MARKERS OF PROFIBROTIC MACROPHAGES AND THEIR RELEVANCE TO SCLERODERMA

IDENTIFICATION OF MARKERS OF PROFIBROTIC MACROPHAGES SHARED BETWEEN HUMAN AND MURINE SYSTEMS, AND THEIR RELEVANCE TO SYSTEMIC SCLEROSIS

By

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

MASTER OF SCIENCE (2017) McMaster University

(Medical Sciences) Hamilton, Ontario

Title: Identification of Markers of Profibrotic Macrophages Shared between Human and Murine Systems, and Their Relevance to Systemic Sclerosis

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Number of Pages: xii; 80

ABSTRACT

Systemic sclerosis (SSc), or scleroderma, is a complex, rare disease of unknown etiology. Macrophages constitute a large portion of the immune cell infiltrate in the skin of patients with SSc, and are an important target of study. Particularly, the M2 macrophage has been implicated scleroderma and other fibrotic diseases as a key contributor to fibrotic processes. However, the definition of an M2 macrophage appears to change with context, and is poorly elucidated in different species. With varying characterizations between species and disease models, there is a need to establish some consensus on how to identify this macrophage in an uniform manner across species. We used a bioinformatic approach to identify a unique gene signature for the M2 macrophage phenotype, which is shared between human and mouse systems. We were able to confirm a 7-gene subset of this theorized signature using human and mouse *in vitro* systems. In addition, we selected one of the identified genes, *CLEC7A*, and characterized its expression at the protein level on different macrophage phenotypes, across several human and mouse models. Our data show that CLEC7A is a more selective marker of murine M2 macrophages than current reference markers, and is useful in human models as well. Using our M2-specific gene signature, we also identified a potential inhibitor of the signature and showed its effects on M2 marker expression. Finally, we showed some preliminary work into CLEC7A expression in skin tissue from patients with scleroderma. Overall, our data suggest that CLEC7A may be a valuable addition to the panel of markers used to

characterize M2 macrophages and distinguish between macrophage phenotypes, and perhaps provide clarity into the development and function of the M2 macrophage. Better understanding of the M2 macrophage would ultimately be useful to the study of fibrotic diseases such as scleroderma, wherein this macrophage phenotype may be a viable target for antifibrotic therapy.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Kjetil Ask. Dr. Ask has been a fantastic mentor and supervisor, providing me with constant encouragement, support and patient guidance. He provided me with a stimulating research environment filled with positivity and creativity, and his enthusiastic support has been integral to my success.

My sincere thanks to my Master's committee members, Drs. Mark Inman, Maggie Larché, and Asghar Naqvi. Their support, commitment, and insightful feedback throughout the past two years is greatly appreciated. I would also like to thank Dr. Nader Khalidi and the clinical team (Barbara Baker, Diane Robins, and Andrea Gardner) without whom I could not have completed the clinical study. The entire team went above and beyond to help me, for which I am very grateful.

I would like to thank my lab members for their technical help and moral support with my project, and for creating an enjoyable work atmosphere. This project was only possible because of the team effort.

Special thanks to my friends and family, for giving me a world of love and happiness always. Thank you for cheering me on, and for providing the humour and love that kept the stress away.

Throughout my graduate studies, my family has been my ultimate rock. The unconditional love and encouragement of my parents and sister has been the foundation for every milestone and achievement in my life. Thanks to my Mother for her incessant strength, patience, and thoughtfulness as I navigated through the ups and downs of graduate school. Thanks to my Father for his motivation, empathy, and kindness, which provided great comfort at stressful times. Thank you to my sister, Prasiddha, for her unwavering faith in my success and for being an excellent role model for me.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACR	American College of Rheumatology
α-SMA	Alpha smooth muscle actin
ARG-1	Arginase-1
ANOVA	Analysis of variance
AP2A2	Adaptor-related protein complex 2 alpha 2 [gene]
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CCB	Calcium channel blocker
CD	Cluster of differentiation
Clec7a	C-type Lectin Domain Family 7, Member A [gene]
Cmap	Connectivity map
Myc	Myelocytomatosis [oncogene]
DECTIN-1	Dendritic cell-associated C-type lectin-1
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDA-FN	Fibronectin spliced variant with extra domain A
EULAR	European Union League Against Rheumatism
FBS	Fetal bovine serum
FFPE	Formalin-fixed, paraffin-embedded
GEO	Gene Expression Omnibus
H&E	Haematoxylin & Eosin
IFN-γ	Interferon gamma
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-13	Interleukin-13
IPF	Idiopathic pulmonary fibrosis
Jak-STAT	Janus Kinase-Signal Transducers and Activators of Transcription
LPS	Lipopolysaccharide
M0	Naïve macrophage phenotype
M1	Phenotype of macrophages treated with LPS and IFN-γ
M2	Phenotype of macrophages treated with IL-4 and IL-13
M2+IL-6	Phenotype of macrophages treated with IL-4, IL-13 and IL-6
MCSF	Macrophage colony stimulating factor
MRC-1	Mannose receptor-1 (CD206)
OLR-1	Oxidized low-density lipoprotein receptor [gene]
P/S	Penicillin/streptomycin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline

PCA	Principal Component Analysis
PFKFB3	6-Phosphofructo-2-kinase [gene]
PICALM	Phosphatidylinositol binding clathrin assembly [gene]
PMA	Phorbol 12-myristate 13-acetate
PMID	PubMed Identifier
PTGS1	Prostaglandin synthase 1 [gene]
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SSc	Systemic sclerosis
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide [gene]

Note: Where abbreviations are used, genes are written in italics with all letters capitalized for human genes and only word-initial letters capitalized for murine genes. Abbreviations for both human and murine protein are fully capitalized. Where both human and murine versions of a gene/protein are mentioned, the human nomenclature is used.

DECLARATION OF ACADEMIC ACHIEVEMENT

The following is a declaration that all of the work presented in this thesis was completed by Pavithra Parthasarathy under the supervision of Dr. Kjetil Ask. Bioinformatic analysis support was provided by Dr. Anna Dvorkin-Gheva.

CHAPTER 1: INTRODUCTION

1.1 Systemic Sclerosis

1.1.1 Overview of Systemic Sclerosis

Systemic sclerosis (SSc), or scleroderma, is an autoimmune, connective tissue disorder affecting nearly 16000 Canadians (Hudson, Steele, and Baron 2007). The word "scleroderma" describes the clinical presentation of thickened, tight skin, which is seen in patients as a result of excessive scarring (fibrosis) (Zulian 2004). Systemic sclerosis (SSc), the systemic form of scleroderma, is a debilitating disease with several internal complications (Gabrielli, Avvedimento, and Krieg 2009). In SSc and other fibrotic diseases, fibrosis leads to destruction of the involved tissues and consequently, impaired organ function (Gabrielli, Avvedimento, and Krieg 2009).

There are two major classifications within this disease, namely "scleroderma" and "systemic sclerosis". Scleroderma, also called morphea, refers to the localized form of the disease, which manifests only in the skin and subcutaneous tissues (Baker et al. 2014). The systemic form of the disease, termed systemic sclerosis, manifests as fibrosis in various other organs, such as the lung, esophagus, and kidneys (Baker et al. 2014). SSc has two major subtypes: limited cutaneous SSc and diffuse cutaneous SSc, which describe the disease based on the extent of skin involvement (Baker et al. 2014). While "scleroderma" and

"systemic sclerosis" are used interchangeably in this report, we focus on systemic sclerosis.

The three major components of SSc are: 1) vascular inflammation and damage of small vessels, 2) autoimmunity with autoantibody production, and 3) excessive extracellular matrix (ECM) protein deposition, or fibrosis, in the skin, visceral organs, and blood vessels (Pattanaik et al. 2015). The systemic complications can be fatal in SSc patients; SSc-associated interstitial lung disease and pulmonary arterial hypertension contribute to more than 50% of all SSc-associated mortalities (Pattanaik et al. 2015). Additionally, when comparing disease-specific mortality amongst autoimmune connective tissue disorders, the highest mortality is seen in patients with SSc (Manetti 2015).

SSc is a very heterogeneous disease, with patients developing a wide variety of clinical presentations. The most recent criteria for the classification of SSc, as put forth by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR), are listed in Table 1 (Pope and Johnson 2015).

The precise etiology of SSc is unknown, but the interplay of genetic predisposition to the disease, and exposure to environmental triggers, is suspected (Gabrielli, Avvedimento, and Krieg 2009). The patient population is largely female, and the age of onset is around 45 years (Hudson and Fritzler 2014). With lowered quality of life as a result of this debilitating disease, patients are currently

faced with few options to control the symptoms of their illness, rather than with therapeutics that would address the underlying issue. To be able to identify a method of preventing the fibrosis in SSc would be of great help to patients.

Table 1: ACR/EULAR classification criteria for SSc (Pope and Johnson 2015)**.** Reproduced from Pope and Johnson (2015). Of note is the integration of criteria that address the three aspects of the disease: fibrosis, vasculopathy, and autoantibody production.

Table 1
The ACR/EULAR criteria for the classification of SSc

Category	Subitems	Weight
Skin[a]	Skin thickening of the fingers of both hands extending proximal to the MCPs[b]	9
	Puffy fingers	2
	Whole finger, distal to MCP	4
Fingertip lesions[a]	Digital tip ulcers	2
	Pitting scars	3
Telangiectasia	—	2
Abnormal nail fold capillaries	—	2
PAH and/or interstitial lung disease	—	2
Raynaud's Phenomenon (RP)	—	3
Scleroderma-related antibodies (any of anticentromere, anti–topoisomerase-I [anti–Scl-70], anti–RNA polymerase-3)	—	3
—	Total score:	

1.1.2 Fibrosis in SSc

Fibrosis is the excessive deposition of ECM proteins such as collagen and fibronectin (Wynn and Ramalingam 2012). The accumulation of such fibrous connective tissue can result in permanent scarring, impairing the function of the organ in which it occurs (Wynn and Ramalingam 2012).

Fibroblasts are primary modulators of wound healing, and synthesize ECM for tissue remodeling (Abraham et al. 2007). Upon receiving cytokine signals, such as Transforming Growth Factor-Beta (TGF-β) with endothelin-1, quiescent fibroblasts differentiate to become myofibroblasts (Abraham et al. 2007; Wynn and Ramalingam 2012). Myofibroblasts also participate in tissue remodeling through ECM synthesis (Gabrielli, Avvedimento, and Krieg 2009; Wynn and Ramalingam 2012). The myofibroblast is the specialized cell that is central to the fibrotic process; persistent activation of myofibroblasts results in fibrosis (Wynn and Ramalingam 2012). While they are differentiated from fibroblasts, myofibroblasts have characteristics of both fibroblasts and smooth muscle cells (Abraham et al. 2007). They can be distinguished from fibroblasts by identifying the presence of α-smooth muscle actin (α-SMA) and a spliced variant of fibronectin that contains extra domain A (EDA-FN) (Abraham et al. 2007; Julier et al. 2015).

Several studies in the skin of patients with SSc have established the importance of myofibroblasts in SSc (Kissin, Merkel, and Lafyatis 2006; Artlett et

al. 2011; Abignano et al. 2011). In a 2006 study, Kissin et al. noted a positive correlation between myofibroblast accumulation and clinical assessment of skin thickness, thus emphasizing the role of myofibroblasts in disease progression (Kissin, Merkel, and Lafyatis 2006).

Current immunosuppressive and immunomodulatory agents are unsuccessful for fibrosis treatment (Nanthakumar et al. 2015). Given that fibroblast-to-myofibroblast differentiation could be an important target for preventing fibrosis, various groups have investigated this process. Macrophages have become an active focus of research, as they produce profibrotic cytokines involved in this differentiation (Higashi-Kuwata et al. 2009; Sindrilaru and Scharffetter-Kochanek 2013).

1.2 M2 Macrophages

1.2.1 Studying the "M2" Macrophage

Macrophages are antigen-presenting cells that play important roles in both innate and adaptive immunity. As phagocytes, they have receptors to respond to ligands from pathogens such as bacteria, viruses, and parasites. Macrophages can originate as tissue-resident cells, or as monocytes in the circulation that infiltrate tissue when needed and differentiate into macrophages (Hashimoto et al. 2013). There is much debate and controversy surrounding the M1-M2 paradigm for classifying macrophages (Martinez and Gordon 2014; Murray 2017). The M1-M2 nomenclature was first proposed by Mills et al. in 2000, and became a second

method of classification following the "classical vs. alternative activation" model established in the 1990s (Mills et al. 2000; Murray et al. 2014). This nomenclature may falsely lead researchers to believe that there are only two categories of macrophages, while macrophages are very diverse in their activation states (Murray et al. 2014).

In this thesis, we have used the M1-M2 nomenclature because of the use of this classification in the Gene Expression Omnibus (GEO)-deposited DataSets (submitted by other research groups) that we have compared. In line with the recommendations made by Murray et al., it is useful to define macrophage phenotypes based on the cytokine stimulation that the cells had received (e.g. a macrophage stimulated with IL-4 would be written as M(IL-4)) (Murray et al. 2014). Therefore, in this thesis, where the labels "Control"/"M0," "M1," "M2," "M2+IL-6" are used, they refer to the following macrophages respectively: M(no cytokine stimulation); M(LPS,IFN-γ); M(IL-4,IL-13); M(IL-4,IL-13,IL-6). The final phenotype mentioned here, M2+IL-6, has not been published previously. Previous work by our research group has led us to characterize this phenotype as a "hyper-M2" macrophage (unpublished data; submitted for publication). Therefore, we incorporated it into this study to compare its phenotype with the Control, M1, and M2 cells.

Within the M1-M2 model, undifferentiated macrophages polarize to the M1 phenotype in response to interferon-gamma (IFN-γ) and lipopolysaccharide

(LPS) exposure, as if exposed to a bacterial pathogen (Stifano and Christmann 2015). M1 macrophages exhibit robust microbicidal and tumoricidal functions, and secrete pro-inflammatory cytokines (Stifano and Christmann 2015). On the other end of this spectrum are M2 macrophages, which are activated by IL-4 and IL-13 addition, and respond to parasitic infection (Chanput, Wichers, and Mes 2013).

The definition of an M2 macrophage appears to change with context, and is poorly elucidated in different species (Murray et al. 2014). For example, arginase-1 (ARG-1) is considered a murine marker of the M2 macrophage, but it has been shown that ARG-1 expression is induced in M1 macrophages as well (El Kasmi et al. 2008). CD206 is considered to be an M2 marker in both mice and humans, but its immune functions have not yet been clearly elucidated (Rőszer 2015). With varying characterizations between species, there is a need to establish some consensus on how to identify this macrophage in an uniform manner across models (Murray and Wynn 2011b; Murray et al. 2014; Murray 2017). While working with the M1-M2 foundation, we have sought to identify markers of M2 activation that are common to both human and mouse systems, and which can be used in conjunction with current markers to better characterize and distinguish this macrophage phenotype. Ultimately, in the context of fibrotic disease, this may help us to distinguish the functional capacity of this macrophage to contribute to scarring, in both models of fibrosis and in samples from patients with fibrosis.

