary fibrotic disease

Flow cytometric expression of Dectin-1 in bleomycin-induced fibrotic model

To determine if Dectin-1 expressing macrophages are increased in the bleomycin-induced experimental model of fibrosis, bleomycin (0.04 units) or the negative control phosphate-buffered saline (PBS) was administered intratracheally to C57BL/6 mice (n=5
for each group). At 7 and 14 days post-administration, typically, there is significant inflammation and the early stage of fibrosis begins in the bleomycin model respectively (Ayaub et al., 2017). Day 21 represents the fibrotic phase which eventually resolves with time by Day 35 (Ayaub et al., 2017). Lung mononuclear cells were isolated from digested lungs on day 7 and day 14. These cells were stained with surface markers of the myeloid lineage including CD11b (monocyte/macrophage marker), F4/80 (macrophage marker), CD64 (expressed on activated macrophages) as shown in Table 2 (Ploeger et al., 2013; Stifano & Christmann, 2016). Our marker of interest, Dectin-1/Clec7a was included in this flow cytometric panel to examine its expression on the surface of cells gated into specific immune cell populations using the FlowJo Software (Table 2). The gating strategy for this experiment was primarily focused on gating macrophages as CD45+, F4/80+CD11b+.

**Figure 9** outlines an example flow plot of lung cells from 14 days post-bleomycin administration which were gated to include the population of naïve macrophages. Approximately 4.88% of all analyzed cells were phenotyped as macrophages (CD45+, CD11b+F4/80+), out of which 21.1% also expressed Clec7A (**Figure 9**). Using this gating strategy, Dectin-1 expressing cells were phenotyped in this experimental model of fibrosis at day 7 and day 14 post-bleomycin administration. Clec7a+ macrophages were found to be significantly increased in mice with bleomycin-induced lung fibrosis at day 14 compared to naïve healthy mice (**Figure 9B**).
Expression of Dectin-1 positive macrophages in a murine model of lung fibrosis

To modulate the activity of alternatively activated macrophages over time, and to specifically understand the nature of the resolving macrophages past the injury phase of the bleomycin model, we first need to characterize the time dependency of this experimental model of fibrosis. In this preliminary experiment, immunohistochemical analysis of mouse lungs exposed to bleomycin at day 7, day 14, day 21 and day 35 were examined. Masson’s Trichrome staining allowed for IHC analysis of the level of extracellular matrix deposition in the lungs, while α-SMA stain allowed for examination of myofibroblast expression in these murine tissue sections. Based on the representative images per sacrifice day post-bleomycin-administration, it was observed that mice exposed to bleomycin for 21 days visibly had the highest level of extracellular matrix (Figure 10) and myofibroblast expression (Figure 11). In both Trichrome and α-SMA stained tissues, the level of fibrosis based on extracellular matrix deposition and myofibroblast expression seems to resolve slightly in mice exposed with bleomycin for 35 days but compared to the naïve mice, there is still a level of established fibrosis (Figures 10 and 11).
DISCUSSION

Macrophages are leukocytes which play an important role as effectors in the innate immune system (Bargagli, Prasse, Olivieri, Muller-Quernheim, & Rottoli, 2011). Profibrotic (M2) macrophages are involved in the wound healing process and have garnered a growing interest in fibrotic research as potential mediator cells upstream of myofibroblast differentiation (Pesce et al., 2009). Considerable research has implicated the role of alternatively activated macrophages in fibrotic diseases through depletion studies which demonstrate that deletion of macrophages attenuates fibrosis (M. G. Kim et al., 2015). Targeting these M2 macrophages proves increasingly difficult as the historically used dichotomous classification of classically versus alternatively activated phenotypes cannot be assigned in vivo where macrophages, which are plastic in nature, are exposed to complex cellular milieus (Murray et al., 2014). Thus, there is a need for a comparative study which fully characterizes a marker for profibrotic macrophages to better target these cells in vivo in both human and murine models of fibrosis.

To identify a shared marker for targeting alternatively activated macrophages, our lab previously conducted a bioinformatic analysis comparing gene datasets of macrophage phenotypes from both human (PBMC-derived) and murine (BMDM) systems. Upon validation of the in vitro based bioinformatic analyses through nanoString® technology, six gene signatures shared in both human and murine systems were found to be significantly upregulated in M2 macrophages. These genes include CLEC7A, OLR1, APA2A2, PICALM, PTGS1, and MYC. While the proteins encoded by these genes have diverse functions, characterization of one of the upregulated genes as a
potential biomarker for M2 macrophages could help develop a characterization system which can be applied to the other markers in the future.

