UNIVERSITY OF CALIFORNIA SAN DIEGO

Single-Cell Sequencing Reveals an IRF3-dependent Immune and Remodeling Response in Wild Type Macrophages and Fibroblasts

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Bioengineering

by

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Signature Page

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University of California San Diego

2019

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Acknowledgements

I would like to express my appreciation and gratitude to Dr. Kevin King for the privilege of working in his lab. Through his mentorship, support, and guidance he allowed me to appreciate research and guide my thesis work in a unique way to truly appreciate the process. I am very grateful for the environment he fostered in the lab and the selfless mentorship, time, and willingness to help he gave me and everyone in lab. It was truly a great experience and a testament to his dedication for research and mentorship.

To all my lab mates, I want to thank you all for great company and always providing your willingness to help. I am always grateful how productive I am in everyone's presence when we can bounce ideas off each other. The lab socials, meetings, and everyday moments we had together were truly great and memorable moments I will always cherish.

I would also like to take this opportunity to thank Tiffany for proofing this thesis and providing additional help in the design of figures and schematics. This work, in addition to all of the other papers you had to endure to help proofread my all of prior work I wrote in undergrad and graduate school has always been greatly appreciated. Thank you.

Lastly, I must thank my family without whom this experience would not have been possible. The unconditional love and support you provide me from thousands of miles away is truly a blessing. Thank you providing me with endless opportunities and inspiration to work hard every day.

Abstract of the Thesis

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University of California San Diego, 2019

Professor Kevin King, Chair

There is general consensus that immune cells, such as macrophages, within injured tissue influence remodeling and fibrosis. At the cellular level, this has been attributed to the communication between macrophages and fibroblasts. However, precisely how this communication is mediated at the molecular level remains unknown. We hypothesized fibroblasts are influenced by type I interferon signaling, either directly or indirectly via macrophages, which is a determining factor in the induction of adverse cardiac remodeling. In this study, differential gene expression analysis at a single cell level was done between wild type

and knockout mice after myocardial infarction. It was found that a subset population of macrophages produce IRF3-dependent type I Interferon genes. Further, in fibroblasts, it was found matrix metalloproteinases are dominantly expressed in wild type. Given these results, this study proposes that the overexpression of matrix metalloproteinases in fibroblasts is linked to the production of the IRF3-dependent type I interferon genes in macrophages.

Introduction

Myocardial infarction (MI), which is characterized by a blockage of blood within the heart, is the leading cause of death in the US [1]. Despite current treatment strategies, up to one-third of those that survive ultimately develop heart failure (HF) [2]. Current therapeutic intervention of HF patients only target symptoms rather than underlying disease progression and pathology [3]. Thus, the progression of MI is an important area of research and development in preventing adverse remodeling in the heart after MI.

MI elicits a strong immune response that involves the recruitment of macrophages, often inducing inflammation and cardiac remodeling shortly after. At the single cell level, this innate immune response in macrophages and fibroblast can be analyzed in adult mice after MI. Immune cells such as macrophages have been attributed to influence remodeling and fibrosis. At the cellular level, this has been attributed to communication between macrophages and fibroblasts. However, precisely how this communication is mediated at the molecular level remains unknown. It was recently shown that IRF3 and the type I interferon response are activated after MI and inhibition of this response, either genetically or pharmacologically, reduces inflammation, limits ventricular dysfunction and dilation, and improves survival by reducing ventricular rupture [4]. We hypothesize fibroblasts are influenced by type I interferon signaling and that differentially expressed genes across conditions at a single cell level would yield:

1. IRF3 leads to decreased expression of collagen and ECM matrix components in the fibroblasts thus increasing susceptibility to rupture after MI.
2. IRF3 leads to increased expression of matrix metalloproteinases by fibroblasts thus increasing susceptibility to rupture after MI.

This thesis investigates the response of fibroblasts in vivo after MI. The thesis begins with an overview and background of foundational knowledge to understand the given problem.

Then, we introduce and describe the extracellular matrix, macrophages, fibroblasts, and single-cell analysis as they relates to cardiac tissue and the immune response. Thereafter, we describe the IRF3-dependent type I interferon pathway and the motivations of this study as they relates to cardiac remodeling. The thesis concludes with the results and proposed model associated with the immune response and involvement of fibroblasts.

Chapter 1: Collagen Remodeling and Reorganization

The Extracellular Matrix (ECM) is a critical component of the body that links individual cells together and ultimately gives way to form tissue and functional organs. The ECM is a three-dimensional network that resides external to the cell and is composed of many protein macromolecules and glycoproteins. The interaction of the cellular membrane components integrin with the ECM component fibronectin is what coordinates cellular communication with the extracellular matrix. Discussed below are the important factors of the ECM related to ECM homeostasis and scar formation, namely collagen and matrix metalloproteinases.

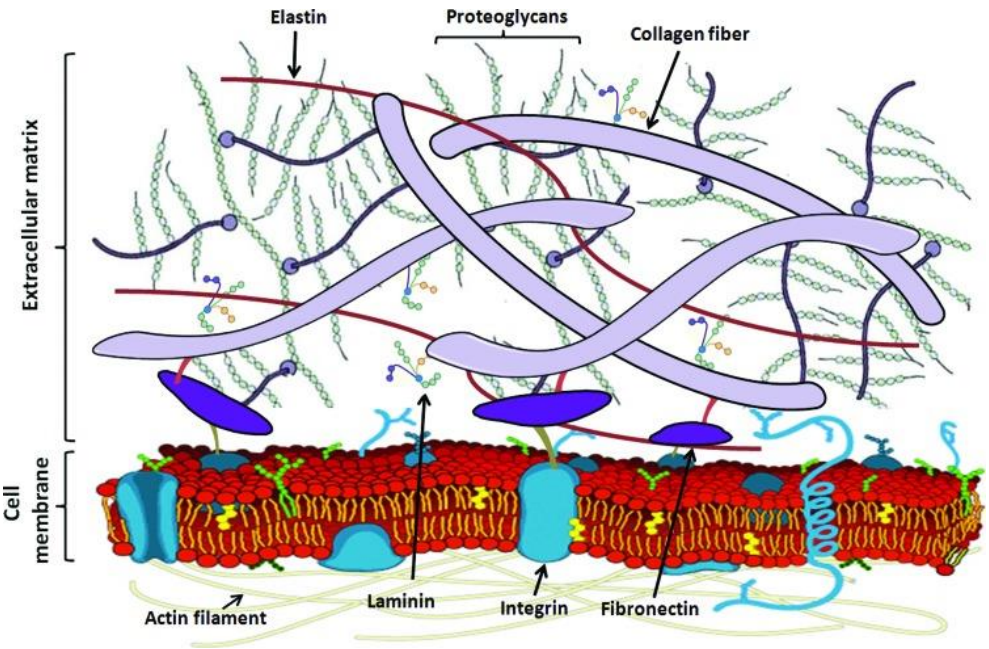

Figure 1.1: The Components of ECM [5].

1.1 - Collagen

There are many different units of protein collagen throughout the body as shown in Table 1.1. Of particular importance in this study is the distribution of fibrillar collagen which exhibits increased tensile strength. Fibrillar collagen is produced primarily by fibroblasts and arranged in a helical structure which cross links together to eventually form a collagen fiber unit. In the skin,

these collagen fibers appear external to the cell within the ECM and are arranged in an interleaved pattern (Figure 1.1). The arrangement in optimized helical structures with hydrogen bonds of fibrillar collagen are uniquely resistant to degradation by proteases such as chymotrypsin and pepsin [5]. On the other hand, matrix metalloproteinases (MMPs) are specific zinc containing proteases that are able to specifically degrade collagen. Classes of MMPs that specifically degrade collagen are called collagenases.

Table 1.1: Classes and Location of Different Collagen [6].

Type	Class	Distribution
I	Fibrillar	Dermis, bone, tendon
II	Fibrillar	Cartilage, vitreous
III	Fibrillar	Blood vessels
IV	Network	Basement membranes
V	Fibrillar	Dermis, bone, tendon
VI	Filaments, 100 nm	Dermis, bone, tendon
VII	Fibers with antiparallel dimers	Dermis, bladder
VIII	Hexagonal matrix	Membrane
IX	Fibril-associated collagens with interrupted triple helices	Cartilage, vitreous
X	Hexagonal matrix	Cartilage
XI	Fibrillar	Cartilage
XII	Fibril-associated collagens with interrupted triple helices	Tendon

1.2 - Matrix Metalloproteinases

Matrix Metalloproteinases (MMPs) play a crucial role in the breakdown of the ECM. These family of proteases break down a wide variety of ECM components from fibronectin, elastin, and collagen proteins [7]. MMPs can be subdivided into different classes depending on the region of ECM they target. For instance, collagenases are MMPs that specifically target the breakdown of collagen while gelatinases specifically target Type IV collagen and gelatin proteins [8]. In total, MMPs are divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other generalized MMPs [8]. Structurally, MMPs

consists of a distinct catalytic domain, which contains the active site of the protein and requires zinc as a cofactor to function.

Tissue inhibitors of metalloproteinases (TIMPs) are a family of protease inhibitors that specifically inhibit MMPs. TIMPs regulate the activity of MMPs and together, the two are carefully regulated at homeostasis such that the net effect is the constant renewal of ECM and other related structural components. An overproduction of TIMPs relative to MMPs results in the overabundance of ECM proteins such as collagen, often resulting in fibrosis [9]. Likewise, an abundance of MMPs results in the excessive degradation of the ECM, resulting in the net effect of matrix proteolysis [9].

1.3 - Matrix Metalloproteinases in Myocardial Infarction and Heart Failure

MMPs play a role both in post MI response as well as subsequent cardiac remodeling [10-12]. In the first 72 hours following MI, the classical wound healing response is observed, which is characterized by the release of cytokines, recruitment of inflammatory cells such as neutrophils and macrophages, and differentiation of resident cardiac fibroblast to myofibroblasts [13]. During this early post MI phase, it was found that inflammatory cells as well as myofibroblasts contribute to the release of MMPs. This increases ECM turnover, thereby contributing to early scar formation [13]. However, at later stages following MI it is believed that an expanded myofibroblast population in the infarct area creates a continuous and deleterious release of MMPs which results in adverse cardiac remodeling [14]. Table 1.2 summarizes MMP substrates and their post MI functions [15].