1.2.2 Macrophages in the Context of SSc

M2-polarized macrophages exhibit a profibrotic, anti-inflammatory profile in the cytokines they secrete (Stifano and Christmann 2015; Ploeger et al. 2013). As a result, M2 macrophages are of particular interest in SSc, especially in the context of fibroblast-to-myofibroblast differentiation.

Macrophages constitute a large portion of the immune cell infiltrate in the skin of patients with SSc (Stifano and Christmann 2015). In a 2010 study of skin from patients with SSc, Higashi-Kuwata et al. noted a significant presence of M2 macrophages (identified by their surface markers, CD163 and CD204), particularly around the collagen fibres in the skin (Higashi-Kuwata et al. 2010). Higashi-Kuwata et al. also discussed the development of $CD163^+$ $CD204^+$ monocyte lineage cells in the circulation of patients with SSc, which may provide a clue as to the origin of the M2 macrophages found in the skin (Higashi-Kuwata et al. 2010). Several other serum studies have shown that $CD163^+$ monocyte counts are elevated in patients with SSc, and that they directly correlate with disease severity (Kowal-Bielecka et al. 2013; Bielecki et al. 2013; Nakayama et al. 2010). Therefore, studying M2 macrophage involvement in SSc may provide many insights in how the fibrosis progresses.

Although the secreted cytokine profile of M2 macrophages results in their classification as profibrotic, some studies provide contradicting evidence suggesting that they may instead suppress fibrosis (Wynn and Ramalingam 2012;

Pesce et al. 2009). A murine study of conditional ARG-1 deficiency indicated that arginase-1-positive M2 macrophages are needed to attenuate and resolve fibrosis (Pesce et al. 2009). Although these macrophages may be serving both pro- and antifibrotic functions, it may also be important to recognize the obstacles in our understanding caused by the lack of a well-characterized, reliable marker for this phenotype.

While the M2 macrophage could be an important target for research, currently there is a lack of a well-characterized M2-related marker that can be commonly measured in both *in vitro* macrophage polarization models and in human samples. Translational research can be strengthened by identifying a direct link between human diseases and models used to simulate these diseases. Therefore, we sought to identify a gene signature unique to the profibrotic M2 macrophage phenotype, and that is common between human and mouse *in vitro* models. Genes thus identified may provide potential markers that strengthen the validity of models in fibrosis research.

1.3 Hypothesis

The identification of gene and protein markers for the profibrotic, M2 macrophage, that are shared between human and mouse systems, will serve as an important tool to understanding and manipulating the M2 macrophage phenotype.

1.4 Objectives

1.4.1 Objective 1: To identify gene-level markers of macrophage polarization common to human and murine model systems

While M1 and M2 macrophages have both been described in fibrotic diseases, the M2 macrophage is of particular interest due to its production of fibrosis-promoting factors such as TGF-β, which are directly involved in the fibroblast-to-myofibroblast differentiation process.

The study of M2 macrophages in fibrotic disease has been impeded by several factors. Firstly, certain fibrotic diseases such as scleroderma do not have adequate animal models that properly mimic the various aspects of the disease *in vivo* (Gerber et al. 2013). Even for those diseases with established animal models, the lack of a well-characterized, common marker between human and animal M2 macrophage phenotypes makes it difficult to study the M2 macrophage in these systems. For example, one of the most widely-used markers for M2 macrophages in mice is ARG-1 (Pesce et al. 2009). However, it is not a marker in the human system (Raes et al. 2005).

The identification of an M2 marker with similar patterns of expression in the human system and in appropriate animal models of fibrosis would be very useful to the study of macrophage biology in fibrosis. Unlike the current situation wherein different markers identify M2 macrophages in animal and human systems, such a finding would strengthen the translational link between fibrotic disease in humans, and the animal models used to study the disease.

1.4.2 Objective 2: To investigate protein expression of markers identified in Objective 1 in human and murine models of macrophage polarization

We decided to investigate each of the genes identified in Objective 1, starting with *CLEC7A*. Our data on *CLEC7A* thus far were at the RNA level, and we wanted to verify whether CLEC7A (protein) was present on M2 macrophages. This would give us more insight into how the functional protein is expressed on this macrophage, and how this expression varied between the different macrophage phenotypes. It was important to investigate this in murine macrophages and in THP-1 human macrophages, but we also extended this to two other sample types. Firstly, to make our findings more relevant to human systems, we looked for $CLEC7A^+$ cells in macrophages differentiated from human peripheral blood-derived monocytes. Secondly, we explored CLEC7A expression in lung cells from a murine model of lung fibrosis. The well-established bleomycin-induced lung fibrosis model would give us insight into whether $CLEC7A^+$ macrophages are present in a fibrotic lung environment.

1.4.3 Objective 3: To investigate the bioinformatically-derived compound radicicol for selective inhibition of the M2 macrophage phenotype

Using a bioinformatics approach with the same DataSets as in Objective 1, we sought to answer a question surrounding M2 macrophage phenotype inhibition. If the M2 macrophage has a unique gene expression pattern (opposite to the M1 macrophage), and there are known compounds that can alter the expression of the genes within the M2 macrophage signature, such compounds may prevent the formation or persistence of this profibrotic phenotype. Specific inhibition of this phenotype alone could be more useful to treating fibrotic disease, rather than inhibiting a variety of macrophage phenotypes.

1.4.4 Objective 4: To conduct pilot clinical study on macrophages in fibrosis in systemic sclerosis

While macrophages have been found in the tissue of systemic sclerosis patients, there is a limited understanding of how they are implicated in the initiation and/or progression of the fibrosis within this disease. Furthermore, there is limited characterization of $CLEC7A^{+}$ cells in this context, which we feel is a gap in knowledge that our work could address.

CHAPTER 2: MATERIALS AND METHODS

2.1 *In silico* Identification of M2-Specific Gene Signature and Connectivity Mapping

Human and mouse gene DataSets were obtained from the Gene Expression Omnibus (GEO) and used to derive macrophage gene expression signatures. The human DataSet (GSE5099), referenced with detailed methods in two different studies (PubMed Identifier (PMID) 20530259 and PMID 17082649), contained global gene expression profiles of M0, M1-polarized and M2-polarized macrophages that were derived from monocytes in the peripheral blood of healthy human subjects (Martinez et al. 2006; Solinas et al. 2010). The mouse DataSet (GSE53321), referenced with detailed methods in one study (PMID 25526089), contained global gene expression profiles of M0, M1-polarized and M2-polarized bone marrow-derived macrophages (BMDMs) (Li et al. 2015). Following differentiation into macrophages with the addition of recombinant macrophage colony stimulating factor (MCSF) (human and mouse, respectively), these cells had been stimulated with LPS and IFN-γ to achieve a proinflammatory, M1 macrophage phenotype, or with interleukins 4 or both 4 and 13 to achieve a profibrotic, M2 phenotype. The DataSets did not have gene expression profiles for M2+IL-6 macrophages; as described in the introduction (see **Studying the "M2" Macrophage**), the M2+IL-6 phenotype has not been published previously.

Similar analyses were performed for both the human and mouse data (separately for each of the DataSets). Firstly, differential expression analysis (limma package, in R environment, https://cran.r-project.org/) was conducted to compare M2 to M0 ("Control") macrophages, as well as M1 to M0 macrophages. Secondly, the list of differentially expressed genes (a gene signature) generated from the first comparison was compared with the list generated from the second comparison with the goal of identifying genes that are unique to the first list. Through this step, we obtained one mouse M2-specific gene signature, and one human M2-specific gene signature. Thirdly, the mouse M2-specific gene signature was compared to the human M2-specific gene signature. This yielded a list of genes with regulation specific to M2 macrophages in both mice and humans. The signature obtained by the methods described above was used to conduct a Connectivity Map (cMap) query, which provided candidate compounds that would be predicted to reverse this signature (Lamb et al. 2006).

2.2 *In Vitro* Macrophage Experiments

2.2.1 Murine Bone Marrow-Derived Macrophages

Female C57BL/6 mice were used for these experiments. Tibiæ and femora were crushed in phosphate-buffered saline (PBS) to extract the bone marrow cells. All of the work was done in a laminar flow hood. The bones were crushed until they appeared clear, to ensure that the majority of the bone marrow content had been extracted. The PBS solution containing the cells was filtered and centrifuged

at 400 rcf for 10 minutes. The pellet was subsequently resuspended in Dulbecco's Modified Eagle Medium (DMEM) F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 2 mM L-glutamine. Recombinant murine MCSF was added for a final concentration of approximately 20 ng/mL (Peprotech). The cells were plated in 10-cm polystyrene petri dishes and cultured at 37° C, under 5% CO_2 conditions. At 72 hours post-plating, additional medium with MCSF was added to the cells. 7 days post-plating, the supernatant was discarded and the adhered cells (monocytes, which had differentiated into macrophages) were washed with sterile PBS chilled to 4° C. A cell-lifting reagent (Accutase or 1x Trypsin) was added to the cells at 37° C for 5-10 minutes. These bone marrow-derived macrophages (BMDMs) were subsequently lifted using a cell lifter, and stored in PBS or medium. The cells were centrifuged, resuspended in medium, and counted using a hematocytometer.

For NanoString experiments**,** the macrophages were seeded in a 96-well plate at a density of 80,000 cells/well. For flow cytometry, the macrophages were seeded at 4-5 million cells/plate in 100-mm petri dishes. The cells were treated with cytokines (Peprotech) to polarize them to the macrophage phenotypes (100 ng/mL LPS and 20 ng/mL IFN-γ for M1, and 20 ng/mL IL-4 and 20 ng/mL IL-13 for M2). IL-6 was added at 5 ng/mL for the M2+IL-6 phenotype. For both RNA analysis and flow cytometry experiments, the cytokine treatment was for 30 hours.

2.2.2 THP-1-Derived Macrophages

THP-1 cells were seeded at either 4-5 million cells/dish in 100-mm polystyrene petri dishes (for flow cytometry) or at 80,000 cells/well in 96-well tissue culture plates (for NanoString). The medium used to treat the cells was Roswell Park Memorial Institute (RPMI)-1640, supplemented with 10% FBS, 1% P/S and 2 mM L-glutamine. THP-1 cells used for experiments were between passages 7 and 25. The cells were treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 hours for differentiation into macrophages. Following this, the cells were subjected to the human polarization cocktails (Stem Cell or Peprotech) (100 ng/mL LPS and 20 ng/mL IFN-γ for M1; 20 ng/mL IL-4 and 20 ng/mL IL-13 for M2; 20 ng/mL IL-4, 20 ng/mL IL-13, and 5 ng/mL IL-6 for M2+IL-6). For RNA analysis, the cytokine treatment was for 30 hours; for flow cytometry experiments, it was for 72 hours. Where radicicol drug treatment was done, the cells were treated with 1 μM of radicicol (Cayman Chemicals) in dimethyl sulfoxide (<0.1% of final volume) along with the cytokines for 72 hours.

2.2.3 Peripheral Blood Mononuclear Cell (PBMC)-Derived Macrophages

Approximately 80 mL of blood was collected from healthy donors into BD Vacutainer sodium heparin tubes (Fisher Scientific). Blood separation was done using Lymphoprep density gradient medium and SepMate tubes (Stem Cell). 24 mL of whole blood was layered over Lymphoprep in a 1:2 volume ratio (for subsequent plasma isolation), while the remainder of the blood was diluted 1:1 in wash buffer (PBS with 2% FBS), and layered over Lymphoprep in a 1:1 volume

ratio. The blood was centrifuged at 1200 rcf for 10 minutes at room temperature, with the brake switched on. The plasma was collected into 1.5 mL Eppendorf tubes for storage. The buffy coat was collected, washed with wash buffer, and re-centrifuged at 300 rcf for 8 minutes at room temperature, with the brake switched on; this step was done twice. The cells were subsequently counted using a Countess Automated Cell Counter, and resuspended into RoboSep buffer (Stem Cell) at $50*10^6$ cells/mL for monocyte enrichment.

2.2.4 Monocyte Enrichment and Differentiation

Monocyte enrichment was performed using a RoboSep automated cell isolation platform (Stem Cell). A $CD14^+$ $CD16^-$ negative selection kit (Stem Cell) was used to isolate monocytes as per the associated protocol. Following isolation, the cells were counted using a hematocytometer or a Countess Automated Cell Counter, and resuspended in RPMI 1640 medium with 10% FBS, 1% P/S, 2 mM L-glutamine, and 25 μg/mL fungizone. The monocytes were plated on 100 mm polystyrene petri dishes and treated with 50 μg/mL human recombinant MCSF (Stem Cell) for 6 days, with a supplementation of medium with MCSF after the first 3 days. On the sixth day, the cells were lifted and used for various experiments.

2.3 NanoString Technology

For all NanoString experiments, the RNA was isolated from the cells using a commercially-available RNA isolation kit. Murine and human NanoString CodeSets consisting of the gene signature derived through *in silico* analysis was were developed. The RNA samples were analyzed using the nCounter® Analysis System for the genes within their respective species' CodeSet.

2.4 Murine Bleomycin-Induced Lung Fibrosis Study

Female C57BL/6 mice aged 6-10 weeks were exposed to 0.04-0.05 U/mouse bleomycin or PBS intratracheally at day 0. At day 7, their lungs were harvested for flow cytometry (see **Flow Cytometry and Analysis**).

2.5 Flow Cytometry and Analysis

Following treatment with cytokines, BMDMs/THP-1 cells/PBMCs were lifted from their petri dishes, and pelleted in a round-bottom 96-well plate. To perform flow cytometry on cells extracted from murine lungs, the lungs were first digested in DMEM F12 media supplemented with 10% FBS, 1% P/S, 2 mM L-glutamine and digestion enzymes; the cells were subsequently filtered through a syringe and pelleted in a round-bottom 96-well plate.

The cells were pelleted and resuspended in FACS buffer (0.3% bovine serum albumin in PBS), and fluorescence-activated cell sorting (FACS) cell

surface and intracellular staining protocols were employed. Non-specific binding of immunoglobulins to Fc receptors was blocked with the addition of Fc block. Following this, the cells were stained with the antibodies found in Table 2.

Flow cytometry data were analyzed using FlowJo® software version X.0.7 (FlowJo, LLC).

Table 2: Flow cytometry antibodies and channels. This table lists the antibodies and their corresponding channels that were used for all *in vitro* macrophage experiments.