*CLEC7A* encoding the protein Dectin-1 or Clec7a was investigated first since Dectin-1 is a transmembrane receptor which has been cited to be widely expressed on macrophage and neutrophil lineages, and on dendritic cells (McDonald, Rosas, Brown, Jones, & Taylor, 2012). Dectin-1 is a non-Toll-like receptor (TLR) which binds specifically to the glucose-based carbohydrates, β-1,3-glucans, derived from the cell walls of fungi and yeast (Herre, Gordon, & Brown, 2004). While Dectin-1 has been shown to play a role in antifungal immunity, it is not well characterized in other disease models (Herre et al., 2004). Upon ligation of β-glucan to Dectin-1, intracellular signaling activated through the spleen tyrosine kinase (SYK)-mediated pathway initiates phagocytosis, produces reactive oxygen species (ROS) and induces the release of cytokines including IL-6 and TNFα (Goodridge et al., 2011). While Dectin-1 has not yet been fully characterized in terms of functionality, since it is a surface receptor for which the ligand, β-glucan, is commercially available, Dectin-1 was chosen to be investigated for the purposes of this study. Furthermore, while the phosphorylation and thus activation of Syk have been implicated in experimental models of renal and scleroderma, this study was conducted to help elucidate the expression of Syk protein in the context of pulmonary fibrosis (Ma et al., 2016; Pamuk et al., 2015). To confirm the bioinformatic analysis from which CLEC7A was suggested to be an upregulated gene for M2 macrophages in both human and murine models, we assessed Dectin-1 expression in both the human system of pulmonary fibrosis using formalin-fixed paraffin embedded lung tissues from patients
with IPF and the murine system using the bleomycin-induced experimental model of fibrosis.

Conducting microscopic examinations using human tissue biopsies has been the standard method for histopathological analyses (Kallioniemi, Wagner, Kononen, & Sauter, 2001). Examining multiple tissue samples in a single tissue microarray (TMA) is advantageous in that high throughput screening is enabled (Kallioniemi et al., 2001). A tissue microarray can, therefore, be used as a tool for target identification through examination of changes in marker expressions and in the microarchitecture of tissues. The TMA previously developed by our lab contains 304 cores from formalin-fixed lung tissues from control patients and patients with a variety of fibrotic diseases which are clinically characterized.

In this study, we assessed the ability of our TMA in examining other markers of interest by first quantifying the level of fibrosis in the fibrotic cores using Masson’s Trichrome and CD206 staining. Aligning with published literature showing that extracellular matrix and alternatively activated macrophages are increased in lung tissues of patients with fibrosis, we found the fibrotic regions of our TMA had increased areas of positively stained trichome (Figure 2C) and CD206 (Figure 3C). This confirmed the possible use of our TMA and quantification software as a potential method for quantifying Dectin-1 expression in the human model of the fibrotic disease.

Consistent with our overall hypothesis, the dectin-1 positive areas were significantly increased in the fibrotic regions of lungs from IPF patients compared to the non-fibrotic regions of these patients and control patients (Figure 4B). Although fibrotic
regions of IPF patients showed increased areas of both CD206+ macrophages and Dectin-1 expression, it cannot be concluded that CD206+ macrophages are also expressing the Dectin-1. In order to determine whether macrophages are expressing this receptor, colocalization studies must be performed. To characterize whether alternatively activated macrophages also express Dectin-1 in fibrotic tissues, as we hypothesize, triple in situ fluorescent hybridization technology can be used to colocalize CD68 (macrophage marker), CCL18 (M2 macrophage marker), and Dectin-1 (target of interest). Despite these results aligning with our hypothesis, we also cannot exclude the fact that other immune cells such as neutrophils and dendritic cells express Dectin-1 as this increase in Dectin-1 expression in fibrotic regions of IPF patients may be due to the typical increase of immune cell infiltrates during fibrotic processes.

