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Single Cell Transcriptomics of *In Vitro* and *In Vivo* bone marrow Nrf2-stimulated macrophages and Pro-inflammatory SiglecF^{High} neutrophils

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

in

Biology

by

Kenneth Huang

Committee in charge:

Professor Kevin R. King, Chair
Professor Dong-Er Zhang, Co-Chair
Professor Stephanie Mel

2021

The Thesis of Kenneth Huang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

I dedicate this thesis to my family, including my father, Jun, my mother, Hong, and my sister, Mei. Thank you all for your words of encouragement, self-care reminders, financial support, and unconditional love. I could not have gotten this far without the relentless support I have been given. In addition, thank you to all my incredible friends and significant other. I have received countless words of encouragement and amazing memories I will cherish forever. Finally, I thank the entire lab including David, Nika, Nicholas, Avinash, Claire and Dr. King for their mentorship, guidance, and support.

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LIST OF ABBREVIATIONS

Nrf2 – Nuclear factor erythroid-2-related factor 2

BMDM – Bone marrow derived macrophage

MI – Myocardial Infarction

IFN-β – Interferon Beta

IFN-α – Interferon Alpha

Irg1 – Immuno-responsive gene 1

ISG – Interferon stimulated gene

NSG – Nrf2 stimulated gene

WT – Wild Type

KO – Knockout

PBS – Phosphate-Buffered Saline

IRF3 – Interferon regulatory factor 3

DAMP – damage associated molecular pattern

PAMP – pathogen associated molecular pattern

TLR – toll-like receptor

NLR – NOD-like receptor

CHD – coronary heart disease

ATM – adipose tissue macrophage

UMAP – Uniform Manifold Approximation and Projection

LPS – lipopolysaccharide

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Next, I would like to thank Dong-Er Zhang and Stephanie Mel for their time and effort to be on my committee. My opportunity to mentor students as both an undergraduate and graduate teaching assistant under the supervision of both Stephanie Mel and Stanley Lo has been crucial in my passion in teaching.

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ABSTRACT OF THE THESIS

Single Cell Transcriptomics of *In Vitro* and *In Vivo* bone marrow Nrf2-stimulated macrophages and Pro-inflammatory SiglecF^{High} neutrophils

by

Kenneth Huang

Master of Science in Biology

University of California San Diego, 2021

Professor Kevin R. King, Chair
Professor Dong-Er Zhang, Co-Chair

Myocardial infarction (MI) is the leading cause of death in the U.S., affecting elderly populations and more recently, younger populations. Treatments thus far have reduced rates of myocardial infarction (MI), but progress has slowed due to downstream consequences of heart failure, especially involving the immune system response to necrotic cells in the heart, causing subsequent inflammation. To elucidate gene expression and specialized subsets of immune cells that exacerbate or ameliorate inflammation after MI we have analyzed the single-cell transcriptomics of mice bone marrow, the source of inflammation of the heart. We have observed differences in single-cell gene expression between bone marrow *in vitro* and *in vivo* of mice four days post-MI, especially in the expression of cardioprotective and proliferative macrophages, marked by genes such as Top2a and Mki67, *in vitro*. Additionally, we compared single-cell transcriptomic differences of *in vitro* bone marrow derived macrophages with *in vivo* bone marrow

macrophages. We have also found specialized neutrophils, SiglecF^{High} neutrophils, that are suggested to exacerbate inflammation in the heart via the IL6 pathway. Lastly, we reported crosstalk between the IRF3 pathway and the Nrf2 pathway, especially between itaconate, a key Nrf2 component and interferon-beta (IFN-B), a component of the IRF3 pathway. In conclusion, single cell revealed new populations of specialized immune cells that can potentially aid or detriment post-MI recovery. Further investigation into specialized immune cells using *in vivo* imaging and functional characterization of neutrophils will allow for higher resolution of immune cell function and potential antibody-based therapeutics designed to reduce SiglecF^{High} neutrophils.

INTRODUCTION

Clinical management of myocardial infarction (MI) and cell-mediated immunity

Coronary heart disease (CHD) is the leading cause of death in many developed countries (Murray & Lopez, 1997). Myocardial infarction (MI), a type of CHD that is commonly known as a heart attack, occurs when there is thrombotic occlusion of blood flow to specific areas of the heart, causing decreased oxygen supply and subsequent necrosis of cardiomyocyte cells (Frangogiannis, 2015). Treatments thus far have reduced rates of CHD by approximately 50% for both men and women in the last 20 years (Ford & Capewell, 2007). However, a central issue still in treating MI is alleviating downstream consequences of heart failure, such as increased stiffness of the ventricle following improvement in clinical management. Currently, typical treatments involve medications that promote reperfusion or reduction of clots formed due to plaque. These include statins, blood thinners, beta blockers and, in more severe cases, medical procedures involving coronary stents and angioplasties (Lu, Liu, Sun, Zheng, & Zhang, 2015).

Despite rapid improvement in clinical management of MI, we still face problems such as adverse remodeling which is defined by cardiac fibrosis or stiffness of the heart and cardiomyocyte hypertrophy or enlargement of cardiac muscles. Ultimately, these symptoms contribute to significant reduction in the heart's pumping efficiency. Also, this often leads to heart failure and recurring events of post-MI fibrosis. (Konstam, Kramer, Patel, Maron, & Udelson, 2011). These downstream events are fueled by an inflammatory response comprised of multiple immune cells including neutrophils and monocytes that promote fibrosis leading to heart failure (Suthahar, Meijers, Sillje, & de Boer, 2017). Currently, clinicians do not focus on the immune system in the context of MI because no immune modulating drugs have demonstrated significant benefit. However, recent clinical research has shown that immune

modulators may attenuate the inflammatory response caused by immune cell recruitment and improve symptoms of heart failure (Aday & Ridker, 2018).