Cells	Antibody	Channel
BMDMs	F4/80	BV605
	CD206	BV650
	Arginase-1	APC
	CLEC7A	PE
THP-1/PBMC-Derived Macrophages	CD206	AF700
	CD163	BV421
	CLEC7A	PE

2.6 Systemic Sclerosis Clinical Study

2.6.1 Overview of Clinical Study

As depicted in Figure 1, we recruited 12 study subjects for this pilot study (approved by Hamilton Integrated Research Ethics Board; study #0434) based on subject age and date since diagnosis. Four subjects were healthy controls, four subjects were systemic sclerosis patients who were taking calcium channel blockers (CCBs) such as nifedipine or amlodipine, and four subjects were systemic sclerosis patients who were not taking CCBs at the time of the study. Clinical data were collected for each subject. Two 4-mm skin punch biopsies were obtained from the non-dominant forearm at a standardized location (regardless of fibrotic skin score at that location), and approximately 30-50 mL of peripheral blood was drawn. The first biopsy was divided vertically into two halves; one half was fixed in 10% formalin for 48 hours and subsequently embedded in paraffin for immunostaining, while the other half was stored in RNALater and used for RNA isolation. Where possible, the second biopsy was cultured in vitro to extract dermal fibroblasts from the tissue. The fibroblasts were subsequently stored in liquid nitrogen for future analysis. From the blood collected, the whole buffy coat was isolated by Ficoll-Paque Plus density gradient centrifugation and stored in liquid nitrogen, while the plasma was stored in -80° C.

2.6.2 Histology Processing and Staining

All histological processing and staining was conducted by the John Mayberry Histology facility within the McMaster Immunology Research Centre.

2.6.3 RNA Isolation

For RNA isolation from skin, skin punch biopsies were transferred from RNALater to TRIzol (Invitrogen), and homogenized using a tissue homogenizer. For RNA isolation from cells, the cells were suspended in TRIzol immediately following treatment. The TRIzol RNA isolation protocol provided by Invitrogen was used to isolate RNA.

2.6.4 Fibroblast Culture from Skin Punch Biopsy

To culture fibroblasts from skin punch biopsies, we adopted the protocol outlined by Vangipuram et al. (Vangipuram et al. 2013). We elected to use DMEM medium, supplemented with 10% FBS, 1% P/S, 2 mM L-glutamine.

2.6.5 PBMC Isolation from Whole Blood

The same protocol as above (see **Peripheral Blood Mononuclear Cell (PBMC)-Derived Macrophages**) was used with some modifications, as outlined here. Approximately 30-50 mL of fresh whole blood was obtained from study subjects. Blood was processed within 2-4 hours of collection. Where the density gradient was established using Ficoll-Paque Plus (GE Healthcare Life Sciences) medium, blood was layered, in a 1:1 volume ratio, on top of Ficoll-Paque Plus in a 50-mL tube. The blood was spun down for 30 minutes at 400 rcf at room temperature, with the brake switched off. The plasma was collected into 1.5 mL Eppendorf tubes for storage, and the buffy coat was isolated into a separate 15-mL tube. The buffy coat was washed in a 1:5 volume ratio with RPMI 1640

medium and centrifuged for 10 minutes at 400 rcf at room temperature, with the brake switched on. The supernatant was discarded, and the pellet was resuspended in PBS. Following a cell count using a hematocytometer or a Countess Automated Cell Counter (Invitrogen), cells were resuspended in 90% human serum (Cedarlane) and 10% DMSO at a final density of $7*10^6$ million cells/mL for freezing.

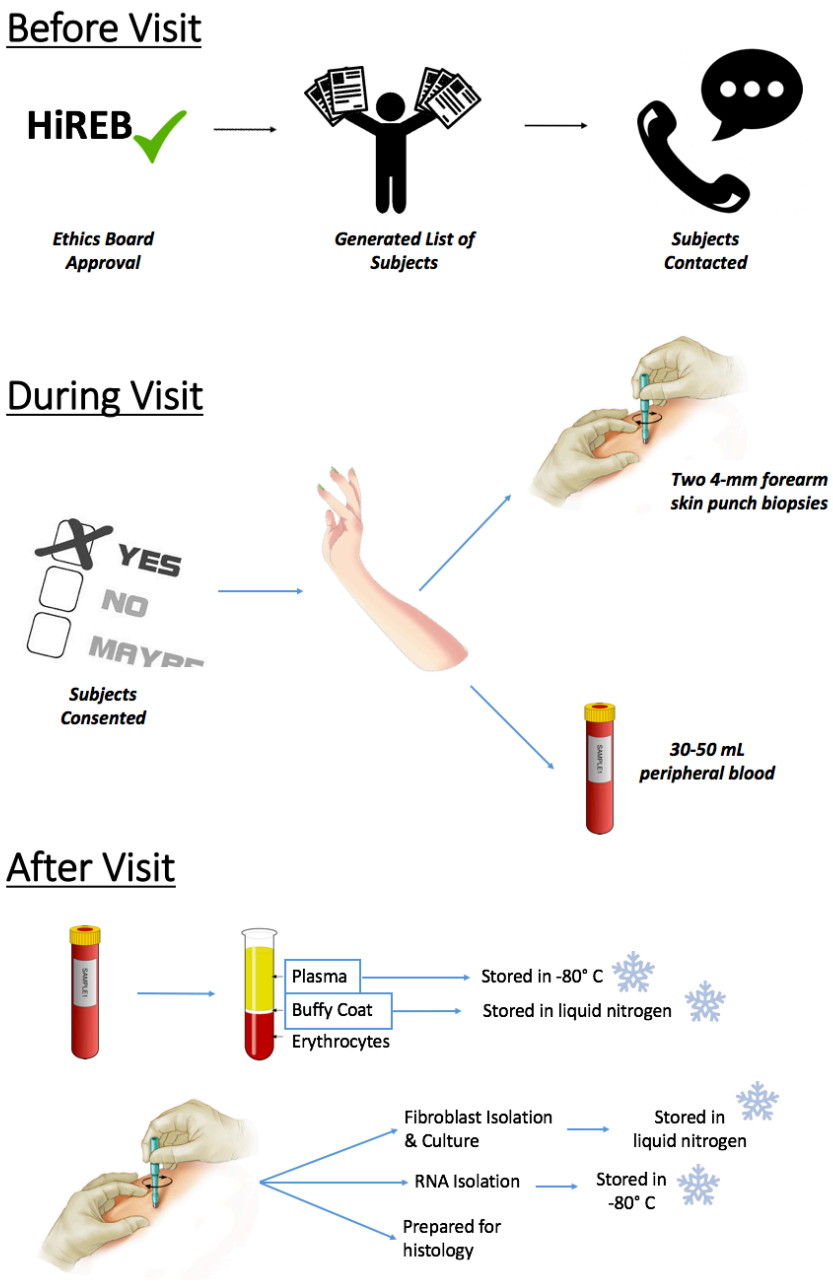


Figure 1: Schematic of clinical study. This diagram depicts the protocol and sample processing order as performed for the clinical study.

2.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 7.0. Data are presented as mean ± SEM. Where only two groups were compared, unpaired Student's T-test was used. In comparing more than two groups, one-way analysis of variance (ANOVA) was used, followed by Tukey's method. A p-value < 0.05 was considered to be statistically significant.

CHAPTER 3: RESULTS

3.1 Objective 1: To identify gene-level markers of macrophage polarization common to human and murine model systems

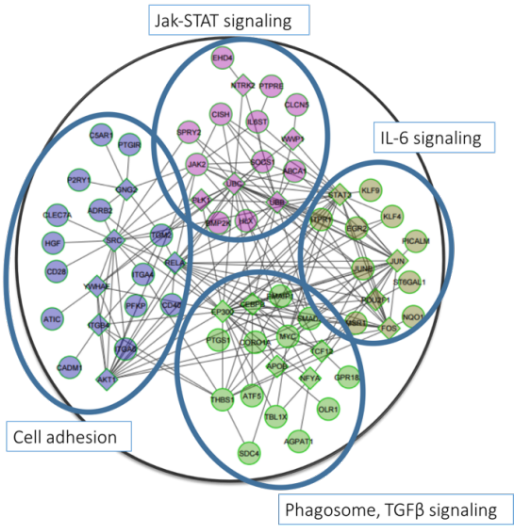
3.1.1 Bioinformatically-derived shared gene signature for M2 macrophage phenotype

From the comparison of the human and mouse macrophage gene DataSets, a list of 34 genes was identified as a signature unique to the M2 macrophage phenotype (Figure 2A). Amongst these genes, 27 were upregulated and 7 were downregulated, in the M2 macrophage in comparison to the Control and M1 phenotypes. We algorithmically mapped the protein-protein interactions between protein products derived from this gene signature. Fig. 2B depicts them in four modules relating to pathways that have different functions, and Fig. 2C indicates whether they are up- or downregulated in the M2 phenotype. The genes were involved in a variety of cellular functions, such as cell adhesion, Janus Kinase-Signal Transducers and Activators of Transcription (Jak-STAT) signaling, IL-6 signaling, phagosome activity, and TGF-β signaling (Harrison 2012).

A

Upregulated		Downregulated
AGPAT1	*PICALM*	*PMAIP1*
AMPD2	*PTGS1*	*ITPR1*
ATIC	*PTPRE*	*FPR1*
AUH	*ST6GAL1*	*ACSL1*
BMP2K	*IL1R1*	*PFKFB3*
CLCN5	*AP2A2*	*PIR*
CLEC7A	*DIP2C*	*GBP2*
EGR2	*DOCK10*	
GPR183	*NFE2*	
IL6ST	*SIPA1L3*	
KLF9	*SLC4A7*	
MYC	*SYS1*	
OLR1	*UBL3*	
P2RY1		

B



C

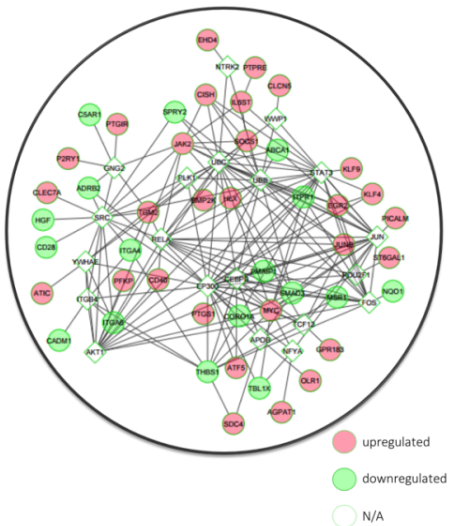


Figure 2: Theorized M2-specific gene signature. (A) The 34 genes identified through bioinformatic analysis of the GEO-deposited DataSets are listed above. The protein-protein interaction for these genes' protein products is also depicted in modules. (B) Four large modules can be categorized based on the pathways in which these proteins are involved, as shown in the map. (C) A second map with a visualization of the regulation for each gene is provided, and indicates that within each module, there are both up- and downregulated genes.

3.1.2 Expression of identified genes in murine BMDMs

Principal Component Analysis (PCA) diagrams provide a visual representation of the data in a format that emphasizes the variance between the data points. In the PCA graph in Figure 3A, each replicate is represented as a single sphere, and the variance in the gene expression patterns for the 36 genes together is visualized (although only 34 genes were shortlisted as mentioned above, a known M2 macrophage marker *Arg-1* and the housekeeping gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide gene (*Ywhaz*) were added to the CodeSet) (Murthi et al. 2008). The Control (M0), M1, and M2/M2+IL-6 gene signatures were distinct from each other. The heat map in Figure 3B shows the expression pattern for each of the 36 genes in each sample using a dendrogram. It can be seen from this diagram that some genes that were upregulated (red) in M2 and M2+IL-6 samples were downregulated (blue) in M1 and/or Control samples, such as *Clec7a* and *Il6st*. However, this does not depict whether the expression patterns of these genes were *significantly* different between the phenotypes. Figure 3C and Figure 3D list the genes that were significantly upregulated were significantly downregulated, respectively, in the M2 phenotype in comparison to all of the other macrophage phenotypes. Of the 28 genes expected to be upregulated (27 as identified bioinformatically, and *Arg-1*), 18 were found to be significantly upregulated; of the 7 genes that were expected to be downregulated, 5 followed this pattern significantly. This empirical verification of the bioinformatics-derived signature

thus provided valuable information about the theorized signature and our systems' expression of these patterns.

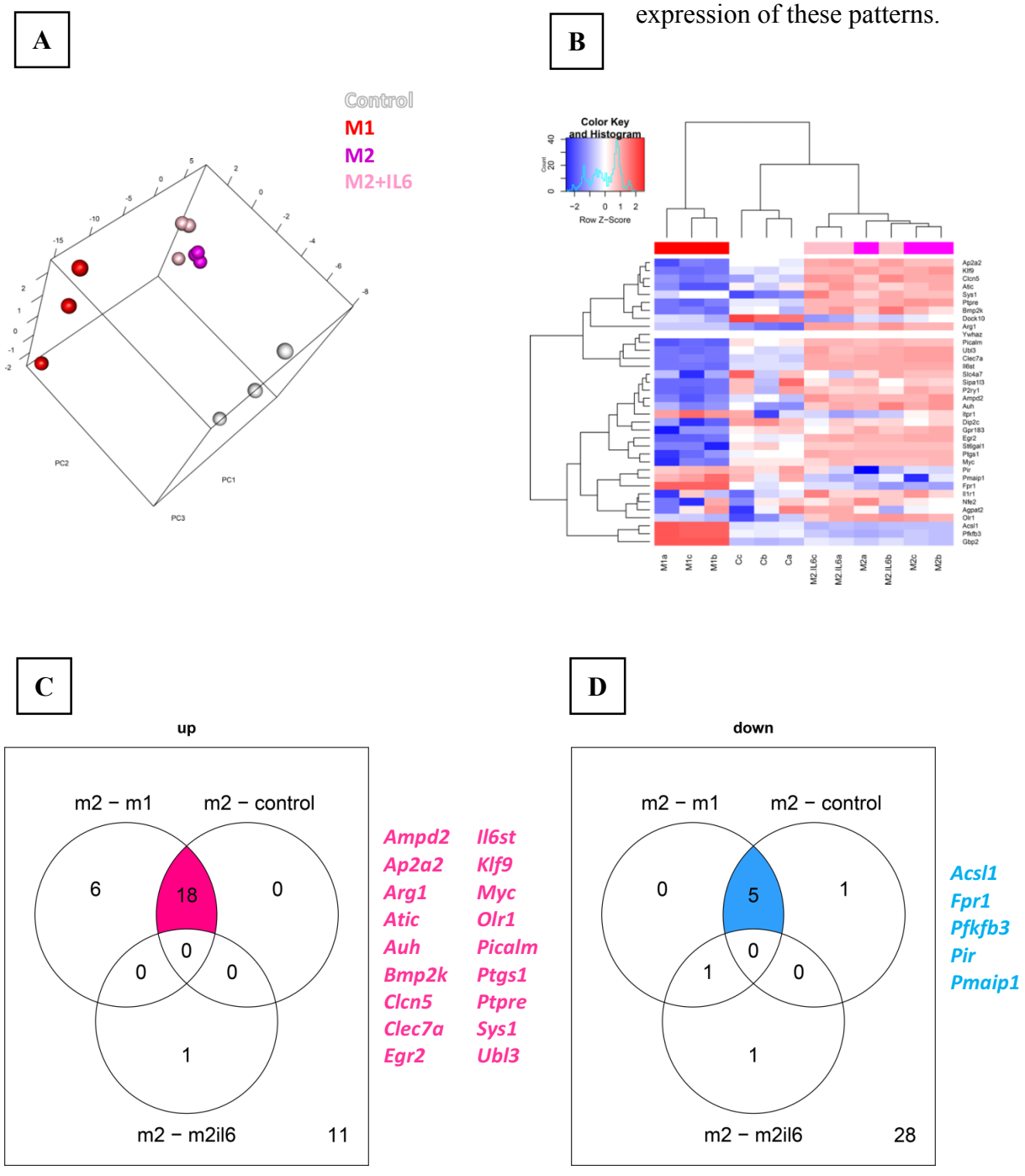


Figure 3: Mouse M2 macrophage RNA signature verification by NanoString. Murine BMDMs were treated for 30 hours without cytokines (Control), or with cytokines to achieve various activation states (100 ng/mL LPS and 20 ng/mL IFN-γ for M1, 20 ng/mL IL-4 and 20 ng/mL IL-13 for M2, 20 ng/mL IL-4, 20 ng/mL IL-13, 5 ng/mL IL-6 for M2+IL-6). RNA from these cells was subsequently isolated, and NanoString technology was used to probe the RNA for the transcripts of interest. (A) The Principal Component Analysis diagram indicates the total variation in the expression of the selected genes, between different phenotypes and between different replicates of the same phenotype. (B) The dendrogram indicates the upregulation (red) or downregulation (blue) of each gene, with Z-score expressed by intensity of colour, in each sample tested. (C) and (D) show that 18 genes were uniquely upregulated and 5 genes were uniquely downregulated, respectively, in the M2 phenotype in comparison to the Control and M1 phenotypes. All gene data were normalized to the housekeeping gene, *Ywhaz*.