Furthermore, while our results may suggest that protein expression in the lungs of patients with fibrotic diseases is increased compared to healthy lungs, we also observed high variability in both non-fibrotic and fibrotic cores from IPF patients in terms of area quantification of Dectin-1 (Figure 4B). As each patient case has been characterized in terms of ILD disease type, gender, average age and other associated risk factors, there is potential to determine whether the cases of high Dectin-1 expression also coincidence with those patients who progressed at a faster rate in their disease progression. Moreover, Dectin-1 expression in these archived lung tissues can be analyzed based on progressors versus non-progressors, as well as disease type, to gain better insight into the variability of this expression in certain patients. Since Dectin-1 is a receptor that is implicated in antifungal immunity, it can be speculated whether these isolated cases with very high
Dectin-1 expression may be due to associated fungal infections in these few IPF patients. The contribution of a fungal infection in the fibrotic process can further be explored by assessing fibrosis in lung tissue via immunohistochemistry or macrophage activity via a CCL18 ELISA upon administration of commercially available beta-glucans in an experimental model of fibrosis or in *in vitro* studies using macrophages from the THP-1 human monocytic cell line respectively. Our results also suggest a potential rationale for assessing fungal infections when creating clinical databases of lung biopsies in the future.

Although our developed TMA is a useful tool in identifying unique markers in human lung fibrotic disease, the quantified expression results from the TMA should be confirmed using additional experiments made up of larger stratified populations due to the clinical heterogeneity presented in the TMA. Furthermore, the protein expression quantification method using the Halo® software of the stained TMA cores should be re-analyzed such that the air space in each core is also accounted for in the quantification algorithm to prevent any over or under-estimation of marker expression and to further confirm our results.

Upon examination of protein expression of Dectin-1 through IHC of our TMA, we examined the transcript level of Dectin-1 using RNA isolated from the FFPE lung tissues of 10 control patients and 12 IPF patients via nanoString® gene analysis technology. We found that CLEC7A or Dectin-1 was significantly downregulated in the fibrotic cores of patients with IPF compared to control patients (Figure 6A). Although this finding was inconsistent with both the overall hypothesis and protein expression levels of Dectin-1 in the fibrotic regions of IPF patients (Figure 4B), the levels of mRNA and protein are not
mutually exclusive. As mRNA is translated into protein, a variety of post-transcriptional mechanisms are involved that have not yet been sufficiently well-defined to accurately estimate or correlate protein concentrations by mRNA transcripts. Several factors could account for this unexpected downregulation of Dectin-1 transcript levels in IPF patients, for instance, proteins may differ in their \textit{in vivo} half-lives which can limit our ability to get a consistent correlation between protein and gene expression. The gene for Dectin-1 in a fibrotic setting may be also downregulated through a negative feedback loop where sufficient protein expression delays further transcription of the gene upstream.

While this study has investigated the protein and transcript expression of Dectin-1 in archived human lung tissues in patients with IPF, there is increasing evidence that there is a circulatory component contributing to pulmonary fibrosis which suggests that tissue-resident profibrotic macrophages are derived from the circulation (Misharin, Morales-Nebreda, & Reyfman, 2017). To determine whether Dectin-1 is expressed in such circulating cells, we assessed the gene expression of Dectin-1 in monocyte-derived primary macrophages from the whole blood of IPF patients. Here, CD14+ monocytes were isolated from four IPF patients and were subsequently differentiated into macrophages with human recombinant M-CSF stimulation. To create a profibrotic milieu for these macrophages, they were additionally stimulated with TGF-β and polarized with the profibrotic cytokines, IL-4 and IL-6. After isolating the RNA from these cultured cells, gene expression analysis through nanoString® technologies indicated that Dectin-1 transcripts increased significantly with combined stimulation of IL-4 and IL-6 (Figure 8A). These results are consistent with those previously examined in our lab where bone-
marrow derived macrophages polarized with IL-4, IL-13 and IL-6 showed the highest expression of Dectin-1 compared to naïve macrophages (unpublished data, Ask Lab, 2017). These findings also suggest that circulating cells may have the capacity to be polarized into profibrotic macrophages through IL-4 stimulation alone (Figure 8A).

As ligand binding of Dectin-1 induces the activation of the Syk pathway, it was important to examine gene expression of the associated SYK gene. Consistent with our findings showing that the mRNA expression of SYK was unchanged in the fibrotic regions of the archived lung tissues of IPF patients (Figure 6B), we also found that SYK transcripts were unchanged in primary macrophages with or without stimulation from TGF-β or profibrotic cytokines (Figure 8B). This may be due to a need for the spleen tyrosine kinase pathway to be activated first through ligand binding of a pathogen such as beta-glucan in order for the SYK gene to be upregulated. To address this issue, our lab is currently in the process of optimizing a phospho-Syk antibody (Abcam, REF# 2711), representing the activated protein from the Syk pathway. This antibody is predicted to react both in the human and murine models where its activation will be examined by assessing changes in its expression in our TMA through immunohistochemistry.