Table 1.2: Family of MMPs studied after myocardial infarction [15].

MMP substrates	MI functional roles	Cleaved by MMP
Angiostatin	Angiogenesis inhibitor, cardiomyocytes death, ↑heart failure	−2, −3, and −9
C-1158/59	increased migration rate of fibroblast cells, ↑ wound healing	−2 and −9
C-terminal telopeptide of collagen I	exaggerated myocardial fibrosis	−1, −2, −8, and −9
CD36	↓ macrophage phagocytosis and neutrophil apoptosis	−9 and −12
Citrate Synthase	↓ mitochondrial function	−9
Endostatin	suppresses proliferation and migration of endothelial cells	−2, −9, and −13
Fibronectin	act as chemoattractant, ↑ inflammation, migration of monocytes	−2, −7, −9, −12, and −13
Galectin-3	↑ collagen deposition, ↓ LVEF	−9
Hyaluronan	↑ inflammation, ↓ neutrophil apoptosis, induce cardiac dysfunction	−9, and −12
Laminin	inhibit migration of macrophages into the inflammatory region	−2
Osteopontin	↑ migration rate of cardiac fibroblast, ↑ wound healing	−2, −3, −7, −9, and −12
Periostin	↑ myocardial fibrosis, ↑ heart failure	−2, −9, −14
SPARC	anti-angiogenic effect, maturation of ECM	−2, −3, −7, −9, and −13
Tenascin-C	unknown	−3, −4, −7 and −9

MMPs are a family of 25 zinc containing endopeptidases that contribute to ECM turnover. Only half have been studied extensively post MI in murine studies (Table 1.2). Early studies in murine models validate the relationship between MMP expression and adverse left ventricular remodeling. For instance, studies conducted by Rohde et al found that administration of a broad spectrum MMP inhibitor after MI decreases left ventricular dilation [16]. Genetic knockout studies by Heymans and colleagues utilizing gene transfer techniques in mice found that increased levels of MMP-9 predispose mice to cardiac rupture while increased levels of TIMP-1 may be cardioprotective [17]. Later studies discovered Col1a1, a major cardiac ECM component, is the substrate of MMP-1, MMP-2, MMP-8, and MMP-9 [18]. Further, epidemiological studies on patients with dilated chronic cardiomyopathy from Chagas Disease showed an increase presence of MMP-2 and MMP-9 [19].

Chapter 2: Macrophage

2.1 - Macrophages

Macrophages are distributed throughout all organs and tissues in the body, which either have origins from circulating blood monocytes or resident tissue macrophages that were established during embryonic development and remain into adulthood. During an immune response, macrophages play a crucial role in the innate immunity and help the recruitment of adaptive and more specific immunity through the recruitment of other immune cells such as white blood cells. Classically, macrophages are highly specialized cells responsible for eliminating cellular debris, pathogens, senescent cells, and other related cellular waste materials through the process of phagocytosis.

2.2 - Cardiac Macrophage

In the heart, resident macrophages are responsible for a wide variety of roles. Traditionally, these resident macrophages engulf and eliminates pathogens and senescent cells. Additionally, macrophages are also involved in heart electrical conduction. It has been shown that resident cardiac macrophages are heavily abundant in the AV node and depletion of these macrophages results in an AV block [20]. Further, these resident cardiac macrophages exhibit transcriptional and cellular heterogeneity [21].

Resident macrophages in the heart are unique in that they contains distinct populations of macrophages arising from unique origins such as monocyte-derived or embryonic-derived macrophages. This allows us to differentially analyze functional subsets of macrophages. In the past, distinct subsets have been characterized by their surface markers such as the (Ly6C+ CCR2+) macrophages [22]. These surface markers have been used extensively to classify and sort populations of macrophages and group them to particular phenotypes. However, the recent emergence of single-cell RNA-seq lays a new groundwork to further classify and characterize

cell types not merely based on their surface markers, but also through their specific expression of unique expression profiles.

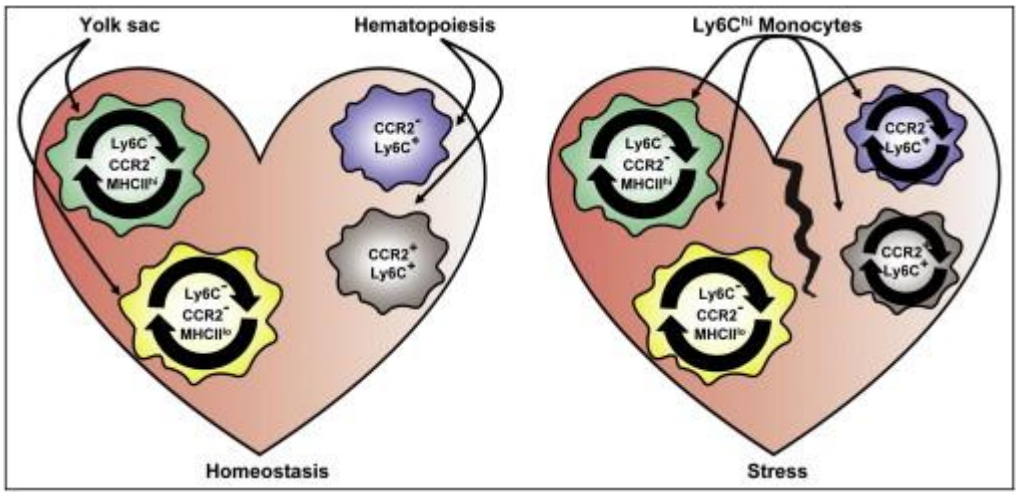

Figure 2.1: Origins and heterogeneity of cardiac macrophages at homeostasis and stress [21].

2.3 - Cardiac Macrophage after Myocardial Infarction

Following Myocardial Infarction (MI), the immune response is initiated and a variety of macrophage subsets play a pivotal role in the acute inflammatory response, remodeling, and reparative phases. In the acute inflammatory response, macrophages secrete a variety of inflammatory cytokines to recruit additional immune cells to the infarcted region. In addition, as previously discussed, macrophages utilize their phagocytic ability to eliminate the senescent neutrophils.

Figure 2.2 demonstrates the origin and response of macrophages following MI. Circulating blood monocytes infiltrate the heart and differentiate into macrophages [23]. Consequently, there are distinct macrophage lineages derived either embryonically or from circulating monocytes that have numerous functional capabilities. For instance, it was demonstrated that depletion of embryonically derived macrophages in neonatal mice resulted in

the loss of myocardial regenerative capacity and ability to form fibrotic scars. However, further studies need to be done to further validate macrophage phenotype and function.

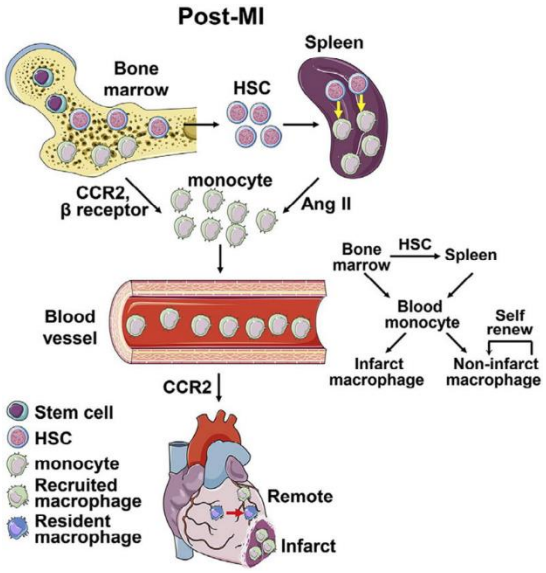

Figure 2.2: Schematic of Macrophage Recruitment after MI [24].

Chapter 3: Fibroblasts

3.1 - Fibroblasts

Fibroblasts are a type of cell that is responsible for producing the extracellular matrix (ECM) components and the collagen necessary for the structural integrity of many organs. During wound healing, for instance, fibroblasts rapidly proliferate and secrete ECM components such as collagen and fibronectin. These procollagen forms aggregate together to form fibrillar helical structures, which arrange to form collagen fibers that provides the structural connectivity of the ECM. Myofibroblast, an activated form of fibroblasts that resembles a mix of fibroblast and smooth muscle cells, contracts to close the wound during the wound healing response. During the repair process, myofibroblast are involved in the inflammatory as well as the remodeling response to injury.

Myofibroblast are large cells that can be distinguished from fibroblast by the presence of smooth muscle actin (Acta2) and an extensive endoplasmic reticulum [25]. During the wound healing response, myofibroblast have the ability to respond and secrete cytokines thereby contributing to the initial inflammatory response [25]. At the later stages of wound healing, as scar tissue matures around 21 to 28 days, smooth muscle actin in myofibroblasts decreases [26]. In vitro, it has been shown the application transforming growth factor beta 1 (TGF-β1) stimulates collagen deposition through differentiation of fibroblast to myofibroblasts [26]. Myofibroblasts are also found to be present in infarcted regions of the heart resembling myofibroblasts studied in vitro [26].

3.2 - Cardiac Fibroblasts

In the heart, resident fibroblasts function to maintain structural ECM homeostasis. During homeostasis, fibroblasts secrete ECM components such as collagen while matrix metalloproteinases (MMPs) degrade existing ECM components or precursor collagen precursors.

The net effect is the constant renewal and maintenance of the extracellular matrix during homeostasis. This maintenance of ECM of the heart provides a stable structural scaffold for which cardiomyocytes can reside [28]. In addition, the ECM meshwork scaffold containing the specific arrangement of cardiomyocytes also results in the proper distribution of mechanical forces during atrial and ventricular contraction and dilation [28].

Morphologically, fibroblasts are flat star-shaped cells that exhibit strong adherence properties in vitro. Within the adult heart of a mice, fibroblast have been proposed to make up less than 20% of the existing cells [29]. However, during development, since the majority of cardiac fibroblast originate from the embryonic epicardium, there is an increased presence of resident cardiac fibroblasts in neonatal mice hearts [30]. This feature allows for the isolation and in vitro analysis of resident fibroblasts not possible in adult cardiac fibroblasts [30]. While the origins and the composition of resident fibroblasts in the heart have been extensively studied, the contribution of activated fibroblast during an immune response is still an active area of research.