3.1.3 Expression of identified genes in THP-1-derived macrophages

Similar to the mouse samples, the RNA from the THP-1 cell line was subject to NanoString analysis. The data obtained from the human cell line samples are shown in Figure 4. The PCA diagram in Figure 4A shows that overall, the M2/M2+IL-6 samples differed from the Control and M1 macrophages in their expression pattern of the 36 genes (although only 34 genes were shortlisted as mentioned above, the housekeeping gene *YWHAZ* and a known M2 macrophage marker *MRC-1* were added to the CodeSet). Figure 4B is a heat map, similar to that in Figure 3. It is evident that the M2/M2+IL-6 macrophages had opposite patterns of expression for certain genes, from the M1 and/or Control macrophages. For example, *CLEC7A* and *SLC4A7* were both upregulated in M2 macrophages (red), but downregulated in M1 (blue). Furthermore, the expression pattern of each gene in every phenotype replicate can be viewed in this diagram. Figure 4C depicts whether there was a *significant* difference in the expression of each gene between the macrophage phenotypes. Of the 28 genes whose expression in M2 macrophages was theorized to be upregulated in comparison to Control and M1 macrophages (27 as identified bioinformatically, and *MRC-1*), 10 were verified to be significantly upregulated. 9 of these genes were only upregulated in M2 macrophages in comparison to Control and M1, while *CLEC7A* was upregulated in M2 macrophages in comparison to M2+IL-6 macrophages as well. Seven genes were expected to be downregulated in M2 macrophages; only 3 of these were downregulated in our system.

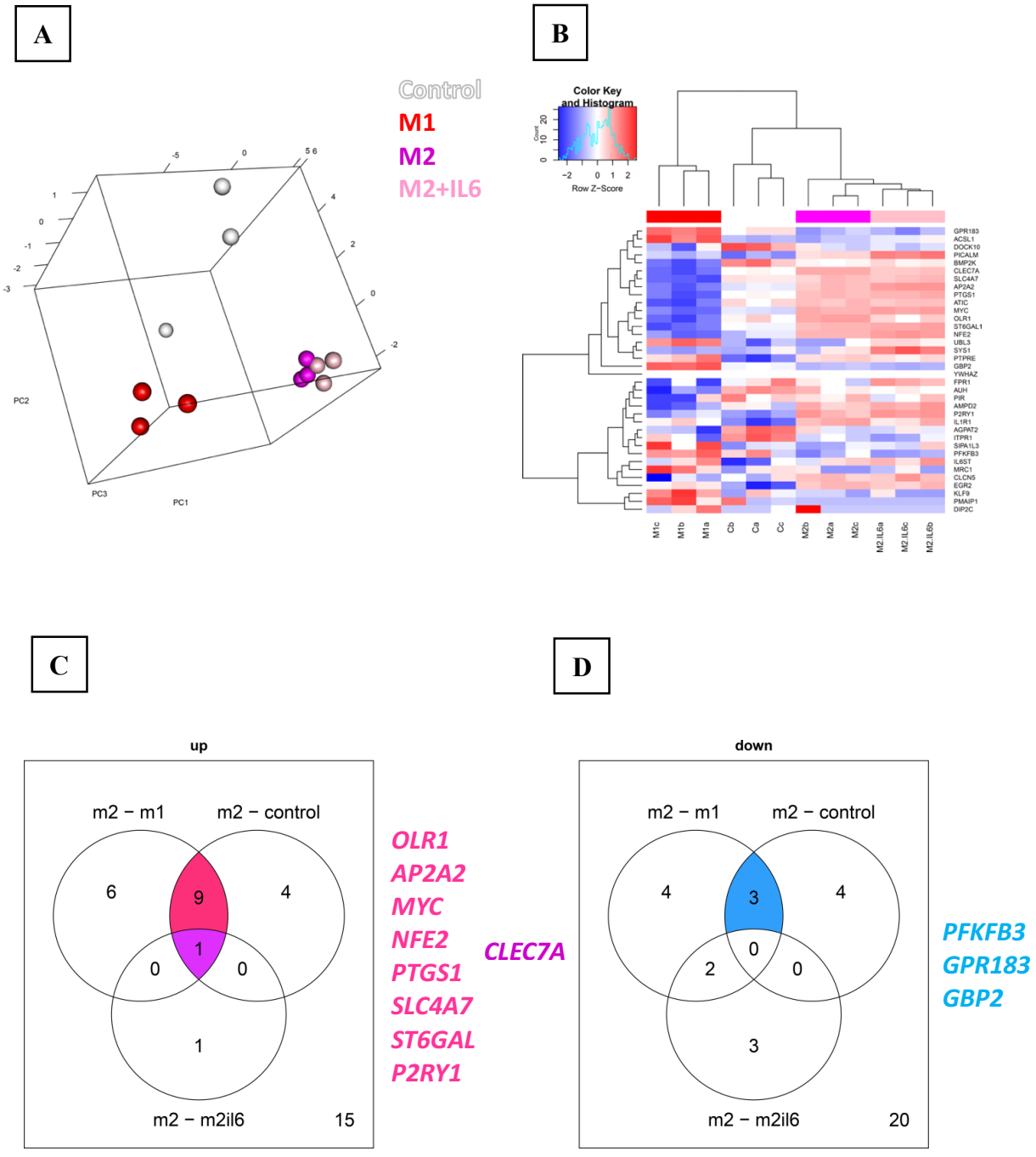
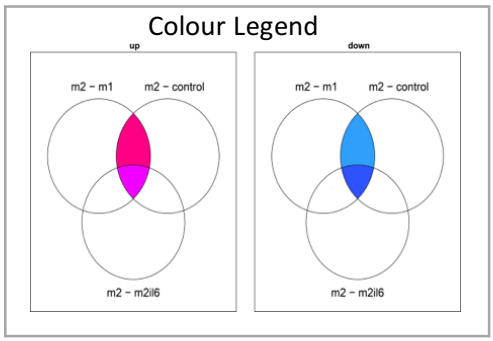

A
Control
M1
M2
M2+IL6
PC1
PC2
PC3
B
Color Key and Histogram
Count
Row Z-Score
GPR183
ACSL1
DOCK10
PICALM
BMP2K
CLEC7A
SLC4A7
AP2A2
PTGS1
ATIC
MYC
OLR1
ST6GAL1
NFE2
UBL3
SYS1
PTPRE
GBP2
YWHAZ
FPR1
AUH
PIR
AMPD2
P2RY1
IL1R1
AGPAT2
ITPR1
SIPA1L3
PFKFB3
ILRST
MRC1
CLCN5
EGR2
KLF9
PMAIP1
DIP2C
M1c
M1b
M1a
Cb
Ca
Cc
M2b
M2a
M2c
M2.IL6a
M2.IL6c
M2.IL6b
C
up
m2 − m1
m2 − control
m2 − m2il6
6
9
4
0
1
0
1
15
OLR1
AP2A2
MYC
NFE2
PTGS1
SLC4A7
ST6GAL
P2RY1
CLEC7A
D
down
m2 − m1
m2 − control
m2 − m2il6
4
3
4
2
0
0
3
20
PFKFB3
GPR183
GBP2

Figure 4: Human M2 macrophage RNA signature verification by NanoString. THP-1-derived macrophages were treated without cytokines (Control), or with cytokines to achieve various activation states (100 ng/mL LPS and 20 ng/mL IFN-γ for M1, 20 ng/mL IL-4 and 20 ng/mL IL-13 for M2, 20 ng/mL IL-4, 20 ng/mL IL-13, 5 ng/mL IL-6 for M2+IL-6). Following 30 hours of exposure to cytokine stimulation, the cells were lysed and their RNA was isolated. NanoString technology was used to probe the RNA for the transcripts of interest. (A) The Principal Component Analysis diagram emphasizes the total variation in the expression of the selected genes, between different phenotypes and between different replicates of the same phenotype. (B) The dendrogram indicates the upregulation (red) or downregulation (blue) of each gene, with Z-score expressed by intensity of colour, in each sample tested. (C) shows that 9 genes were uniquely upregulated in the M2 cells in comparison to Control and M1 only. Another gene, *CLEC7A*, was upregulated in M2 cells compared to all of the other phenotypes (Control, M1, M2+IL-6). (D) show that 9 genes were uniquely upregulated and 3 genes were uniquely downregulated, respectively, in the M2 phenotype in comparison to the Control and M1 phenotypes. All gene data were normalized to the housekeeping gene, *YWHAZ*.

3.1.4 Combined analysis of expression of identified genes in BMDMs and THP-1-derived macrophages

The above data show the expression of the bioinformatics-derived gene signature in the human and mouse systems separately. Since the goal of this investigation was to identify gene(s) that have unique patterns of expression in the profibrotic M2 phenotype, and are shared between human and mouse systems, it was necessary to do a comparison of the genes listed in Figure 3C and Figure 4C. This is shown in Table 4. A total of six genes were upregulated in M2 macrophages in both the human and mouse systems. This included *AP2A2*, *OLR1*, *MYC*, *PICALM*, *PTGS1* and *CLEC7A*. *PFKFB3* was the only gene significantly downregulated in both murine and human M2 macrophages. *CLEC7A* is shown separately in the table because the levels of *CLEC7A* expression differed significantly between M2 and M2+IL-6 human macrophages, but did not differ significantly in the murine M2-M2+IL-6 comparison. However, solely comparing the well-established Control, M1, and M2 phenotypes, *CLEC7A* appears on both mouse and human columns, and is thus considered a shared marker for our purposes. The functions of each of these genes is listed in Table 4.

Table 3: Combined analysis of mouse and human M2 macrophage signatures. The genes listed in Figure 3 and Figure 4, which were uniquely regulated by the M2 macrophage, are listed in this table. Genes with unique regulation in only murine M2 macrophages are listed in the left-most column, and genes with unique regulation in only human M2 macrophages are listed in the right-most column. The middle column lists the genes with unique regulation in both human and murine M2 macrophages. *CLEC7A* (outlined by a green box) was an exception to the shared gene list in the third (pink) row. It is written on the right-most column because it was the only gene that is uniquely upregulated in human M2 macrophages vs. Control, M1, M2+IL-6 phenotypes. However, it appears on the mouse list as well, as a gene that was upregulated in M2 macrophages compared to Control and M1, but not M2+IL-6, macrophages. Therefore, in this investigation, *CLEC7A* was given equal consideration alongside the shared upregulated genes outlined by the green box.



MOUSE	Shared	HUMAN
		CLEC7A
Ampd2 *Arg1* *Atic* *Auh* *Bmp2k* *Clcn5* *Clec7a* *Egr2* *Il6st* *Klf9* *Ptpre* *Sys1* *Ubl3*	*AP2A2* *OLR1* *MYC* *PICALM* *PTGS1*	*NFE2* *P2RY1* *SLC4A7* *ST6GAL*
Acsl1 *Fpr1* *Pir* *Pmaip1*	*PFKFB3*	*GBP2* *GPR183*

Table 4: Genes with significant up-/downregulation in M2 macrophages and their protein functions. The human-mouse shared genes with unique regulation in M2 macrophages produce proteins with diverse functions. Six of the seven genes in the gene signature were upregulated genes, and encode proteins with diverse functions.

Regulation	Gene	Function of Protein
Up	*CLEC7A* (C-type Lectin Domain Family 7, Member A gene)	Membrane receptor for fungi (binds to β-glucans) (Taylor et al. 2007)
	OLR1 (Oxidized low-density lipoprotein receptor gene)	Endocytosis receptor, involved in apoptosis, *CLEC7A* homolog (Tatsuguchi et al. 2003; Huysamen, Brown, and Sullivan 2009)
	AP2A2 (Adaptor-related protein complex 2 alpha 2 gene)	Participates in rapid endocytosis (Boehm and Bonifacino 2001)
	PICALM (Phosphatidylinositol Binding Clathrin Assembly gene)	Clathrin assembly (Ishikawa et al. 2015)
	PTGS1 (Prostaglandin synthase 1 gene)	Prostaglandin synthesis (Langenbach et al. 1995)
	MYC (Myelocytomatosis oncogene)	Regulates cell proliferation, induces apoptosis (G. I. Evan et al. 1992; Gerard I. Evan and Vousden 2001; Wang et al. 2013)
Down	*PFKFB3* 6-(Phosphofructo-2-kinase gene)	Cell cycle advancement, prevents apoptosis, promotes glycolysis (Bolaños, Almeida, and Moncada 2010; Yalcin et al. 2014)

3.2 Objective 2: To investigate protein expression of markers identified in Objective 1 in human and murine models of macrophage polarization

3.2.1 CLEC7A expression on murine bone marrow-derived macrophages

Figure 5 shows the intensity of CLEC7A expression by Control, M1, M2, and M2+IL-6 macrophages. From Figure 5A, it can be seen that the approximately the same number of events was collected for each of the samples. Normalized to mode, the M2 and M2+IL-6 macrophage samples had a noticeable rightward shift, indicating increased expression of CLEC7A when compared to Control and M1 macrophage samples. The sample data are shown individually in the dot plots in Figure 5B, wherein the frequency of $CLEC7A^{+}$ cells is 30-35% on M2 and M2+IL-6 macrophages, in comparison to the 2.5-3.5% in the Control and M1 macrophage samples.