A major clinical implication that stems from our research in studying circulating cells revolves around being able to better target profibrotic macrophages through therapeutic interventions. A circulating population of monocytes implicated in the process of fibrosis may be found to be more easily targetable compared to be tissue-resident macrophages in terms of administering systemic drug therapeutics. Our analysis of gene expression in monocyte-derived macrophages should also be confirmed by examining
protein expression in circulating cells through flow cytometry to phenotype the populations of Dectin-1+ immune cells in the circulation which contribute to fibrosis. However, at least one million monocytes would be required to run a successful flow cytometry study, whereas 20mL of whole blood collected from patients allows us to isolate approximately 450,000 CD14+ monocytes. Thus, although protein expression in circulating cells via flow cytometry can be accomplished in principle, we are limited in the volume of whole blood that can ethically be collected and processed from patients.

Since the bioinformatic analysis identified Dectin-1 to be a potential shared marker for profibrotic macrophages in both human and murine systems, we then sought to examine Dectin-1 expression in the murine system. The bleomycin model of experimental lung fibrosis is well-characterized and since macrophages have been implicated in the injury phase (day 7 post-bleomycin administration) and fibrotic phase (after day 14 post-bleomycin administration) of this experimental model, we used this model of lung fibrosis to characterize the proportion of Dectin-1+ macrophages at these time points (Ayaub et al., 2017). To determine whether Dectin-1 is expressed on the surface of macrophages, we assessed its expression in CD45+/Cd11b+/F4/80+ macrophages from lung mononuclear cells isolated from digested lungs in mice exposed to bleomycin. Dectin-1+ macrophages were found to be significantly increased in mice with bleomycin-induced lung fibrosis at day 14 compared to naïve healthy mice (Figure 9B). In the future, specific cell populations gated for all profibrotic macrophages (CD45+, F4/80+CD11b+, CD64+MHCII+, CD206+Arg-1+ cells), activated macrophages (CD45+, F4/80+CD11b+, CD64+MHCII+ cells) and alveolar macrophages (F4/80+CD11b-CD11c+SiglecF+ cells)
should be examined to investigate more subsets of macrophage phenotypes using a more specific myeloid flow panel for better target identification of cells expressing Dectin-1.

Despite this, this finding aligns with literature demonstrating that M2 markers have been upregulated past the injury phase of this model and suggests that future intervention studies should administer therapeutics before day 14 post-bleomycin exposure to better characterize the resolution of fibrosis in this experimental model (Ballinger et al., 2015).

While the bleomycin-induced model of pulmonary fibrosis is widely cited, the mechanisms revolving around the resolution phase which occurs by day 35 post-administration of bleomycin have yet to be clarified. Thus, a full assessment of Dectin-1 expression and modulation of the activity of profibrotic and resolving macrophages demands the need for the time dependency of this experimental model of pulmonary fibrosis to be elucidated. As a preliminary step, we began to assess the level of extracellular matrix and myofibroblast expression through Masson’s Trichrome and α-SMA staining via IHC from mouse lungs exposed to bleomycin at day 7, day 14, day 21 and day 35 (Figures 10 and 11). Quantification of these stains through Halo® software is required to make conclusive observations based on the changes of fibrosis through the time course of this experimental model.

While the results derived from the experiments performed in this paper may suggest that Dectin-1 is expressed in certain regions of archived tissues or cell populations, the functionality of Dectin-1 as a receptor needs to be determined. This can be addressed by conducting depletion or inhibition studies where the progression of fibrosis upon bleomycin administration is examined in knock-out mice (Dectin-1−/−). By
validating the functional role of Dectin-1 in an experimental model system *in vivo*, its utility as a translational biomarker is strengthened. Furthermore, therapeutic targeting of the Syk pathway by administering mice with a Syk inhibitor after being exposed to bleomycin would be required to gain insight into the requirement and functionality of the Dectin-1 associated pathway in the fibrotic process. A commercially available Syk inhibitor was purchased via Boehringer Ingelheim (BI 1002494) and has only yet been examined in the context of arterial thrombosis (van Eeuwijk et al., 2016). As the inhibitor is reported to be highly potent with low toxicity, it may serve as a powerful tool to examine the requirement of the Syk pathway in advancing pulmonary fibrosis. Our lab has currently set up *in vitro* assays where the effect of the Syk inhibitor on the polarization of macrophages via a CCL18 ELISA for macrophages derived from a human cell line, and an arginase assay for murine bone-marrow-derived macrophages will be examined.