3.3 - Cardiac Fibroblast Response after Myocardial Infarction

Upon disruption of homeostasis in the heart, fibroblasts play a crucial role after injury to replace necrotic cardiomyocytes with fibrotic scar tissue. This scar tissue is characterized by cross-linked collagen aligned in a single direction. This is different from ECM collagen that interleaves collagen thereby forming a basket weaving pattern [31]. As a consequence, the scar tissue changes the structural, mechanical, and electrophysical properties of the heart. Since the body does not have the capacity to regenerate muscle tissue, this feature of cardiomyocyte replacement is crucial to retain the functional capacity of a beating heart. However, if this process of scar tissue generation by fibroblast persists, adverse remodeling of the heart can take place.

Prolonged activity of activated fibroblast following MI has been found to excessively release profibrotic factors such as collagen and maladaptive proinflammatory signals [32]. As a consequence, post MI often results in excessive cardiac fibrosis which adversely remodels the heart. These pathological and adverse remodeling effects result in left ventricular dilation, cardiomyocyte hypertrophy, and eventually heart failure [33].

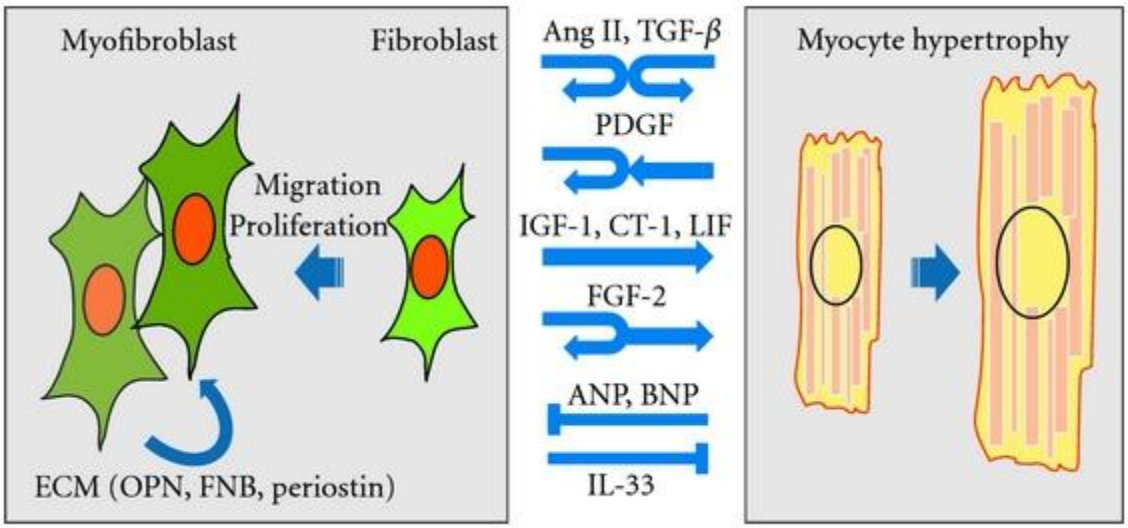

Figure 3.1: Fibroblast transdifferentiation to Myofibroblast and myocyte hypertrophy [31].

In canine models of reperfused myocardial infarcted hearts, it was found myofibroblast proliferation peak 3 to 7 days following reperfusion [34]. Post MI, the cellular content of myofibroblast has been found to have an increased expression of alpha-smooth muscle actin in highly fibrotic (scarred) areas [34]. Myofibroblasts are believed to be associated with fibrosis and contribute to scar formation [36]. However, further studies into molecular markers of myofibroblasts and cellular contents remain under active investigation [35].

Chapter 4: Single Cell RNA-Seq

Single Cell RNA Sequencing (scRNA-seq) is a method of separately analyzing the transcriptome expression profile of individual cells. This method allows for a finer resolution of analyzing RNA-sequencing data. That is, rather than averaged expression patterns classically present when bulk RNA-Seq is performed, a more refined and thorough picture is obtained often presenting multiple lineages and special cell type signatures that would not have otherwise been detected through bulk RNA sequencing [37]. Methods of obtaining scRNA-seq data often involve the separation of cells with the use of microfluidics or the use of combinatorial barcoding to identify individual cell expression bioinformatically [37]. In this thesis, I outline my analysis pipeline to find differentially expressed genes of fibroblasts and technical considerations to ask biologically driven questions.

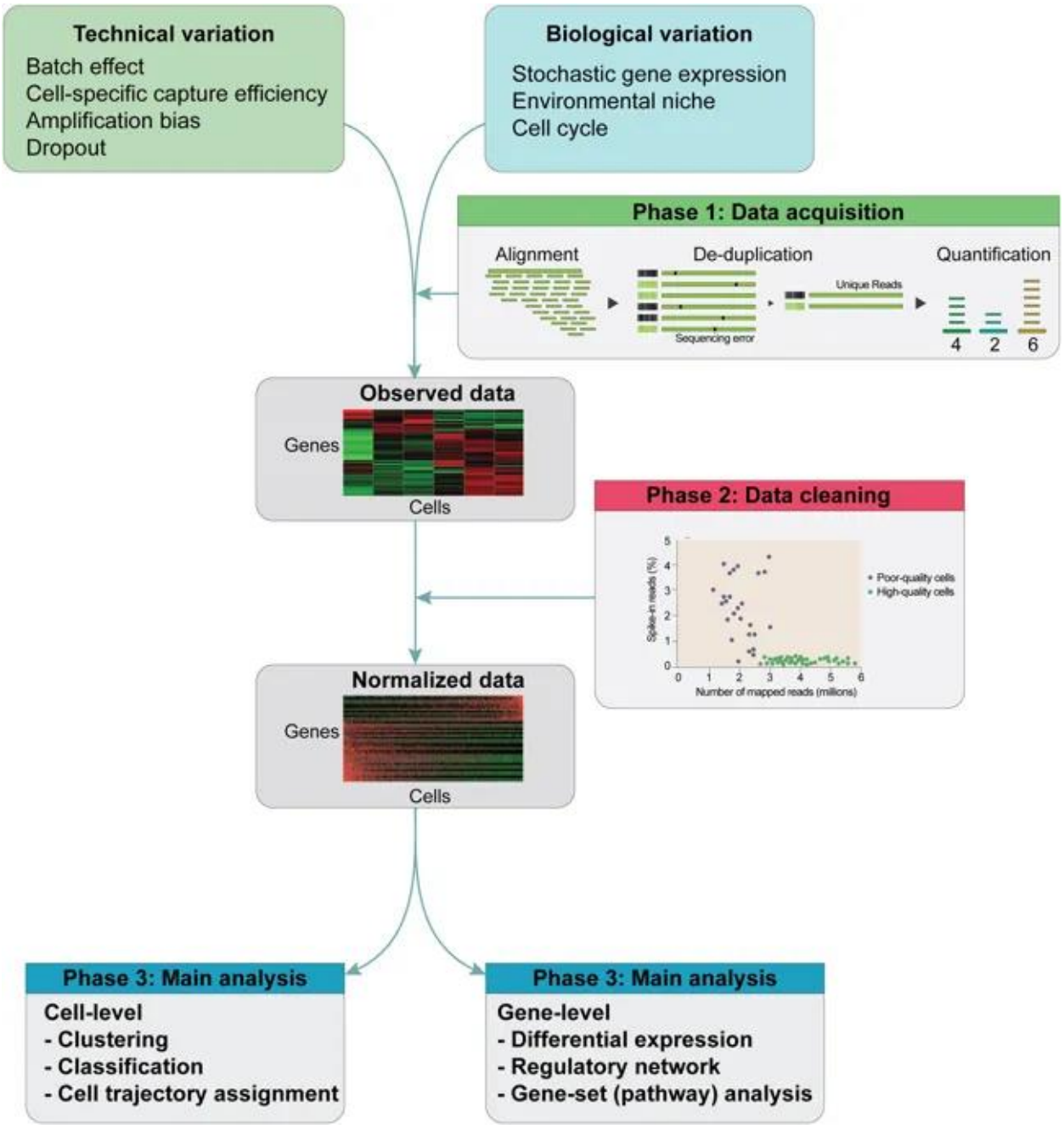

Figure 4.1: scRNA-seq overview and analysis pipeline [37].

4.1 - Cell Type Identification

As previously discussed, cell surface markers have been predominantly used in identification and classification of immune cells. In macrophages, distinct phenotypes have been

assigned due to the expression or lack of expression of CCR2 and Ly6C. With the advent of scRNA-seq, characterization of cell type involves the use of transcriptional expression profiles and hierarchical clustering to group cells together. The result is the potential to classify heterogeneous samples, find rare cell types, and analyze particular subsets of cells based on their expression profiles.

To identify cell types across a given sample of single cell data, an unbiased clustering algorithm must be implemented after quality control and normalization of the data. Clustering algorithms group common cell types together based on unique gene expression signatures that distinguish a cluster of cells from the other cell types in the sample. Due to the multitude of cells and genes each cell expresses, dimensional reduction algorithms is used to reduce the dimensionality of the dataset into linear principal components. These principle components can then used to apply a graph based clustering algorithm such as K-nearest neighbors (KNN) to identify similar cell types based on distances on the reduced principal component space. To visualize the clusters, other nonlinear dimensionality reduction techniques such as t-Distributed Stochastic Neighbor Embedding (tSNE) or Uniform Manifold Approximation and Projection (UMAP) may be applied.

After dimensional reduction and clustering, all the cells of a given sample can be grouped together based on underlying transcriptional signatures. In fibroblast, this would mean the expression of remodeling genes such as Collagen I and Periostin. It is important to note that while the given set of genes that distinguish a cluster of cells from the rest of the cells often gives known canonical markers, the cell identification and classification is determined holistically by the unique transcriptional profile of that cluster. Thus what arises is an unsupervised method to cluster unique and known groups of cells together for further downstream analysis.