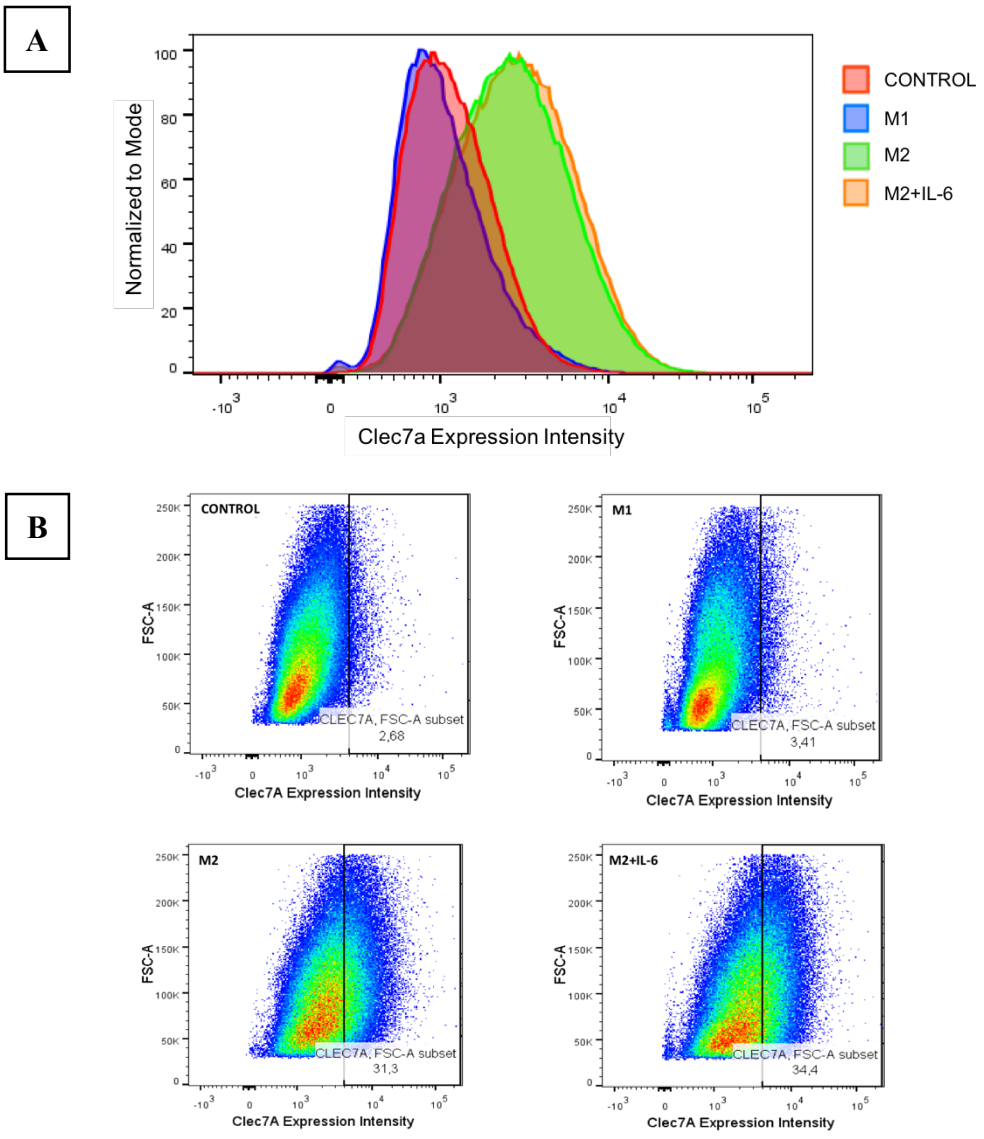


Figure 5: Flow cytometry analysis of CLEC7A expression by BMDMs. BMDMs were stimulated with cytokines for 30 hours and stained for CLEC7A expression. (A) Histogram showing intensity of CLEC7A expression by the different macrophage phenotypes. (B) shows separate dot plots for each individual

phenotype, with the percentage of total cells that were CLEC7A-positive written within each dot plot.

3.2.2 Comparison of CLEC7A expression with arginase-1 and CD206 expression

Arginase-1 activity is a reliable marker of murine M2 macrophages (Figure 6A). However, when arginase-1 was measured by flow cytometry, arginase-1 was expressed at 20-30% frequency in all four macrophage phenotypes (Figure 6B). Therefore, in our murine BMDM system, we compared the expression of CLEC7A with the expression of arginase-1 and CD206, two markers of M2 macrophages. Control and M1 macrophages expressed CD206 on 50%-55% of cells, while M2 and M2+IL-6 macrophages expressed it on 82% and 94% of cells, respectively. Figure 6C compares the expression of CLEC7A, arginase-1, and CD206 by the different macrophage phenotypes.

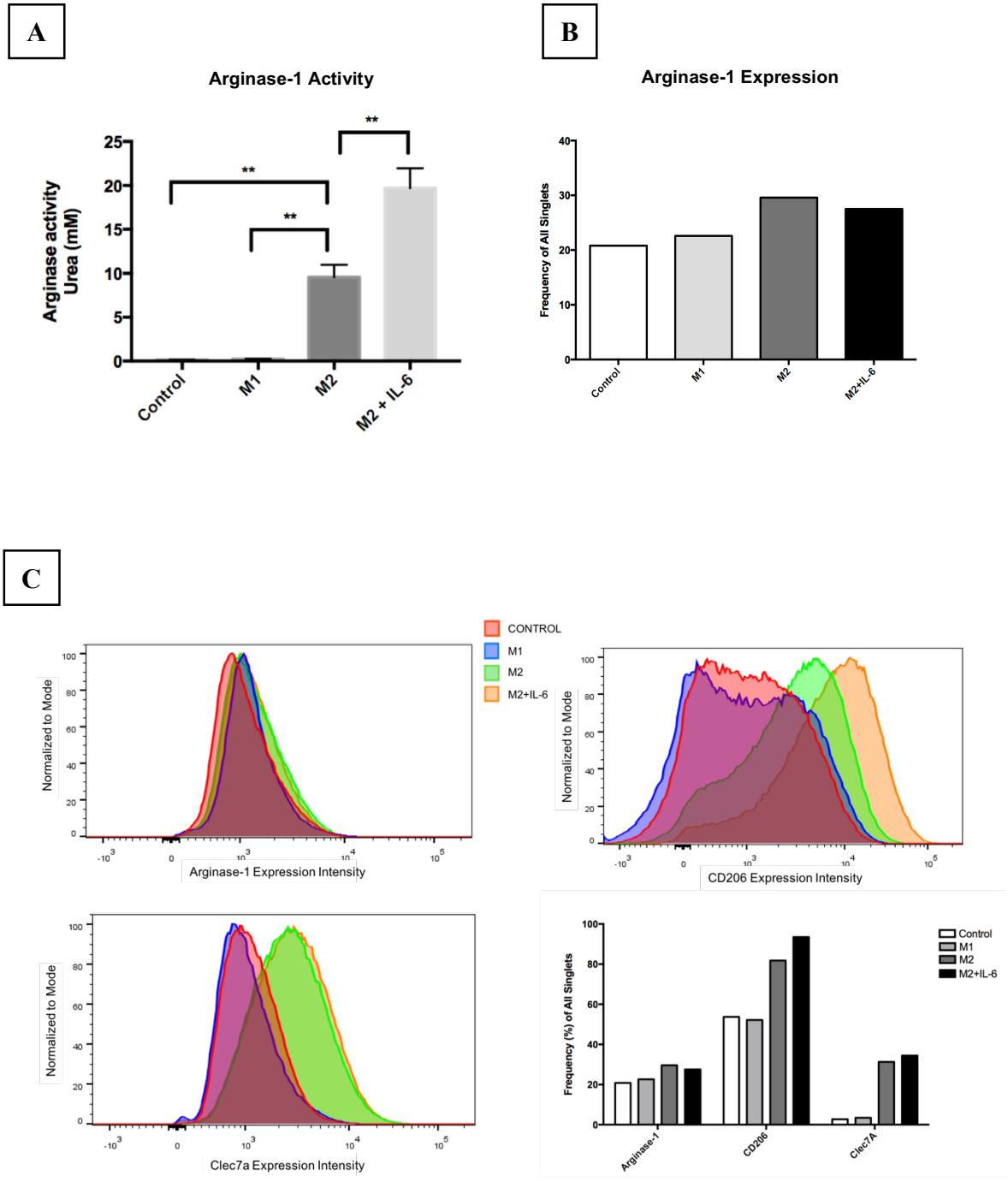
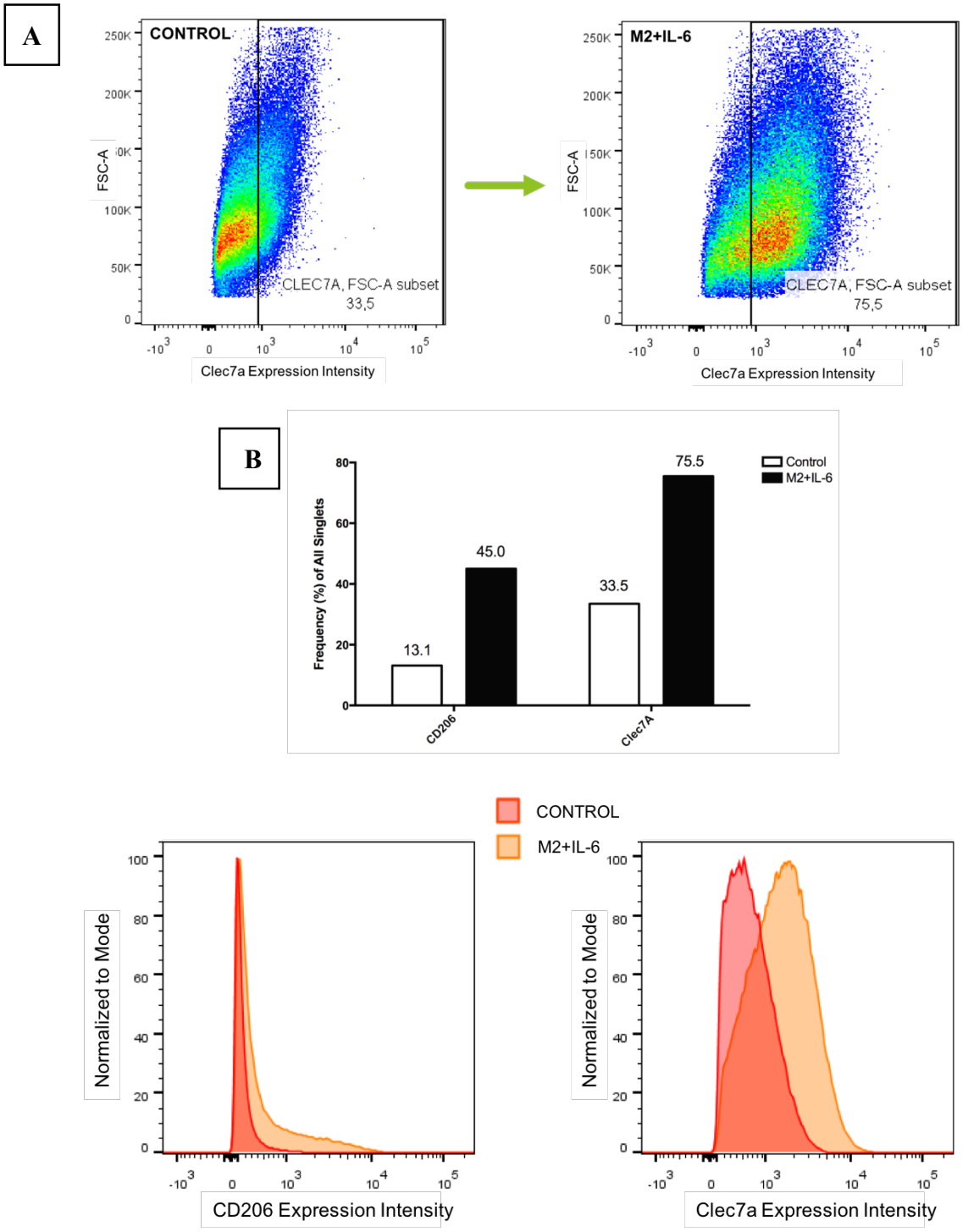

A
Arginase-1 Activity
Arginase activity
Urea (mM)
25
20
15
10
5
0
**
**
**
Control
M1
M2
M2 + IL-6
B
Arginase-1 Expression
Frequency of All Singlets
40
30
20
10
0
Control
M1
M2
M2+IL-6
C
CONTROL
M1
M2
M2+IL-6
Normalized to Mode
Arginase-1 Expression Intensity
CD206 Expression Intensity
Clec7a Expression Intensity
Frequency (%) of All Singlets
Control
M1
M2
M2+IL-6
Arginase-1
CD206
Clec7A

Figure 6: Comparison of macrophage expression of current M2 markers and CLEC7A in BMDMs. BMDMs were stimulated with cytokines for 30 hours, then stained for arginase-1, CD206, and CLEC7A. (A) Arginase-1 activity assay data are shown for reference, as the assay is a standard measurement to identify M2 macrophages. (B) The bar graph depicts frequency of arginase-1 expression as quantified by flow cytometry on macrophages. The data indicated that despite arginase-1 activity assay measurements, arginase-1 expression could not be used easily to distinguish M2 macrophages. (C) The histograms and bar graph provide different visualizations of flow cytometry data on expression of arginase-1, CD206, and CLEC7A by the different macrophage phenotypes, and show CLEC7A as a more selective marker of M2 macrophages than arginase-1 or CD206. The analysis performed in (A) was one-way ANOVA followed by Tukey's method. (**$p<0.01$; data presented as mean ± SEM.)

3.2.3 CLEC7A expression on THP-1-derived macrophages

Figure 7 depicts the results of two different trials of THP-1-derived macrophage treatment and CLEC7A expression evaluation by flow cytometry. It can be seen in both trials (Figure 7A/B being the first trial, Figure 7C being the second trial) that the expression of CLEC7A by the Control cells was approximately the same between the trials; this was the case for M2+IL-6-treated cells as well. 28-33.5% of Control cells expressed the receptor, while this value was 75.5-78% in M2+IL-6 cells. Figure 7B compares CD206 and CLEC7A expression intensity between Control and M2+IL-6 cells, using the CLEC7A quantification from Figure 7A. CD206 expression was 13.1% in Control cells, and 45% in M2+IL-6 cells. Figure 7C provides a complete picture of CLEC7A expression in all four phenotypes. It demonstrates that CLEC7A expression on Control and M1 cells was between 10-30%, and on M2/M2+IL-6 macrophages it was between 70-80%.