Full characterization of the Dectin-1 pathway mediated by Syk activation in both human and mouse models of fibrosis will add insight into the molecular machinery and role of alternatively activated macrophages in the fibrogenic process. These studies can allow for the development of targeted biological therapeutics which would be used to modulate and prevent the profibrotic macrophage phenotype in the progression of fibrotic lung disease. The increased expression of beta-glucan receptor Dectin-1 in both murine and human M2-like macrophages as demonstrated through this work provides a unique prospect to use the ligand beta-glucan to develop innovative nanoscale delivery vehicles.
which could have the potential to be specifically internalized by various target cells, such as profibrotic macrophages, through receptor-induced endocytosis.
REFERENCES


Karatzas, E., Bourdakou, M. M., & Kolios, G. (2017). Drug repurposing in idiopathic pulmonary fibrosis filtered by a bioinformatics-derived composite score. 7(1), 12569. doi: 10.1038/s41598-017-12849-8


FIGURES & TABLES
Figure 2. Area quantification of Trichrome stained TMA. A) Full TMA block stained with Masson’s Trichrome B) Representative image of markup by Halo® software where only red stained areas are selected for C) Percent total area stained positive for collagen (green stain) out of total area per core comparing TMA cores from control, IPF NF (non-fibrotic regions) and IPF F (fibrotic regions) patients. Significance was established with a One-way ANOVA using Tukey’s post hoc test on GraphPad Prism 7.0.
Figure 3. Cytonuclear quantification of CD206 stained TMA. A) Representative images showing a stained core out of a full TMA block. B) Image markup using the Halo® software where individual cells are grouped based on their degrees of positive staining. C) The percent of CD206 positive stained cells out of the total number of cells per core, compared to control and IPF patients. D) The percent of CD206 positive stained cells per core comparing fibrotic vs. non-fibrotic regions in a sample of nine IPF patients. Significance was established with a One-way ANOVA using Tukey’s post hoc test on GraphPad Prism 7.0.
Figure 4. Area quantification of Clec7a stained TMA. A) Representative images showing visible variability in Clec7a staining in a representative control core vs. fibrotic IPF core. B) Area of positively stained Clec7a per core when comparing controls, non-fibrotic (NF) and fibrotic (F) regions of IPF patients. Variability by each patient case is shown when quantifying positive stained area in controls, NF and F regions of IPF patients. Significance was established with a One-way ANOVA using Tukey’s post hoc test on GraphPad Prism 7.0.
Figure 5. Gene Expression in Typical Fibrotic Response. mRNA isolated from 10 control patients and 12 IPF patients were assessed using a 48 gene code set through nanoString® technologies. Expression of genes relating to extracellular matrix deposition (MMP-7, Col1A1, Col3A1) and alternatively activated macrophages (CCL18) which are typically upregulated in fibrosis were quantified. Nanostring counts less than 5 counts were not detected “ND”. Significance was established using a non-parametric independent Student’s t-test on GraphPad Prism 7.0.
Figure 6. Dectin-1 Associated Gene Expression. mRNA isolated from 10 control patients and 12 IPF patients were assessed using a 48 gene code set through nanoString® technologies. **A) Gene expression for the receptor Dectin-1/Clec7a and B) the associated inactivated Syk gene expression. Nanostring counts less than 5 counts were not detected “ND”. Significance was established using a non-parametric independent Student’s t-test on GraphPad Prism 7.0.