Currently, there is no known unsupervised way to determine the resolution of clustering done for a given sample. Too low of a resolution would cluster distinct groups of cells together, while too high of a resolution would be too sensitive to known variability within a the same cell type. Background knowledge and familiarity of known molecular immune cell type markers are needed to determine what resolution would be the best for a given sample.

4.2 - Batch Effects

With the increased resolution of detecting unique molecular markers within a given sample, comparative analysis of scRNA-seq data across samples and conditions become increasingly difficult. That is, it is difficult to distinguish variability in experiments and laboratory processing with actual biologically relevant differences between two samples. Typically, scRNA-seq has been used within a sample to identify unique cell types and transitional states between given cell types. However, to ask what drives certain differences between different samples and conditions requires integration of datasets and, more importantly, proper processing of such data sets for accurate comparisons.

Within Seurat, novel computation strategies have been implemented for the proper integration and batch correction of multiple datasets. The process of integration, as described by Stuart and Butler et al is based on the use of shared populations across both conditions. The process maps the shared populations together so that the these shared populations cluster together upon non-linear dimensional reduction [38]. This algorithm allows for the integrated analysis of multiple single-cell datasets across different conditions. Integration removes differences across samples such that similar clusters of cells align together. Once similar clusters of cells are aligned, differentially expressed genes between these shared populations can be identified.

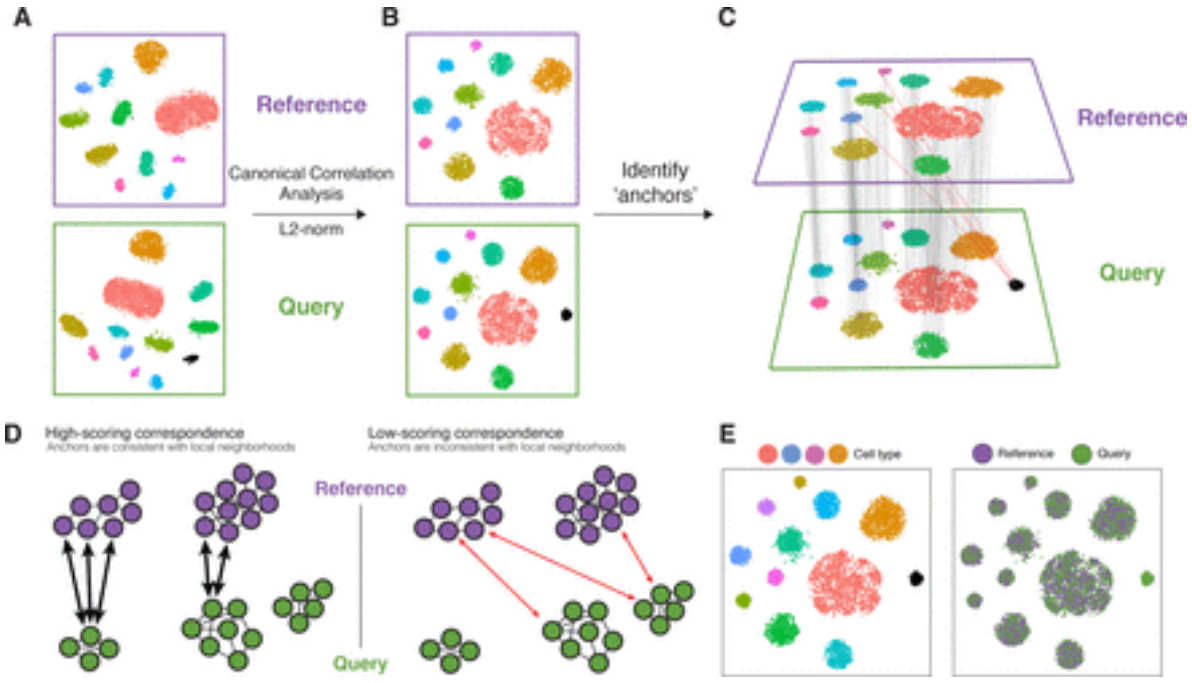

Figure 4.2: Schematic representation of sample integration and canonical correlation analysis [35].

Chapter 5: cGAS-STING Antiviral Cytosolic DNA Sensing Pathway

The cGAS-STING is an ancient antiviral pathway that senses foreign cytosolic DNA and triggers the production of Type I Interferon (IFN) genes. This process occurs when the DNA sensing enzyme cyclic GMP-AMP synthase (cGAS) senses foreign cytosolic DNA which triggers the formation of cyclic GMP-AMP (cAMP) [39]. cAMP activates the transmembrane protein, stimulator of interferon genes (STING), which results in the activation of the transcription factor Interferon regulatory factor 3 (IRF3), thereby triggering the host cell to express Type I IFN genes [40]. Cells known to have this pathway available are known as interferon-inducible cells (IFNICs). These IFNIC release type I IFN cytokines, which bind to the cell membrane protein Interferon-α/β receptor (IFNAR), triggering the expression of Type I IFN genes. This pathway is a component of the innate immune response that triggers defenses against viruses or bacteria that infect host cells by inserting pathogenic DNA (Figure 5.1).

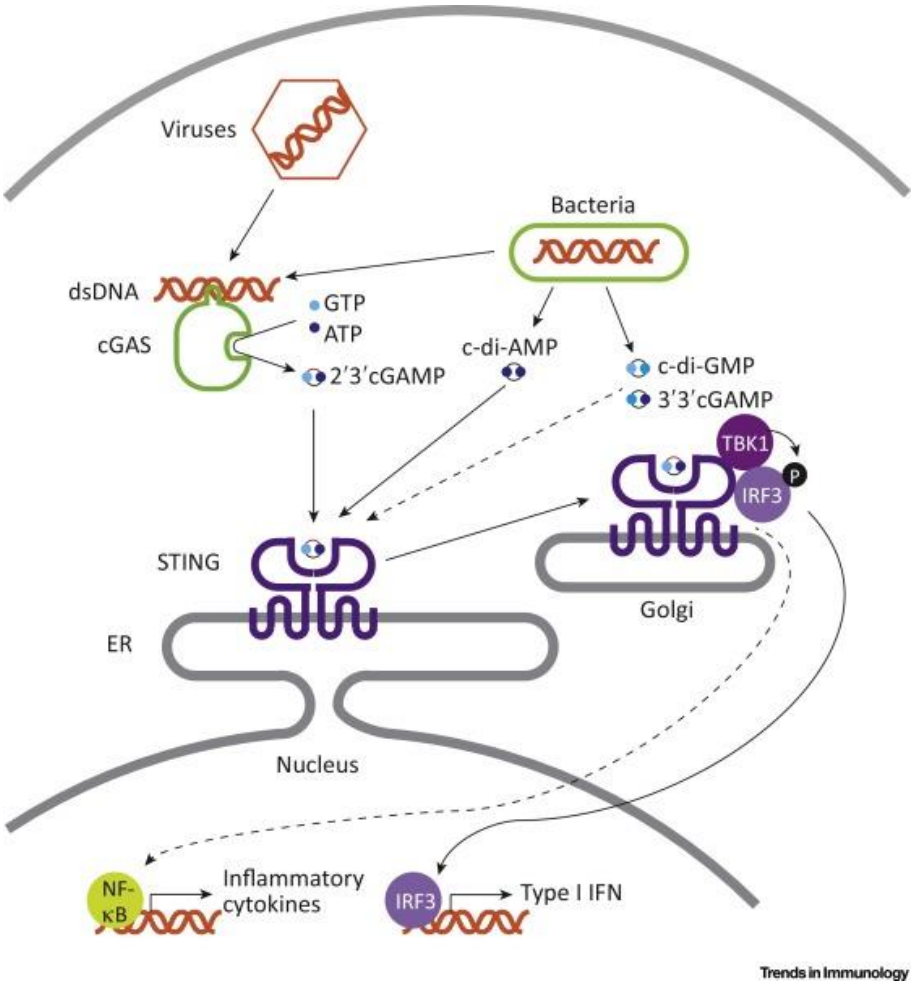

Figure 5.1: The cGAS-STING Pathway [41].

5.1 - Post Myocardial Infarction and Inhibition of the IRF3 Dependent Pathway

It is well known myocardial infarction complications often result in inflammation shortly after MI [42]. Furthermore, it was found that macrophages uptake cellular debris, which causes the induction of the Type I IFN response [43]. More importantly, inhibition of this response, either genetically with the knockout of IRF3 gene in mice or pharmacologically with an IFNAR-neutralizing antibody reduces inflammation, limits ventricular dysfunction and dilation, and improves survival rates in mice unable to elicit the antiviral IFN pathway [43].

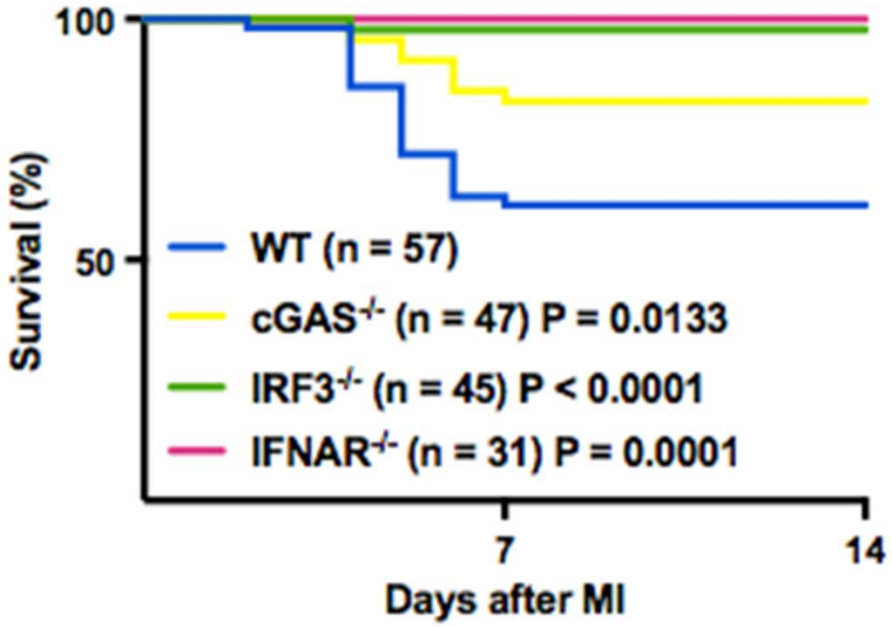

Figure 5.2: Survival rates of wild type mice and mice with a knocked out IRF3 type I interferon pathway after myocardial infarction [4].