A
CONTROL
FSC-A
CLEC7A, FSC-A subset
33,5
Clec7a Expression Intensity
M2+IL-6
CLEC7A, FSC-A subset
75,5
Clec7a Expression Intensity
B
Frequency (%) of All Singlets
Control
M2+IL-6
13.1
45.0
33.5
75.5
CD206
Clec7A
CONTROL
M2+IL-6
Normalized to Mode
CD206 Expression Intensity
Clec7a Expression Intensity

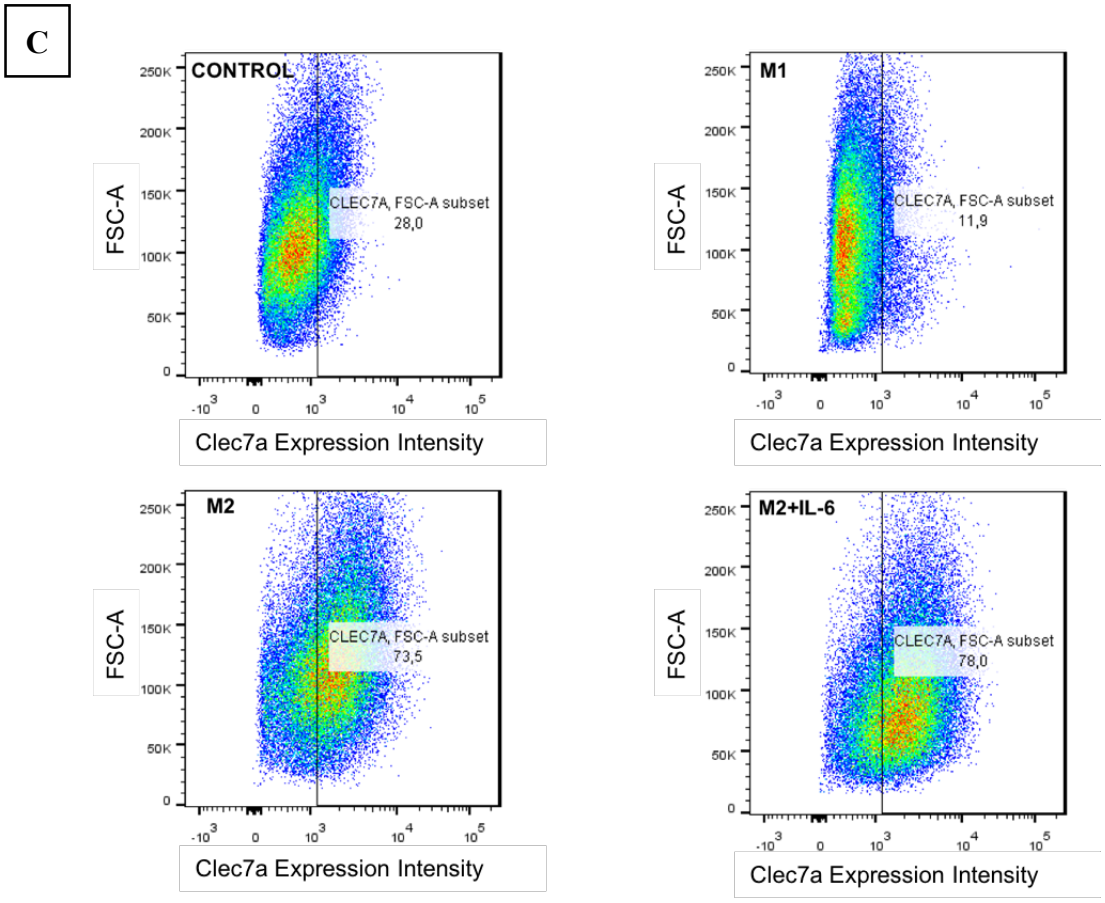


Figure 7: Comparison of macrophage expression of CLEC7A and current M2 marker CD206 in THP-1 cells. THP-1-derived macrophages were exposed to cytokine stimulation for 72 hours. Following stimulation, the cells were stained for assessment by flow cytometry. (A) The dot plots show the difference in frequency of CLEC7A expression between Control and M2+IL-6 macrophages. (B) The bar graph and histograms compare frequency and intensity, respectively, of CD206 and CLEC7A expression by Control and M2+IL-6 macrophages. (C) This set of dot plots provides CLEC7A expression data across all four macrophage phenotypes under study.

3.2.4 CLEC7A expression on PBMC-derived M2 macrophages

Figure 8 shows flow cytometry data of CLEC7A expression on PBMC-derived macrophages. Blood was drawn from a healthy donor and the monocytes were enriched and cultured with MCSF to produce macrophages. Approximately 50% of Control macrophages stained positive for CLEC7A. This value was reduced to 15.4% in M1 cells, while M2 and M2+IL-6 cells showed 63-68% positivity.

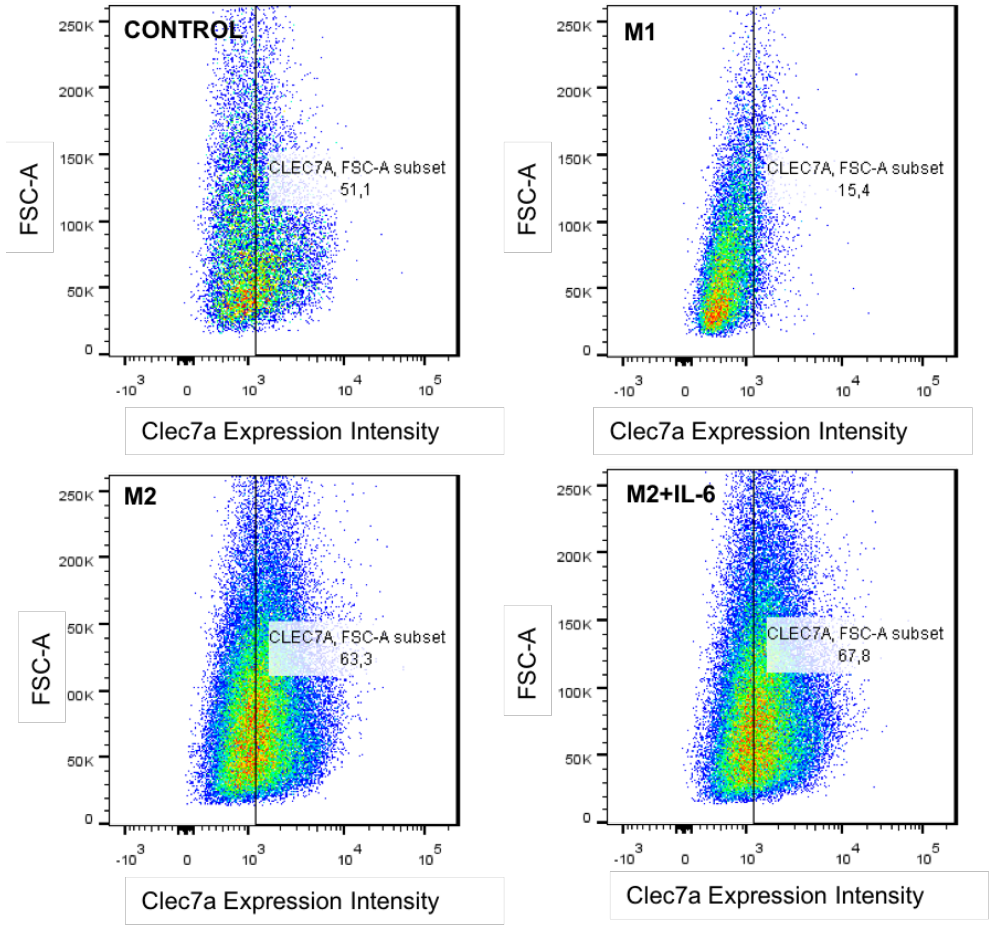


Figure 8: Expression of CLEC7A on PBMC-derived macrophages. PBMC-derived macrophages were stimulated with cytokines for 72 hours prior to flow cytometry. The dot plots provide CLEC7A expression data across all four macrophage phenotypes.

3.2.5 CLEC7A$^+$ macrophages in lungs of mice with bleomycin-induced lung fibrosis

Figure 9 shows a comparison of CLEC7A$^+$ macrophage populations in the lungs of C57BL/6 mice after 7 days of intratracheal exposure to either PBS or bleomycin. CD11b is a monocyte/interstitial macrophage marker, F4/80 is a macrophage marker, and CD64 is found on macrophages and monocytes (Yu et al. 2016). Selecting this triple-positive population helps us to identify the relevant macrophage population in a robust way, out of the total lung cell population which consists of various cell types. 9.52% (± 2.699%, n=3) of the CD11b$^+$ F4/80$^+$ CD64$^+$ cells in the lungs of control mice were CLEC7A$^+$, and 27.00% (± 2.787%, n=5) of the same subset of cells were CLEC7A$^+$ in the lungs of mice with lung fibrosis.

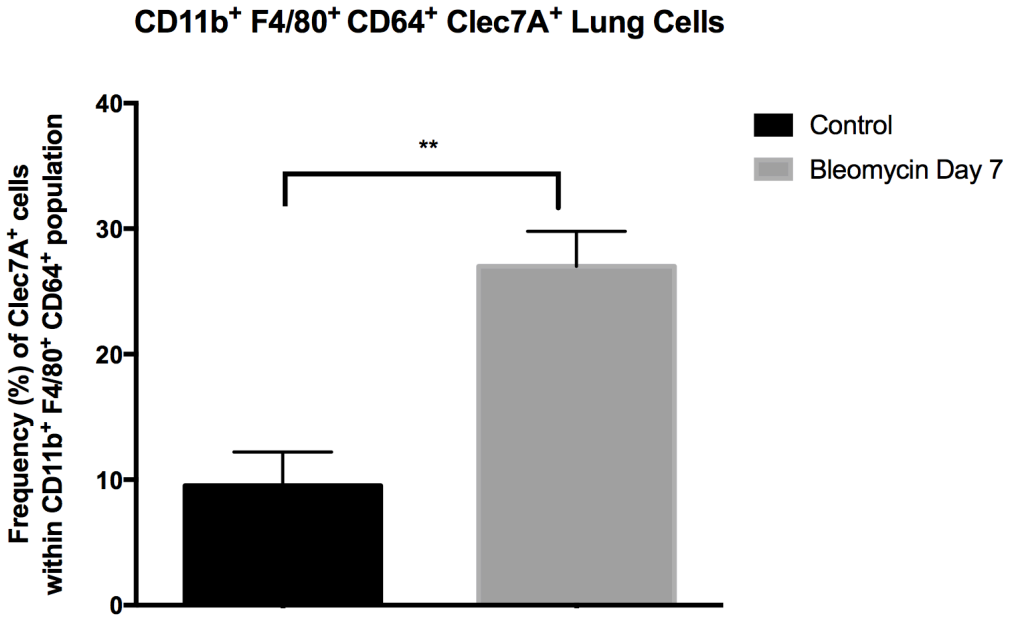


Figure 9: CLEC7A$^+$ macrophages in the lungs of mice with pulmonary fibrosis. Flow cytometry was performed on whole lung digests from mice following 7 days since exposure to bleomycin or saline. The frequency of CLEC7A$^+$ cells within the subset of lung cells that were CD11b$^+$ F4/80$^+$ CD64$^+$, i.e. macrophages, is shown. ($p<0.01$; n=3 for Control group and n=5 for Bleomycin Day 7 group; data presented as mean ± SEM.)

3.3 Objective 3: To investigate the bioinformatically-derived compound radicicol for selective inhibition of the M2 macrophage phenotype

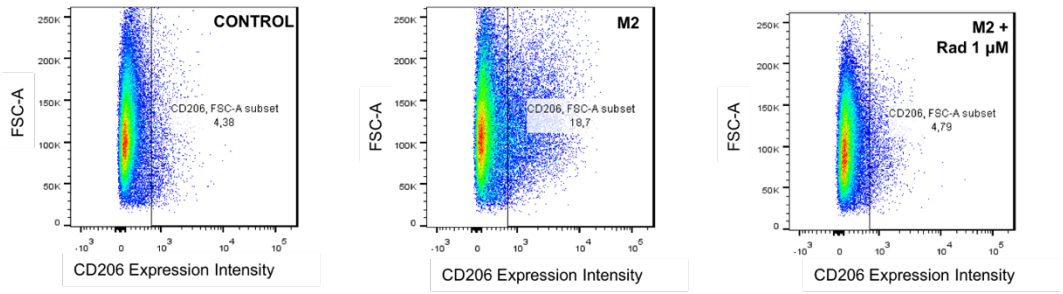
3.3.1 Identification of radicicol

The Connectivity Map (cMap) contains profiles of cells treated with 1,300 small molecules, and was therefore used to find small molecules that would induce or reverse the same signature as the one characteristic to the M2 phenotype. Known drugs that could reverse the expression of these genes (e.g. decrease expression of upregulated genes and increase expression of downregulated genes) were assessed. Through such a query, one of the compounds we identified as a potential inhibitor of this signature was the Heat Shock Protein 90 (HSP90) inhibitor radicicol (monorden).

3.3.2 The effect of radicicol on M2 macrophage marker expression

In THP-1-derived macrophages exposed to IL-4 and IL-13 to induce an M2 phenotype, the addition of 1 μM radicicol reduced the frequency of $CD206^+$ cells from 18.7% in IL-4/IL-13-treated cells to 4.79% in IL-4/IL-13 and radicicol-treated cells. The proportion of $CLEC7A^+$ macrophages also decreased, from 73.5% to 33.6% for the same samples (Figure 10). CLEC7A data are shown as *CLEC7A* was one of the genes upregulated in the M2 phenotype through our gene analysis, and CD206 data are shown as CD206 is a known M2 macrophage marker.

CD206



CLEC7A

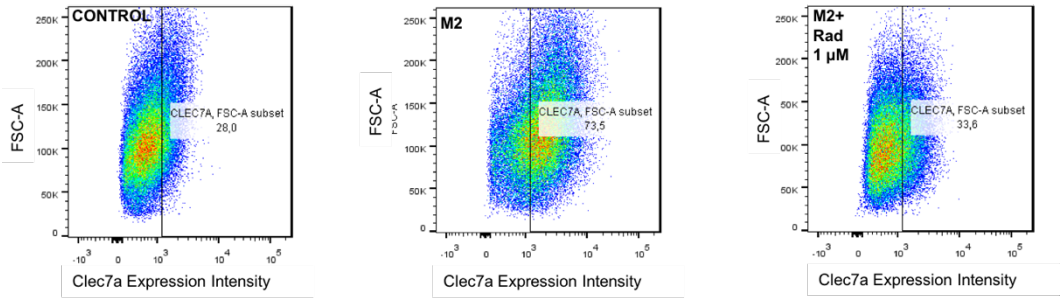


Figure 10: Inhibition of the M2 phenotype by radicicol. Flow cytometry data of macrophages stimulated with cytokines and treated simultaneously with 1 µM of radicicol for 72 hours. Radicicol addition was associated with decreased expression of M2 markers CD206 and CLEC7A. The CLEC7A Control and M2 data were the same as those shown in Figure 7.