Figure 7. CCL18 transcript levels in cytokine-stimulated human primary macrophages derived from patients diagnosed with IPF. 50,000 CD14+ isolated monocytes per well were cultured in a 96-well plate from whole blood of four IPF patients. Monocytes were differentiated with 50ng/ml human MCSF for 6 days, primed with M-CSF (50ng/ml) +/- TGF-β (10ng/ml) for 24hr, then stimulated with either IL4, IL6 or IL4/IL6 (10ng/ml) for 24hrs. RNA was isolated from cultured cells and gene expression was performed by nanoString® technologies. Transcripts were normalized with housekeeping genes: ACTB, B2M, PGK1, POLR2A, PPB1, RPLP2, UBC. Significance was established with a One-way ANOVA using Tukey’s post hoc test on GraphPad Prism 7.0.
Figure 8. CLEC7A and SYK transcript levels in cytokine-stimulated human primary macrophages derived from patients diagnosed with IPF. 50,000 CD14+ isolated monocytes per well were cultured in a 96-well plate from whole blood of four IPF patients. Monocytes were differentiated with 50ng/ml human MCSF for 6 days, primed with M-CSF (50ng/ml) +/- TGF-β (10ng/ml) for 24hr, then stimulated with either IL4, IL6 or IL4/IL6 (10ng/ml) for 24hrs. RNA was isolated from cultured cells and gene expression was performed by nanoString® technologies. A) CLEC7A transcripts and B) SYK transcripts were normalized with housekeeping genes: ACTB, B2M, PGK1, POLR2A, PPBP1, RPLP2, UBC. Significance was established with a One-way ANOVA using Tukey’s post hoc test on GraphPad Prism 7.0.
Table 2: Myeloid Lineage Flow Panel. A panel of surface makers from the myeloid lineage which aims to characterize subsets of macrophage phenotypes in single mononuclear lung cells isolated from digested lungs in the bleomycin-induced model of pulmonary fibrosis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Quantity (per 50uL)</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>BV650</td>
<td>M1/70</td>
<td>0.5uL</td>
<td>Biolegend 101259</td>
</tr>
<tr>
<td>Clec7a</td>
<td>APC</td>
<td>RH1</td>
<td>0.5uL</td>
<td>144305</td>
</tr>
<tr>
<td>F4/80</td>
<td>PE-CF594</td>
<td>T45-2342</td>
<td>0.5uL</td>
<td>BD 565613</td>
</tr>
<tr>
<td>CD45.2</td>
<td>APC-Cy7</td>
<td>104</td>
<td>0.5uL</td>
<td>Biolegend 109824</td>
</tr>
<tr>
<td>CD64</td>
<td>PE-Cy7</td>
<td>X54-5/7.1</td>
<td>0.5uL</td>
<td>Biolegend 139314</td>
</tr>
<tr>
<td>MHCII</td>
<td>PerCp-eFluor 710</td>
<td>AF6-120.1</td>
<td>0.5uL</td>
<td>46-5320-82</td>
</tr>
<tr>
<td>Arginase-1</td>
<td>PE</td>
<td></td>
<td>5uL</td>
<td>R&amp;D systems IC5868P</td>
</tr>
<tr>
<td>CD16/CD32 (Fc block)</td>
<td>PE</td>
<td></td>
<td>1:20 dilution</td>
<td>Biolegend 101302</td>
</tr>
<tr>
<td>CD206</td>
<td>Alexa Fluor 700</td>
<td>C068C2</td>
<td>0.5uL</td>
<td>Biolegend 141734</td>
</tr>
<tr>
<td>Mouse IgG1 k Isotype control</td>
<td>Alexa Fluor 647</td>
<td></td>
<td>0.5uL</td>
<td>Biolegend 400136</td>
</tr>
<tr>
<td>Sheep IgG isotype control</td>
<td>PE</td>
<td></td>
<td>0.5uL</td>
<td>R&amp;D Systems IC016P</td>
</tr>
<tr>
<td>Rat IgG2a isotype control</td>
<td>Alexa Fluor 700</td>
<td></td>
<td>0.5uL</td>
<td>Biolegend 400528</td>
</tr>
</tbody>
</table>
Figure 9. Dectin-1 expression in macrophages phenotyped in the bleomycin-induced fibrotic model via flow cytometry analysis. A) Flow plot demonstrating gating strategy for macrophages B) Kinetic profile of Dectin-1 expression on CD11b+F4/80+ macrophages at day 7 and 14 post-bleomycin compared to naïve mice. Significance was established with a One-way ANOVA on GraphPad Prism 7.0.
Figure 10. Masson’s Trichrome Assessment in Time Course of Bleomycin Model of Lung Fibrosis. Representative images showing trichrome staining in a preliminary time course experiment of bleomycin-induced pulmonary fibrosis (n=5 per group).
Figure 11. α-SMA Assessment in Time Course of Bleomycin Model of Lung Fibrosis. Representative images showing alpha-smooth muscle actin staining in a preliminary time course experiment of bleomycin-induced pulmonary fibrosis (n=5 per group).