Thus, it was shown that inhibition of Type I IFN associated inflammation can be reduced by the inhibition of the IRF3 dependent pathway. However, little is known about why inhibition of the IRF3 dependent prevents the adverse remodeling in mice. That is, why is a pathway that disrupts immune cells also affecting rupture?

Fibroblasts are the known drivers of ECM and collagen driven remodeling, but little is known about how and why the inhibition of the IRF3 pathway results in the prevention of adverse remodeling after myocardial infarction. In this study, we will investigate how the activation of the IFN response in macrophages communicate with fibroblasts to elicit adverse remodeling in the heart. This will be done by utilizing scRNA-seq to find differentially expressed genes between macrophages and fibroblasts in wild-type and IRF3 knockout mice and proposing possible mechanisms based on biologically relevant remodeling markers.

Chapter 6: Experimental Design

To study the potential links between the IRF3 pathway and adverse cardiac remodeling after MI, mouse models and scRNA-seq were used. The use of scRNA-seq allows for classification of cells across all cell types by looking at each cell's unique transcriptional profile and subsequent clustering, subsetting, and analysis. Previous in vitro trials of adult mice cardiac fibroblasts have been proven difficult. Active in vitro studies on fibroblasts utilize neonatal cardiac fibroblasts which are more abundant during the early stages [43]. However, such an approach in cardiac MI mouse model cannot be reasonably done as mouse neonatal hearts may be too small to perform permanent coronary ligation. Likewise, upkeep as well as timing the harvest to obtain neonatal cardiac fibroblast adds another degree of difficulty. Lastly, fibroblasts may have inherent heterogeneity that may not be captured or seen with the use of bulk genomic techniques such as qPCR. For all the reasons mentioned above, scRNA-seq was used to determine potential reasons why a pathway that affects immune cells also affects cardiac rupture.

This thesis investigates these questions utilizing data previously prepared by King et al which utilized inDrop scRNA-seq to analyze the involvement of immune cells in wild type and IRF3 knock mice 4 days after permanent coronary ligation [4]. inDrop was developed by Klein et al and utilizes droplet microfluidics technology which allows for a high-throughput approach for single cell labelling [43]. Single cells are loaded into droplets containing beads with unique oligos that is able to capture mRNA transcripts of the cell upon lysis. This process allows for each cell's transcript to be specifically labelled with a unique barcode upon reverse transcription for sequencing.

Adult C57BL/6J mice was used for the wild type and IRF3 knockout mice were gifted from T. Taniguchi of Tokyo University and provided by M. Diamond of Washington University

School of Medicine. Maintenance of mice environment and subsequent experimentation followed pathogen and animal research care protocols set forth by Massachusetts General Hospital and University of Massachusetts animal facilities [4]. Mice experimentation and harvest was done between 10 - 25 week old adult mice.

To model myocardial infarction, permanent suture ligation of the left anterior coronary artery of the heart was done via thoracotomy. Infarcted heart tissue samples of wild type and knockout was collected 4 days after surgery. To remove red blood cells, hearts were first punctured and flushed with cold saline. Infarct tissue was then collected, stained with DAPI, and flow sorted to exclude dead cells. Finally, collected and processed samples underwent inDrop following previously published protocol by Zilionis et al [44]. Figure 6.1 below illustrates the workflow used in sample collection and subsequent single cell analysis. The output of all this are counts expression matrix, gene list, and unique barcodes that can be inputted in Seurat, developed by Satija lab, which is an R package designed for the filtering, quality control, and exploration of scRNA-seq data [45].

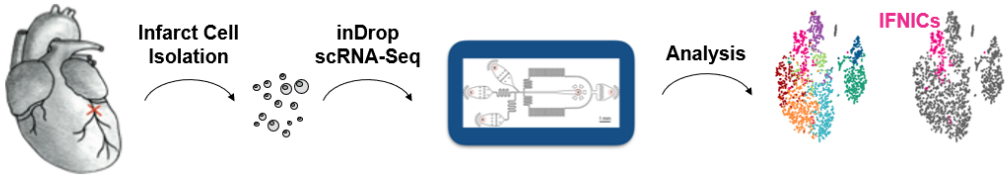

Figure 6.1: Workflow of infarct tissue sample collection to scRNA-seq analysis [4].

Chapter 7: Analysis Design

To prepare inDrop scRNA-seq data for use in Seurat, the inDrop data was converted to 10X single cell genomics data input which composed of a counts matrix, barcodes for cells, and gene lists. The results are two mice scRNA-seq samples of day 4 post MI knockout and wild type mice. To address many of the considerations mentioned previously in Chapter 4 such as cell type identification and batch effects, multiple strategies were employed in this study as discussed below.

In the analysis of fibroblasts and other immune cells at the single cell level, this study aims to characterize not only fibroblasts, but also macrophages. Furthermore, after appropriate characterization of these cell types, subsequent isolation and differential gene expression within each cell type were performed to determine potential links between the disruption of the IRF3 pathway in immune cells and the rupture of cardiac tissue.

As previously mentioned, to classify unique sets of cells that share the same expression profiles, unsupervised clustering must be done. After quality control and normalization of the dataset for faster processing, clustering algorithms were used to group common cell types together. That is, for each scRNA-seq dataset of wild type and IRF3 knockout, initial clustering would give broad cell types characterizing all the cell types within sampled infarcted tissue such as macrophages, neutrophils, and fibroblasts. Subsequent isolation of a particular cluster of cells, such as macrophages, would result in observing more unique macrophage subsets that distinguish a particular macrophage subset (i.e: phagocytic) from the other another macrophage subset (i.e proliferative).

In this study, the process of unsupervised clustering and classification followed by further subsetting of a particular cluster and further analysis were extensively used to analyze

macrophages and fibroblasts. It was shown that macrophages are one of the primary drivers of the innate inflammatory response after MI by King et al, while fibroblasts have been associated with the remodelling [4]. It is for this reason that individual comparisons of macrophage wild type versus IRF3 knockout macrophages and similar parallel comparisons of fibroblasts must be done. The primary goal of such comparisons is to find differentially expression genes between the two cell types first to confirm the observed effect that the IRF3 dependent response is dominant in macrophage and second to determine potential links of how this response affects the wild type and knockout fibroblasts.

Comparisons on a cell type level (i.e: WT-Fibroblast vs. KO-Fibroblasts) are done after appropriate data integration. Appropriate data integration is done by utilizing a novel computation strategy developed by Stuart and Butler et al based on shared populations across conditions (ie: wild type and IRF3 knockout condition) [38]. As previously mentioned, upon integration of both the wild type and IRF3 knockout data, shared populations of cells are determined. Once shared populations and cell types are identified, subsequent subsetting and differential gene expression can be applied within that shared population. To ensure no unwanted cells adversely drives differential gene expression within a subsetted cluster, additional filtering may be done to eliminate obvious extraneous cell types after finer clustering as shown in Figure 7.1. Through this process, it minimizes the effect of batch effects and allows for the emergence of biologically significant differentially expressed genes.

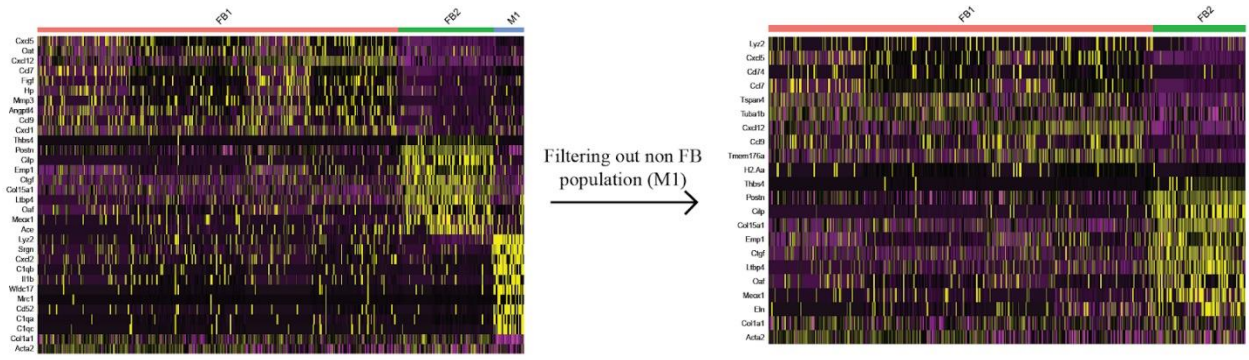

Figure 7.1: Filtering out non fibroblast populations. Depicts the removal of macrophage population. Col1a1 and Acta2 added as canonical fibroblast reference markers not present in the M1 population.

In summary, this thesis aims to utilize inDrop scRNA-seq data of wild type and IRF3 knockout mice to find differentially expressed genes across these conditions in fibroblasts and macrophages. The study utilizes novel algorithms to integrate datasets together allowing biologically significant genes to emerge. Figure 7.2 summarizes the overall experimental and analysis workflow in this study. The hope is that we can find potential links between the IRF3 dependent type I interferon response and adverse remodeling by studying the potential links between macrophage activation and fibroblasts at a single cell level.