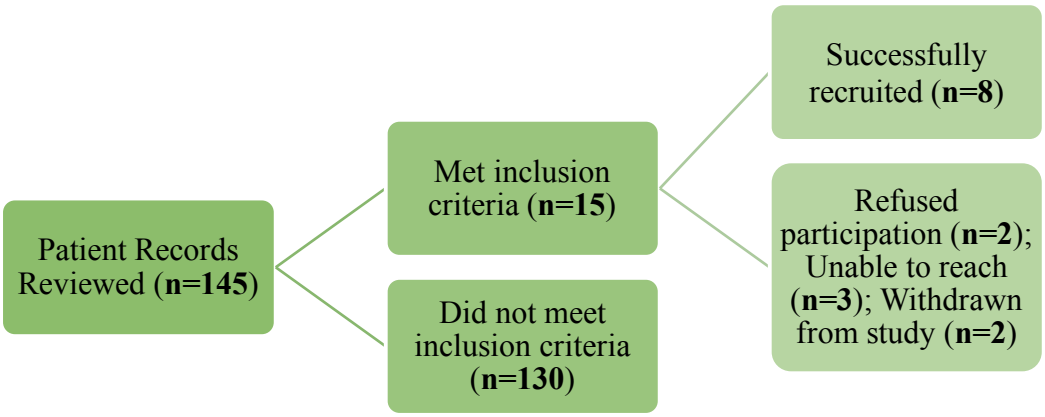
3.4 Objective 4: To conduct pilot clinical study on macrophages in fibrosis in systemic sclerosis

3.4.1 Demographic information

As shown in Figure 11, 145 unique patient records were evaluated as part of this study. All subjects were between 54 and 68 years of age. All healthy subjects were female, while amongst the patients with SSc, there were 6 females and 2 males. As the study focused on early stages of the disease, patients who had been diagnosed more than 5-10 years prior to the date of file review were excluded from the study.

3.4.2 Histology on skin punch biopsies

Figure 12 shows the haematoxylin & eosin (H&E) stain and DECTIN-1 immunohistochemistry on skin punch biopsies. Each letter corresponds to a different subject in the study. With a 4-mm skin punch biopsy, we were able to capture the epidermal and dermal layers. The H&E provides a visualization of the cell types and extracellular components that were present within the skin, and the DECTIN-1 stain indicates which cells were positive for CLEC7A within the skin. The H&E was also used to verify that the staining of DECTIN-1 was not an artefact but a true stain.



Parameter	Healthy Subjects (n=4)	Subjects with SSc (n=8)
Age (years)	55-65	54-68
Female:Male	4:0	3:1
Limited:Diffuse SSc	N/A	1:1

Figure 11: Patient identification and demographic information. 145 patient records were reviewed to arrive at a final set of 8 patients with SSc for study recruitment. Subjects were all within the a 14-year age range of 54 to 68 years. Demographic information for both subject groups is provided.

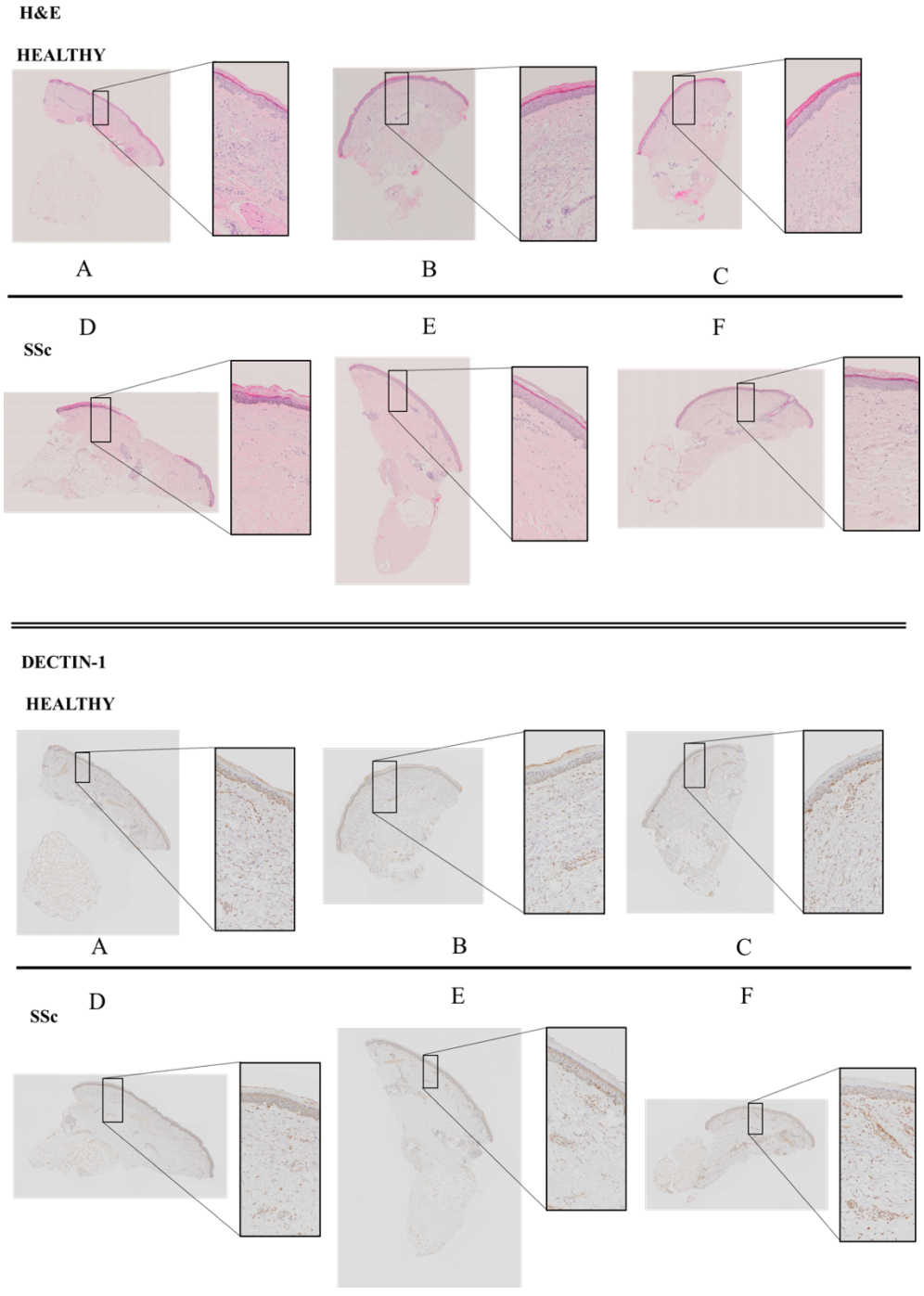


Figure 12: Histochemistry and immunohistochemistry on skin biopsy tissues. H&E and DECTIN-1 staining are shown on the skin biopsies taken from six subjects (3 healthy subjects, 3 with SSc).

CHAPTER 4: DISCUSSION

4.1 A 7-gene subset of a bioinformatically-theorized gene signature for human and murine M2 macrophages is experimentally validated

4.1.1 A distinct, 34-gene signature for human and murine M2 macrophages is obtained through bioinformatic analysis

The M2 macrophage is involved in wound healing functions and is thus of considerable interest in fibrosis research (Pesce et al. 2009). Without a robustly characterized gene signature for this phenotype, studying this cell type *in vivo* in humans and mouse models of fibrotic disease is a significant challenge. In particular, comparative study of the M2 macrophage in humans and mice would be strengthened with the identification of a common marker in both systems, but such a marker is not currently well-known (Murray and Wynn 2011b; Murray et al. 2014; Murray 2017). To address this issue, we conducted a bioinformatic comparison of publicly deposited gene DataSets of human (PBMC-derived) and murine (BMDM) macrophage phenotypes. We identified 27 genes that were uniquely upregulated in the M2 macrophage, in comparison to Control and M1 macrophages, in both human and murine systems. Similarly, 7 genes were downregulated in the M2 macrophage in both systems. This 34-gene signature consisted of genes involved in various cellular processes, notably in TGF-β signaling, which is important for fibrogenic processes (Lech and Anders 2013).

4.1.2 Murine BM-derived M2 macrophage phenotype confirms majority of predicted gene signature

Murine BMDMs have been studied extensively in various areas of research. These cells generate strong responses to stimuli *in vitro*, and serve as a primary tool for studying macrophage phenotypes (Zhang, Goncalves, and Mosser 2008). We isolated RNA from BMDMs that had been polarized to the different macrophage phenotypes and used NanoString technology to verify our predicted gene signature unique to the M2 macrophage. As shown in Figure 3, of the 28 genes that were predicted to be upregulated, 18 were significantly upregulated. Similarly, of the 7 genes that were expected to be downregulated, 5 were significantly downregulated. These genes may or may not be integral to the M2 phenotype in the mouse system, but they could at least serve as a marker of this phenotype. As seen in the PCA in Figure 3A, the replicates for each treatment clustered somewhat closely, but some differences in gene expression were seen in the Control and M1 macrophage replicates. This variation amongst replicates could have contributed to a non-significant expression pattern for a given gene, removing it from the list of genes following a significant upregulation/downregulation pattern between the phenotypes. It also follows that the genes determined as significantly up-/downregulated despite this variation must indeed be very differently expressed between the phenotypes.

4.1.3 THP-1-derived M2 macrophage phenotype confirms subset of predicted gene signature

The THP-1 cell line is a human cell line derived from the acute monocytic leukemia of a 7-year-old male patient (Tsuchiya et al. 1980). First described by Tsuchiya et al. in 1980, it has since been in standard use for monocyte and macrophage *in vitro* studies (Auwerx 1991; Daigneault et al. 2010). Following treatment with polarization cytokines to induce the different phenotypes, THP-1-derived macrophages were lysed to isolate RNA. As shown in Figure 4, when probed using NanoString technology for the predetermined 36-gene CodeSet, 10 of the 28 genes anticipated to be upregulated followed that pattern significantly. Amongst the 7 genes expected to be downregulated, there were 3 genes that followed that pattern significantly.

In the PCA (Figure 4A), there was some unexplained variability between the Control replicates, which may have had some impact in the analysis of the results. It is interesting to note that while many genes were similarly expressed in all of the replicates of a given phenotype (e.g. *PICALM, ATIC*), there were some genes that were not similarly expressed in the replicates of a phenotype (e.g. *PFKFB3* in Control samples, *DIP2C* in M2 samples). These genes may have contributed to the lack of clustering between replicates in Figure 4A. Some genes, which may have been expected to show significant, unique regulation based on the bioinformatic analysis, may have been omitted through this approach because the GEO human DataSets contained data on PBMC-derived macrophages, and not

using a THP-1 model. Conversely, this notion may provide further confidence into the genes that were confirmed to have significant upregulation, as they were maintained across the two different model systems.

4.1.4 A 7-gene signature unique to the M2 macrophage is validated to be shared between human and mouse systems

In the previous two sections, the theorized gene signature was experimentally tested in a murine system and in a human system. A subset of genes that could be confirmed as significantly unique in expression pattern to the M2 macrophage was obtained in each system. For the mouse system, 24 of the theorized 34 genes constituted this subset. In the human system, 13 genes fell into this category. A comparison of these two subsets is shown in Table 3, and the functions of the genes that were shared between both systems are described in Table 4. Most of the genes associated with a unique, M2 macrophage signature were upregulated. The functions of the proteins encoded by these genes were very diverse. Several of these proteins were involved in endocytic roles, whether as a receptor (*CLEC7A*, *OLR1*), in vesicular transport (*AP2A2*), or in clathrin assembly (*PICALM*) (Boehm and Bonifacino 2001; Taylor et al. 2007; Tatsuguchi et al. 2003; Ishikawa et al. 2015). This suggests that these genes may be directly relevant to the M2 phenotype for macrophages.

4.2 CLEC7A (DECTIN-1)

4.2.1 Overview of CLEC7A

CLEC7A was one of the genes identified to be uniquely upregulated in both human and murine M2 macrophages. We decided to begin our investigation into the gene signature at *CLEC7A* for several reasons. *CLEC7A*, which stands for "C-type lectin domain family 7, member A" encodes the C-type lectin receptor (CLR) known as DECTIN-1 (Zhou et al. 2010; Dambuza and Brown 2015). This CLR is expressed on macrophages, dendritic cells, and neutrophils (Taylor et al. 2002).

DECTIN-1 (dendritic cell-associated C-type lectin-1) is a non-Toll-like receptor (TLR) type II membrane receptor (Brown 2006; Zhou et al. 2010). This pattern recognition receptor binds to β-1,3-linked and β-1,6-linked glucans, which are carbohydrates found on fungal cell wall (Brown 2006; Zhou et al. 2010). Upon ligand binding, DECTIN-1 activates intracellular signaling through a cytoplasmic integral immunoreceptor tyrosine-based activation (ITAM)-like motif (Dambuza and Brown 2015). Through a spleen tyrosine kinase (SYK)-mediated response, DECTIN-1 induces the production of reactive oxygen species and initiates phagocytosis (Goodridge et al. 2011).

In the context of macrophage biology, DECTIN-1 is known to be an M2 macrophage marker in human and mouse systems separately (Murray 2017). However, it has not been widely reported as a shared marker for M2-like cells

between human systems and murine models of fibrosis. It has also not been widely reported in the clinical literature as a marker for fibrosis in systemic sclerosis.

4.3 BMDMs, THP-1s and PBMCs show similar patterns of expression of the membrane receptor encoded by the *CLEC7A* gene

4.3.1 Murine bone marrow-derived M2 and M2+IL-6 macrophages upregulate CLEC7A expression

In murine BMDMs, CLEC7A expression intensity was significantly upregulated on M2 and M2+IL-6 macrophages. The Control and M1 macrophage samples had approximately the same, low frequency of $CLEC7A^{+}$ cells in comparison the M2/M2+IL-6 phenotypes. This helped differentiate the M2 macrophages from both Control and M1 macrophages, which was a confirmation of CLEC7A as a marker with an expression pattern *unique* to the M2 phenotype amongst murine BMDMs.

4.3.2 CLEC7A is a more selective marker of murine M2 macrophages than arginase-1 or CD206

Although arginase-1 activity is a reliable marker of murine M2 macrophages (Figure 6A), arginase-1 expression as measured by flow cytometry was a poor tool in differentiating between Control, M1, M2, and M2+IL-6 macrophages (Figure 6B). Thus, we compared CLEC7A expression with the expression of arginase-1 and CD206, two well-known markers of murine M2

macrophages. Compared to arginase-1, CD206 expression was more separated between Control/M1 and M2/M2+IL-6 macrophages. However, we reasoned that 50-55% of Control/M1 cells expressing this marker was a high fraction of non-M2 cells expressing what is used as a marker for M2 macrophages. Figure 6C shows that CLEC7A was a more selective marker of M2/M2+IL-6 macrophages than either of arginase-1 and CD206.