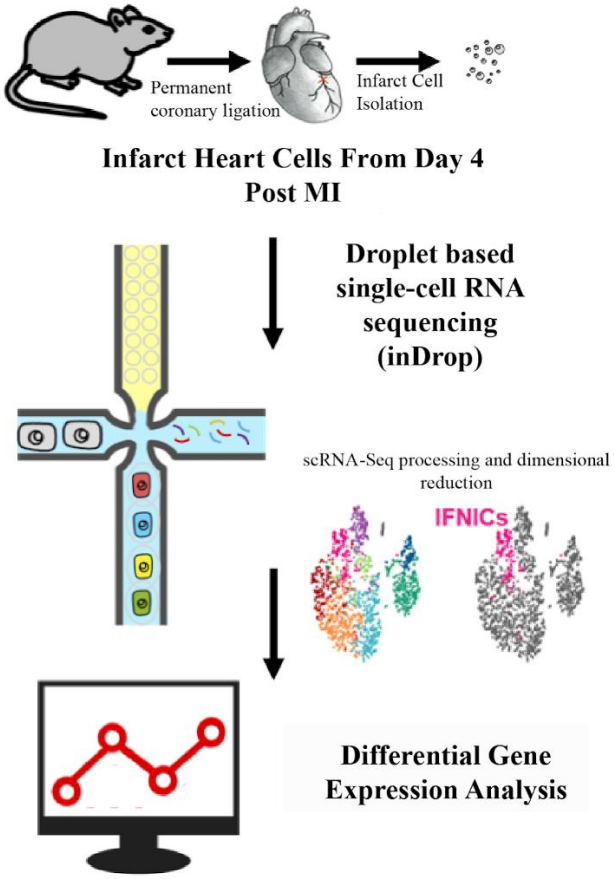

Figure 7.2: Experimental and Analysis Workflow of Wild Type and IRF3 Knockout Mice [4, 46].

Chapter 8: Results

To determine the differences between wild type and knockout mice, the wild type and knockout post MI scRNA-Seq data samples were combined and integrated. Proper integration of the two datasets across different conditions was done based on shared populations in both samples using Butler et al's canonical correlation analysis algorithm[38]. Figure 8.1 shows the major cell populations of the integrated data and highlights the heterogeneity of cell types present following MI. Further, these results demonstrate populations of single cells between wild type and IRF3 knockout mice clustering together based on shared populations such as monocytes and fibroblast present in both cells. This finding is a crucial requirement for further downstream analysis as finding separate distinct cluster of cells for each sample would represent differences due to batch effect rather than biological differences.

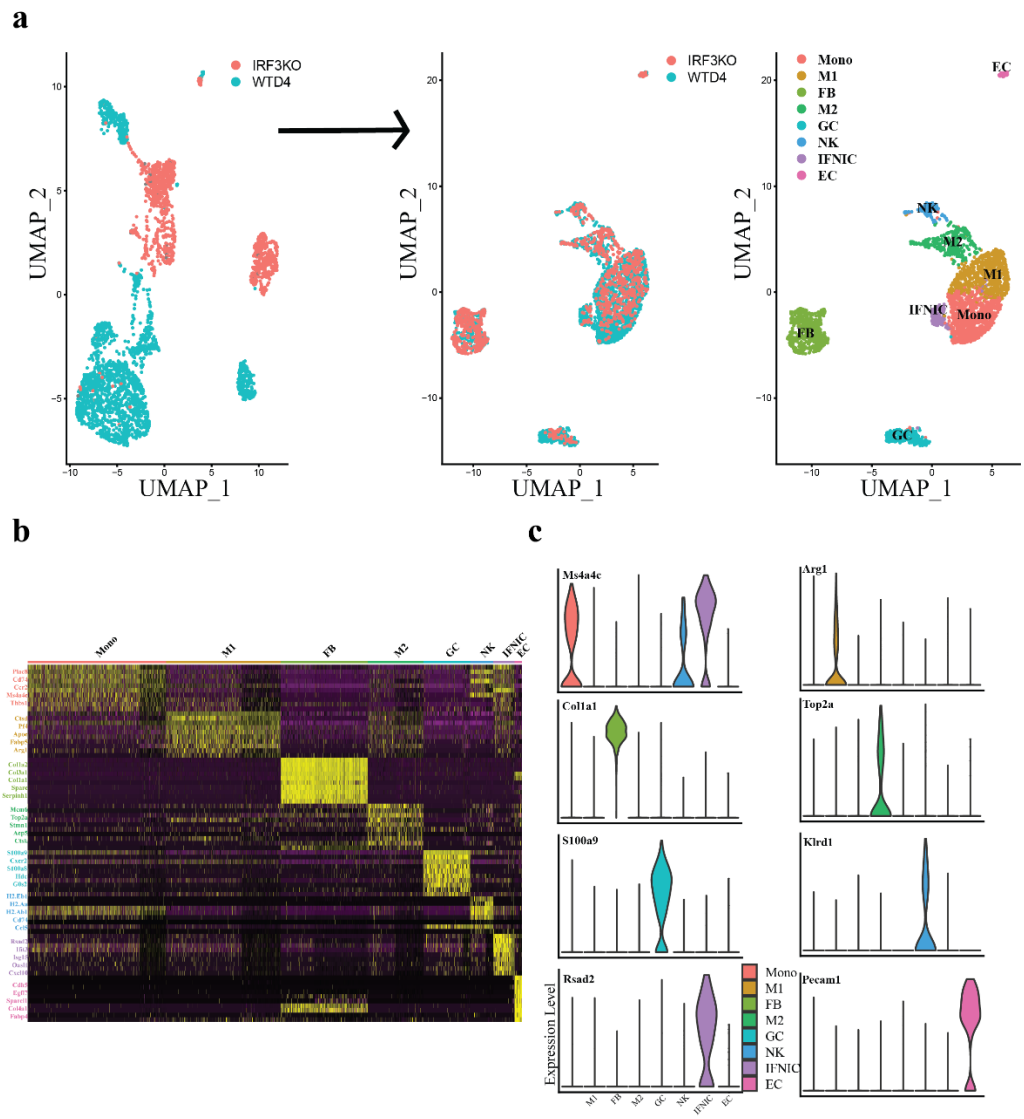

Figure 8.1: scRNA-Seq Analysis of Major Cardiac Cell Types of Integrated IRF3KO and Wild Type Mice after Myocardial Infarction.

(a) Data integration strategy before (left) and after (right) batch correction featuring minimal overlap across conditions before (left) and shared populations of cells across conditions after batch correction (right). Uniform Manifold Approximation and Projection (UMAP) was utilized to reduce the data to 2 dimensions such that each point in this plot represents a single cell, colored based on cluster identity.
(b) Heat map plotting the top ten marker genes (y axis) for each cluster with single cells grouped by cluster (x axis) representing the major cardiac cell populations of both wild type and IRF3 knockout mice four days after myocardial infarction. Mono, monocytes; M1, macrophages; FB, fibroblasts; M2, proliferating macrophages; GC, granulocytes; NK, NK cells; IFNIC, IFNIC macrophages; EC, endothelial cells.
(c) Violin plots depicting the probability density expression of canonical markers for each of the cell types present after cardiac MI and data integration.

Once data has been integrated together, macrophages and monocytes were isolated to determine the dominant differentially expressed genes across conditions. Figure 8.1a shows the presence of interferon-inducible macrophages. Figure 8.2b shows these interferon genes predominantly expressed in the IFNIC population of macrophages. Further, differential gene expression reveals these as IRF3-dependent signaling in Wild Type macrophages (Figure 8.2c).

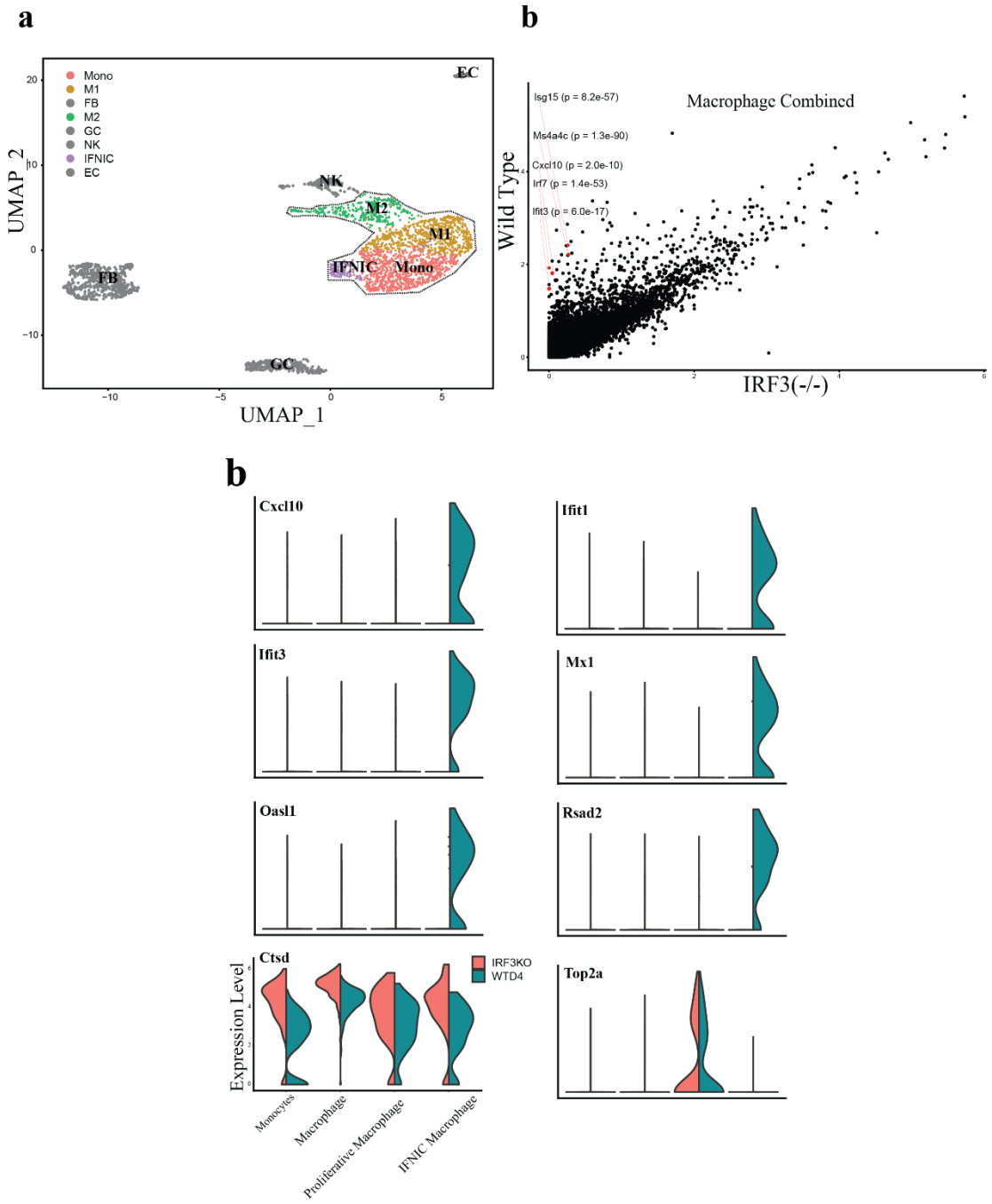

Figure 8.2: Differential Gene Expression Across Conditions Reveals Myocardial Infarction Activates IRF3-Dependent Signaling in Wild Type Macrophages.
(a) UMAP plot depicting which clusters of monocytes and macrophages were isolated to determine the differentially expressed genes dominant in wild type after MI.
(b) Scatter plot representing of differentially expressed genes of both wild type and IRF3 knockout mice. Each point represents a gene with average expression of both wild type (y axis) versus knockout (x axis) highlighting the dramatic IRF3-dependent response after myocardial infarction found on wild type mice.
(c) Violin plots depicting the probability density expression of interferon signaling genes dominant in the IFNIC macrophage cluster. IFNIC; interferon-inducible cells.

The same workflow and algorithm were applied to fibroblast cells. Given the hypothesis that IRF3 knockout fibroblast expressed a greater amount of extracellular matrix components such as collagen and fibronectin to prevent adverse remodeling in the heart, differential gene expression reveals these genes as not a significant marker.

Given the hypothesis that knockout fibroblast expresses a greater amount of extracellular matrix component, a scatter plot featuring differentially expressed genes demonstrate these ECM component genes highly expressed in both knockout and wild type mice (Figure 8.3b-8.3c). Figure 8.3b also demonstrates that there are not many genes found predominantly expressed in the knockout populations over the wild type. This suggest the differences in survival rates and adverse remodeling lay in the genes predominantly expressed in wild type mice. Filtering for differentially expressed genes found exclusively in fibroblasts and having a functional role in ECM remodelling, It was found that matrix metalloproteinases were highly expressed in wild type fibroblasts when compared to knockout mice (Figure 8.3d).

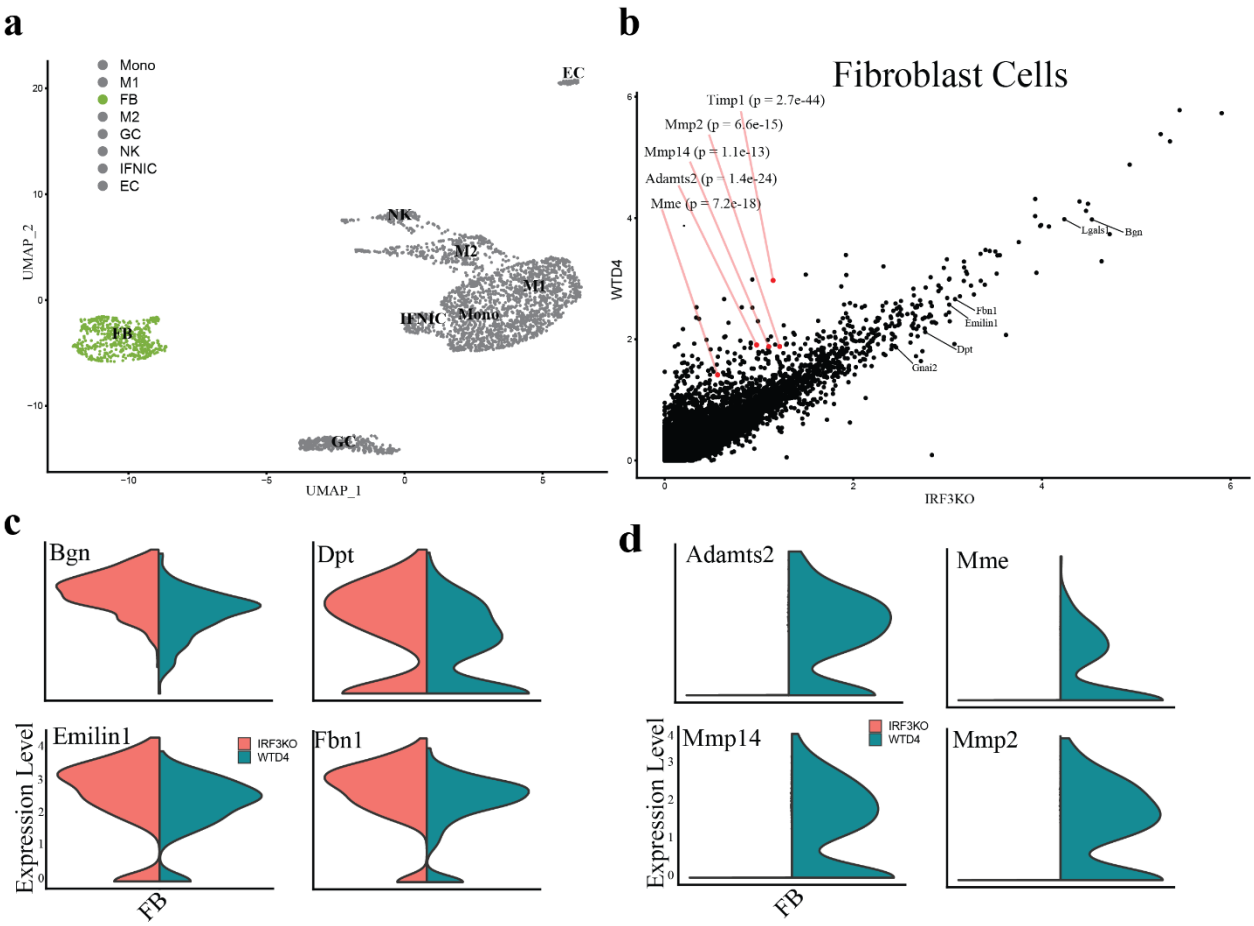

Figure 8.3: Differential Gene Expression Across Conditions Reveals Matrix Metalloproteinases Dominant in Wild Type Macrophages after Myocardial Infarction.

(a) UMAP plot depicting which cluster of fibroblasts were further isolated to determine the differentially expressed genes dominant in wild type mice after MI.
(b) Scatter plot depicting that it is the over expression of matrix metalloproteinases in wild type rather than extracellular matrix components that may explain differences in survival and structural integrity of IRF3 knockout over wild type mice.
(c) Violin plot depicting the probability density expression present in both wild type and IRF3 knockout mice.
(d) Violin plot depicting the probability density expression present predominantly in wild type mice.

To further validate these findings, subsequent subsetting and clustering was done to determine which cluster of fibroblasts drive the expression of matrix metalloproteinases in wild type (Figure 8.4). Unsupervised clustering reveals two distinct populations of fibroblasts classically known in literature as myofibroblasts and fibroblasts (Figure 8.4a). Expression of

alpha smooth muscle actin was added in the heat map to guide classification of fibroblasts in Figure 4a. It was found that these subpopulations of fibroblasts exists both in the wild type and knockout cells (Figure 8.4b) and that wild type myofibroblasts predominantly expression MMPs not found in knockout mice (Figure 8.4c).

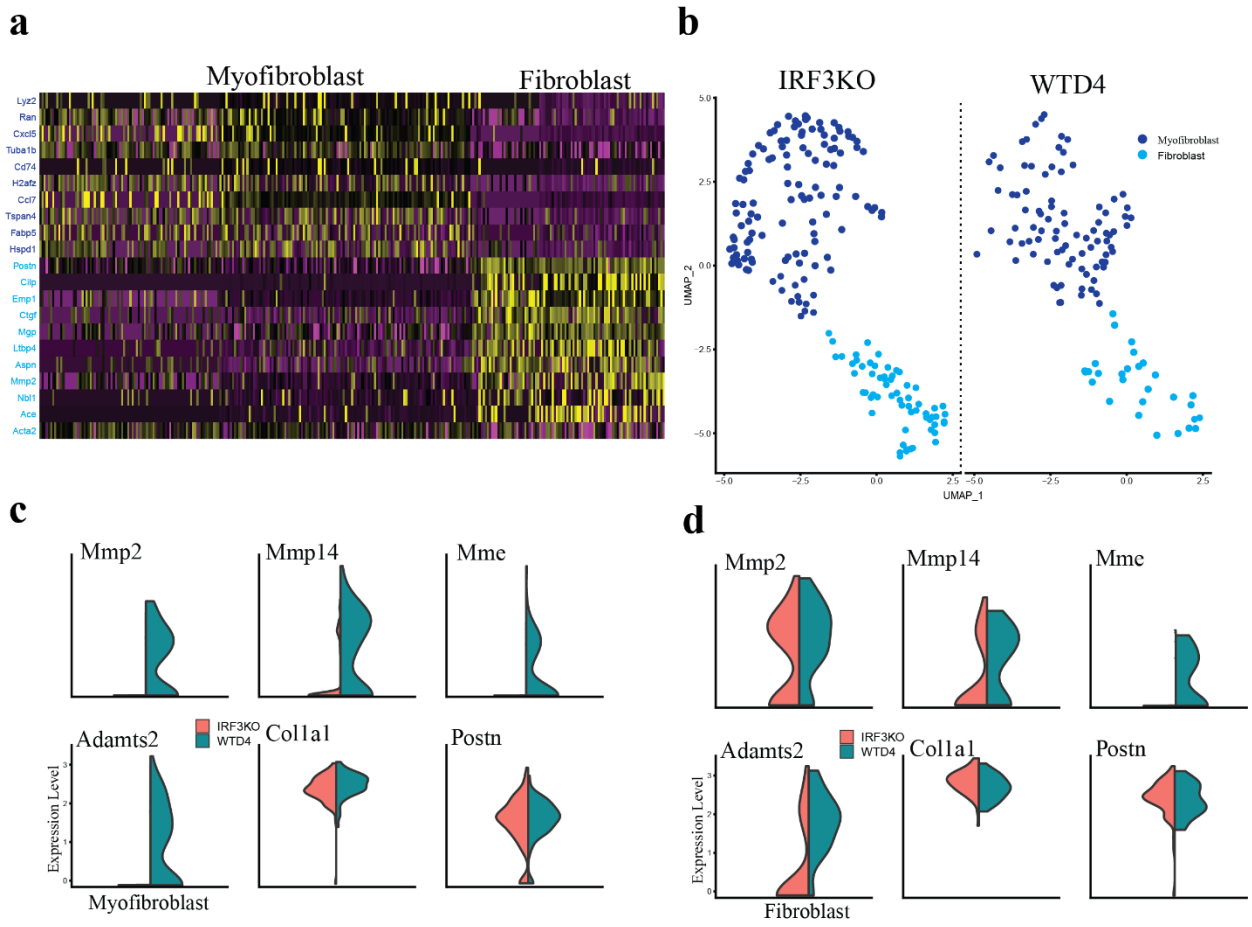

Figure 8.4: Subclustering of Integrated Data Reveals Differences in Expression of Matrix Metalloproteinases in Myofibroblast.