4.3.3 THP-1-derived M2 and M2+IL-6 macrophages upregulate CLEC7A expression

Using macrophages derived from the THP-1 human monocyte cell line, Figure 6 shows the expression of CLEC7A in human macrophage phenotypes. CLEC7A was significantly upregulated on the M2 and M2+IL-6 macrophage phenotypes, in comparison to both Control and M1 cells. The comparison of CLEC7A with the M2 macrophage marker CD206 (Figure 7B) indicates that CLEC7A was as robust as CD206 – the marker widely used to identify M2 macrophages – in discriminating between Control and M2+IL-6 cells. While the expression of CD206 increased by 31.9% from Control to M2+IL-6 macrophages, the expression of CLEC7A increased by 42% for the same samples (Figure 7B). However, it is important to recognize that 28-33.5% of Control cells expressed CLEC7A, which may be too large a proportion for CLEC7A to be used as a singular marker for THP-1-derived M2 cells.

4.3.4 PBMC-Derived M2 macrophages have higher expression of CLEC7A

PBMC-derived macrophages were the human cells used in the GEO DataSets used for the bioinformatic portion of this study (Martinez et al. 2006; Solinas et al. 2010). This made it further interesting to identify whether the protein product of *CLEC7A* would be significantly upregulated in PBMC-derived macrophages. Approximately 50% of the Control cells here stained positive for CLEC7A, and this value increased to 63-68% in M2 and M2+IL-6 cells. The low CLEC7A expression on M1 macrophages fit our hypothesis, but the relatively high expression on Control cells raised some questions. This could be a product of technical error, or PBMC-derived macrophages could naturally be more skewed toward an M2 phenotype. Further optimizations need to be conducted before CLEC7A can be used in a PBMC-derived macrophage system to differentiate the macrophage phenotypes as robustly as it does in the murine and THP-1 systems.

4.3.5 CLEC7A$^+$ macrophages are increased in the lung cell population in mice with bleomycin-induced lung fibrosis

The bleomycin-induced pulmonary fibrosis model is a well-characterized and widely-used model for the study of fibrosis within the lung. As macrophages have been implicated in the lung injury phase of this model, we sought to characterize the proportion of CLEC7A$^+$ macrophages in this model (Ayaub et al. 2016). Day 7 is of particular interest because it is within the injury phase for this model. We sought to identify whether CLEC7A$^+$ macrophages were present at day 7, which was the same phase during which M2-like cell markers have been

previously reported to be upregulated (Ballinger et al. 2015). We demonstrated that mice with pulmonary fibrosis had a marked increase in the proportion of $CD11b^+$ $F4/80^+$ $CD64^+$ cells that were $CLEC7A^+$, in comparison to PBS-treated control mice (Figure 9). This further provided support for the potential scope of CLEC7A as a marker for a subset of macrophages in lung fibrosis studies. Further studies should identify whether this particular subset is pathogenic, and whether $CD11b^+$ $F4/80^+$ $CD64^+$ $CLEC7A^+$ macrophages are distinct or similar to the macrophages identified by using other M2 macrophage markers.

4.4 The HSP90 inhibitor radicicol inhibits the M2 macrophage phenotype

4.4.1 Radicicol is identified as a potential inhibitor of the M2 gene signature

In addition to the bioinformatic identification of an M2 macrophage gene signature, we conducted a cMAP query with this gene signature. The cMAP contains the transcriptional profile of cells post-treatment with 1,300 different small molecules. Our signature was loaded into the database in order to query for drugs that could induce a signature opposite to that which we loaded. Thus, we identified the HSP90 inhibitor radicicol (monorden) as a potential candidate to prevent expression of our M2 macrophage gene signature. As our signature describes a cell phenotype which is implicated in fibrotic disease, finding pre-existing compounds that can be repurposed for therapeutic purposes in this context is of significant value.

4.4.2 Radicicol inhibits CD206 and CLEC7A presentation on THP-1-derived M2 macrophages

Our data indicated that inhibiting HSP90 may be useful for preventing the formation of an M2 macrophage phenotype. Through flow cytometry on THP-1-derived macrophages, we showed that the addition of radicicol at 1 μM prevented the formation of an M2 phenotype. In Figure 10, the flow cytometry data indicated that in cells exposed to IL-4 and IL-13 to induce an M2 phenotype, the addition of 1 μM radicicol reduced the frequency of $CLEC7A^+$ macrophages from 73.5% to 33.6%, which was close to the Control cells' level of 28% CLEC7A positivity. The data for CD206 also followed a similar pattern, but it was remarkable that even the M2 phenotype had poor expression of CD206 (18.7%). While this may suggest that CLEC7A is a better marker of the phenotype than CD206, this phenomenon may also be a shortcoming inherent to the THP-1 cell line, and may indeed be different in macrophages taken from healthy donors.

4.5 Systemic sclerosis clinical study

4.5.1 Detection of DECTIN-1-positive cells in skin

Figure 12 shows the images of six subjects' skin punch biopsies, as stained for H&E and DECTIN-1. To verify the utility of DECTIN-1 as a marker for a profibrotic macrophage in a clinical setting, we stained for DECTIN-1 in the skin tissue of healthy donors (three subjects) and in that of patients who were diagnosed with SSc (three subjects). As shown in Figure 12, there were several

cell types that had a positive stain for DECTIN-1. As DECTIN-1 is expressed on other cells apart from macrophages, such as dendritic cells and neutrophils, it is important to perform a colocalization stain. Immunofluorescent staining of a pan-macrophage marker such as CD68 along with DECTIN-1 would help identify the proportion of macrophages that expresses for DECTIN-1. This can be further correlated to fibrotic skin score, to explore any association of DECTIN-1-positive-macrophages with fibrotic outcome in the skin. This correlation is important for making conclusions because the standardized location of the biopsy may have had varying degrees of fibrosis between subjects.

4.6 Implications of findings

4.6.1 Overview of findings

There is a great deal of variation in our understanding of the M2 macrophage in different settings, and there remains a lack in our characterization of this cell across species (Murray et al. 2014). The M1-M2 model is useful for *in vitro* study, wherein stimulation with specific cytokines can elicit clearly-defined phenotypes. However, *in vivo*, where such isolated stimulation is unlikely, the resulting macrophage phenotypes may be termed as "M1-like" and "M2-like" based on whether they share characteristics with the M1 or M2 *in vitro* phenotypes, respectively (Satoh et al. 2013). Given these inter-system and interspecies discrepancies, there is an urgent and significant need to develop tools with which we can identify and characterize M2-like macrophages with

uniformity across systems and species (Murray and Wynn 2011b; Murray et al. 2014; Murray 2017).

In this project, we used an *in silico* method to derive an M2-specific gene signature common to murine and human macrophages and verified this signature through *in vitro* experiments and RNA analysis. The verification process provided us with a 7-gene subset of the original signature. Subsequent protein-level verification showed CLEC7A to be a promising marker for M2 and M2-like macrophages in murine *in vivo*, and murine and human *in vitro*, systems. CLEC7A as a marker for M2-like cells, shared between human systems and murine models of fibrosis, has not been cited in the literature thus far. In addition, M2-like cells have been implicated in fibrosis, but CLEC7A has not been widely explored as a shared human-mouse marker in this context, especially within SSc (Murray and Wynn 2011a). Therefore, our work provides a novel perspective on the potential of CLEC7A to serve as a shared marker for human and mouse M2 (*in vitro*) and M2-like (*in vivo*) macrophages, with an extension into the specific context of fibrotic disease.

4.6.2 DECTIN-1 and galectins

Investigation into DECTIN-1 in the context of fibrosis is potentially very relevant to current clinical approaches taken to address fibrosis. In particular, in January 2017, a Phase Ib/IIa randomized control trial of an inhaled GALECTIN-3 inhibitor for the treatment of idiopathic pulmonary fibrosis (IPF) was completed,

with results from the phase I study showing promise for this drug (Ford et al. 2015; Clinicaltrials.gov 2017). Galectins represent a subclass of lectins, and GALECTIN-3 is considered a key regulator of pulmonary fibrosis (MacKinnon et al. 2012). A study by Punt et al. demonstrated that in formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients with squamous cervical cancer, the immune cell with highest expression of GALECTIN-3 was the $CD163^+$ type 2 macrophage (Punt et al. 2015). A separate study has shown that DECTIN-1 and GALECTIN-3 interact with each other in RAW 264.7 macrophages (Esteban et al. 2011). The same study by Punt et al. indicated that the majority of $CD163^+$ macrophages were also positive for GALECTIN-9, which is a member of the GALECTIN-3 subgroup (Punt et al. 2015; Cummings et al. 2009). Recently, GALECTIN-9 was shown to activate DECTIN-1 on splenic macrophages (Daley et al. 2017). Taken together, given that DECTIN-1 and galectins are all lectins, an anti-galectin therapeutic is showing clinical benefit for lung fibrosis, DECTIN-1 is upregulated in certain M2 macrophages similar to certain galectins, and GALECTIN-3 and GALECTIN-9 both have recognized interactions with DECTIN-1, there is potentially significant utility in exploring DECTIN-1 as a target for future anti-fibrotic therapies.

4.6.3 Recruited macrophages and circulating monocytes

Furthermore, recruited macrophages and circulating monocytes are increasingly being recognized for their role in fibrotic disease (Mathai et al. 2010; Satoh et al. 2016). Our findings provide a better foundation for the study of

recruited macrophages, as our findings are based on murine bone marrow-derived and human PBMC-derived macrophages. A circulating monocyte population may also be more easily targetable than a tissue-resident macrophage population for the systemic administration of drug therapeutics. Identification of our M2-like cell markers in circulating monocytes may provide important insight into the programming of cells in the circulation towards an M2-like phenotype.

4.6.4 Future directions and considerations

We believe that our investigation into a shared gene signature for human and murine M2 macrophages is very useful to the study of macrophage biology and fibrotic disease. As we have done with CLEC7A, the other genes within our signature could be individually explored at the gene and protein levels within M2-like cells, particularly in fibrosis. Our bioinformatically-identified drug, radicicol, could be tested in several *in vitro* and *in vivo* systems for therapeutic benefit and cytotoxicity. We have also established a platform for studying M2-like cells and our shared gene signature in human fibrotic disease, using skin and PBMC samples from our systemic sclerosis clinical study.

The M2+IL-6 angle in this project has not been previously described in the literature, but is interesting in SSc, as serum IL-6 levels have been shown to correlate positively with skin thickening score in patients with SSc (Sato, Hasegawa, and Takehara 2001). An anti-IL-6 therapeutic, Tocilizumab, has been shown to have an inhibitory effect on M2 macrophage-related genes in a phase 2

systemic sclerosis clinical trial (Khanna et al. 2016). Thus, the study of the M2+IL-6 macrophage is potentially useful within the SSc environment.

Through this project, we have been able to identify markers for M2 macrophages. While we believe that this is an important contribution to understanding a cell type that is implicated in fibrotic disease, this is only a first step. Our work is limited in that uses CLEC7A identifies a cell phenotype, but does not yet establish whether CLEC7A$^+$ macrophages are contributing to disease. To elaborate, M2-like cells have been implicated in fibrosis (i.e. they are profibrotic cells) and our work identifies CLEC7A as a marker associated with M2-like cells. However, *in vivo*, where a multitude of heterogeneous macrophage phenotypes may exist and express CLEC7A (e.g. macrophages differing in their cytokine exposure or location in the body), it remains to be known whether CLEC7A is a marker for those macrophages which contribute to fibrogenesis and/or fibrosis progression, regardless of whether or not these cells fit the "M2-like" label. We have used an M1-M2 framework to identify CLEC7A, but it remains to be known whether the CLEC7A$^+$ subset of cells is directly involved in causing fibrosis. This investigation is necessary to take our work beyond the M1-M2 model and find direct implications for fibrotic disease.

CHAPTER 5: CONCLUSION

The purpose of this study was to identify markers of the M2 macrophage phenotype that are shared between human and mouse systems, using a bioinformatic approach. There are several complexities surrounding the identification and characterization of macrophage phenotypes, and a recognized need exists for more specific markers for the different phenotypes, especially across human and mouse systems (Murray et al. 2014; Murray 2017). Firstly, using an *in silico* method, we obtained a theorized signature of M2 macrophages from both human and mouse data. We empirically verified this signature through RNA analysis by NanoString technology. We have shown that of the 27 genes theorized to be upregulated, only 6 were significantly upregulated in both our human and mouse systems. Only 1 of the 7 genes theorized to be downregulated was significantly downregulated in our systems. These 7 genes, particularly the six upregulated genes, should be further explored individually, to investigate their relevance to the M2 macrophage phenotype.

Secondly, we characterized the presence of the DECTIN-1 (CLEC7A) membrane receptor on murine and human *in vitro* and *in vivo* systems. We have shown that in murine BMDMs and human THP-1-derived macrophages, CLEC7A expression is markedly upregulated on M2/M2+IL-6 phenotypes, compared to Control and M1 phenotypes. In the mouse system, it is a more discriminatory marker than current reference markers ARG-1 and CD206. In the

THP-1 system, CLEC7A is as selective as CD206 in identifying M2 macrophages, and the addition of radicicol prevents the formation of such $CLEC7A^+$ and/or $CD206^+$ cells. The PBMC characterization of CLEC7A expression on these cell phenotypes needs to be further explored before any conclusions can be drawn about its utility in this context. Through our investigation into CLEC7A in the murine lung, we have shown that mice with lung fibrosis have an increased frequency of $CD11b^+$ $F4/80^+$ $CD64^+$ macrophages that are $CLEC7A^+$, in comparison to healthy control mice.

To relate our findings on CLEC7A within the context of fibrosis and make the findings more relevant to humans, CLEC7A colocalization within macrophages must be confirmed on fibrotic tissue, such as skin from patients with scleroderma, or lung biopsy tissue from patients with IPF. If we can detect $CLEC7A^+$ macrophages by immunohistochemistry and use $CLEC7A^+$ as a tool to track these cells in tissue, it could be very useful to both develop and utilize *in vivo* models of macrophage-related diseases. If $CLEC7A^+$ macrophages are shown to be involved in disease pathogenesis, this marker may be used as a tool to diagnose patients. From recent work in our lab and others', the M2 macrophage has been shown to play an important role in the early stages of lung fibrosis. Identifying a population of $CLEC7A^+$ macrophages in a fibrotic organ would give us further insight into the type of macrophage and environment present, whereby we may be able to modulate the presence of such macrophages to reduce progression of such complex and devastating diseases.

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