(a) Heat map plotting the top ten marker genes for each cluster of subsetted fibroblasts. Unsupervised clustering reveals myofibroblast (Acta2 high) and fibroblast clusters.
(b) UMAP plot depicting the presence of both cell types present in knockout and wild type populations.
(c) Violin plot depicting the expression of matrix metalloproteinases dominant in wild type myofibroblasts. Col1a1 and Postn added as reference canonical fibroblast markers.
(d) Violin plot depicting the expression of matrix metalloproteinases in fibroblasts. Col1a1 and Postn added as reference canonical fibroblast markers.

Chapter 9: Discussion

In summary, the results found demonstrate support for the initial hypothesis that MMP genes would be increased in the wild type compared to the knockout. However, it was also found that the initial hypothesis that IRF3 knockout mice exhibit a dramatic increase in expression of collagen and ECM related components was untrue. The initial rationale for this hypothesis was the increased survival rates of knockout may be due to increased expression of ECM components that strengthen the material properties of the heart to prevent adverse remodeling such as ventricular dilation. Likewise, the increased MMPs would be expected as the degradation of ECM related components such as collagen and elastin degrade the heart cell wall leading to ventricular dilation and an oversized heart.

The integrated data of wild type and IRF3 knockout scRNA-Seq data demonstrates that the cardiac cells within both samples cluster together within shared populations (Figure 9.1). This validates the process of proper integration as samples that cluster separately based on experimental condition would indicate significant batch effects which would greatly distort real and significant biological differences. The heat map shown in Figure 6.1a features a population of macrophages producing IRF3 dependent interferon genes indicating that this pathway is strongly activated after MI.

Figure 9.2 demonstrates the increased presence of IRF3-dependent type I interferon genes which validates the process of data sample integration across conditions and differential gene expression. The scatter plot featured on Figure 8.2c demonstrates that many of the type I interferon antiviral genes are not expressed at all in the knockout. Likewise, it is shown that there are many pathways and genes expressed in wild type when compared to knockout. However, the

converse is not true; there are not many genes or pathways activated exclusively in IRF3 knockout macrophages.

Applying this analysis methodology to fibroblasts, it was found that there was a dominant expression of MMPs in wild type compared to knockout (Figure 8.3). Further subsetting and unsupervised clustering shows that the myofibroblasts (Acta2 high fibroblasts) express these MMPs at a greater level than IRF3 knockout fibroblast. However, this difference is not as great as those found in macrophages mentioned previously with the type I interferon genes. The pathway and expression of MMPs is still present in the knockout, just at a lower proportion. Likewise, expression of collagen and ECM component genes was found to lie along the diagonal of the scatter plot (Figure 8.3b) indicating that these genes are not significant differentially expressed genes across conditions.

Figure 9.1 summarizes the proposed mechanism by which the IRF3 dependent response of fibroblasts may occur. Based on the results, it is proposed that ECM component genes such as collagen and fibronectin are still being expressed, but an increased expression of MMPs by myofibroblasts relative to TIMPs degrade the ECM of the wild type contributing to adverse ventricular remodeling. That is, the production of type I interferon genes is associated with the increased prevalence of MMPs in wild-type mice, which degrade crucial components of the cardiac ECM, thereby leading to insufficient scar formation when compared to IRF3 knockout mice post MI. Future studies of adverse cardiac remodeling in the IRF3 type I interferon response can be done to specifically inhibit (genetically and pharmacologically) MMPs such as MMP-2, MMP-2, or MMP-14. Further, to study the relationship between ISG expression and MMPs, ISGs can be applied exogenously to IRF3 knockout macrophage and fibroblasts to see if MMP expression can be induced.

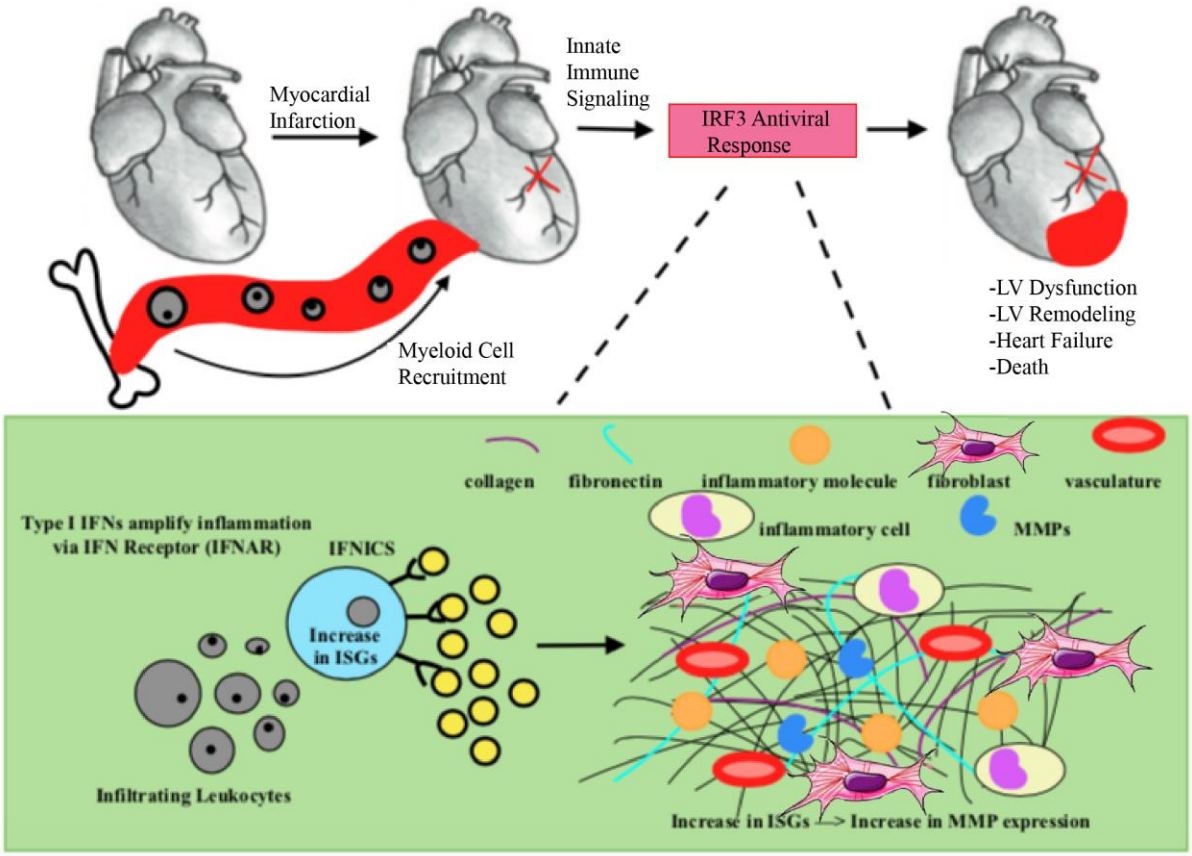

Figure 9.1: Proposed Model of the potential link of Type I IFN response and adverse cardiac remodeling [2, 4].

Materials and Methods

Study Animals and Cardiac Single-Cell Preparation

All samples were obtained from previously available unsorted scRNA-seq prepared by King et al based on IRF3 and Type I Interferon response to MI [43]. IRF3 knockout mice was gifted from T. Taniguchi (Tokyo University) and provided by M. Diamond (Washington University School of Medicine). Wild-type and IRF3-/- mice were anesthetized with 2% isoflurane and permanent coronary artery ligation was performed. The mice were kept post MI until day 4 where samples of heart tissue were then collected and processed to collect scRNA-Seq data through microfluidic inDrop encapsulation. Data was processed through inDrops software (https://github.com/indrops/indrops; accessed April 2017) [44].

Sequencing and Statistical Methods for Analysis of scRNA-Seq Data

Seurat suite version 3.0.0 was used for downstream analysis. Cells were filtered such that the features detected are present in at least 5 cells and greater than 200 features were detected in each cell. Feature counts for each cell were then natural-log normalized and subsequently scaled. For clustering, linear based principal component analysis was performed and used for KNN graph based clustering. The non-linear based dimensional reduction UMAP was then used to visualize the clusters graphically.

Integrated Data Analysis

Seurat suite version 3.0.0 was used to integrate wild-type and IRF3-/- knockout scRNA-Seq datasets as described by Stuart and Butler et al integration strategy [38]. scRNA-Seq processing workflow was then filtered, normalized, scaled, and clustered as previously described. Cell type identification was based on key underlying genetic signatures of each

clusters. Differentially expressed genes across macrophage and fibroblast were determined by subsetting each cluster and performing differential gene expression on the integrated and identified common cell types across conditions. Additional filtering of contaminant cells in macrophage or fibroblasts were done by additional subsetting and data processing thereby eliminating clusters of cells not expressing canonical genetic markers for fibroblast or macrophages. Gene ontology (GO) enrichment analysis of differentially expressed genes for each cluster of macrophage and fibroblast was then used to identify biologically relevant inflammatory and remodelling genes. Only significant genes exhibiting a p-adjusted value less than 0.05 were considered. The p-values were adjusted based on the bonferroni correction.

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