Recently, there is growing evidence of immune cell recruitment and potential therapeutics that function to reduce inflammation in the heart (Frangogiannis, 2014; Swirski & Nahrendorf, 2018). For instance, inflammatory signals composed of cytokines are released from necrotic cells in the infarcted myocardium and immune cells including neutrophils and macrophages from the bone marrow infiltrate to clear the dead cells (Frangogiannis, 2014). This inflammatory response is followed by a reparative phase, defined by the disappearance of neutrophils and the appearance of specialized macrophages that promote angiogenesis and fibrosis (Nahrendorf et al., 2007). Clinically, the first studies associated elevated leukocyte numbers with early stages of inflammation leading to adverse outcomes in acute MI (Barron, Cannon, Murphy, Braunwald, & Gibson, 2000). Moreover, studies also emphasized the role of immune cells including interferon stimulating cells, plasmacytoid dendritic cells (pDCs), and macrophages in MI. For instance, depletion of pDCs proved to reduce hyperplasia in lymphoid organs (Rowland et al., 2014). Additionally, when macrophages were reduced by active treatment, mortality due to MI was increased (Frantz et al., 2013). Thus, reducing levels of macrophages post-MI through immune modulating drugs may benefit patients' recovery.

Historically, immune modulators have typically only targeted cancer-related diseases and function by stunting tumor growth or controlling immune checkpoints (Naidoo, Page, & Wolchok, 2014). Modulating drugs targeted cancer diseases because chronic inflammation was found to be a major contributor to promoting cancer (Coussens et al., 2002). Armed with the knowledge that the immune response increases inflammation post-MI, the first breakthrough came with the use of immune modulators under the CANTOS trial. This trial utilized

canakinumab, an IL-1B monoclonal antibody designed to reduce the effects of downstream activation of IL-6, a pro-inflammatory cytokine and inflammasome integrating proteins. This trial was an important landmark in recognizing inflammation as an attractive therapeutic target for cardiovascular diseases. (Aday & Ridker, 2018).

DAMPs and PAMPs contribute to immune cell heterogeneity post-MI

On the molecular level, it is generally accepted that the immune system response to MI involves inflammatory stimulation by heterogenous populations of neutrophils and macrophages and recognition of damage associated molecular patterns (DAMPs) (Mann, 2011). However, recent research shows there is increased levels of pathogen associated molecular patterns (PAMPs), defined as pathogen lipoproteins or nucleic acids, present post-MI (Mogensen et al.,2009). Broadly speaking, PAMPs originate from microorganisms due to microbial infection and DAMPs originate from the host's cells in response to tissue damage, ischemia, or trauma (Tang et al., 2012). These molecules both signal the innate immune response to become active and destroy intruding or necrotic cells.

PAMPs typically involve neutrophils and two subgroups of macrophages, M1 and M2 (Martinez & Gordon, 2014). Previous research conducted by King and colleagues noted that DNA released by necrotic cardiac cells in MI act as PAMPs, stimulating tissue resident M2 macrophages to produce cytokines (King et al., 2017). The cytokines promote neutrophil recruitment, and the neutrophils migrate toward the site of infection and recruit monocytes. Migrated monocytes differentiate into M1 macrophages and proceed to engulfing foreign pathogens. Following engulfment, M1 macrophages bind to neutrophils to induce apoptosis and M1 macrophages polarize towards an M2 phenotype to resume homeostasis (Prame Kumar,

Nicholls, & Wong, 2018). Sterile injury due to MI can introduce DAMPs in the form of necrotic cells that alarm the immune system to recruit heart shock proteins (HSPs) and high-mobility group box-1 (HMGB1). (Timmers et al., 2012). The HMGB1 stimulates macrophages, neutrophils, monocytes, and cytokines to the site of injury, which ultimately increases inflammation (de Haan, Smeets, Pasterkamp, & Arslan, 2013).

Both these patterns, DAMPs and PAMPs, bind to pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). These receptors are differentially expressed by immune cells such as macrophages and neutrophils and when activated, the cells are transported from the bone marrow to the heart (Mann, 2011). Due to the involvement of DAMPS, PAMPs, and unique populations of neutrophils as well as macrophages, determining which cell population to target with drug therapy becomes difficult.

Immune cell heterogeneity and single cell analysis

Due to the heterogeneity of immune cells involved in inflammation after MI, single cell-RNA sequencing techniques have evolved to characterize heterogenous populations and identify novel distinct immune cell subsets. Knowledge of heterogenous or novel populations of immune cells can lead to precise therapeutic strategies that combat inflammation after MI. Research on immune cell transcriptomics techniques have been established to identify differential genes expressed by the immune cells including macrophages, monocytes, and neutrophils. Previously, researchers used flow cytometry to characterize for immune cell subsets within heterogenous cell populations. However, this technique is limited to the number of fluorophore-conjugated antibodies available (Pockley, Foulds, Oughton, Kerkvliet, & Multhoff, 2015).

With the advent of single cell techniques, identification of new immune subsets and temporal patterns within the heart is possible without limitations due to antibodies. This technique is also designed to identify new immune cell mouse post-MI samples at a higher resolution and define cells by their differentially expressed genes (Hwang, Lee, & Bang, 2018). For instance, Delile and their group discovered new progenitor and neuronal markers in the spinal cord of a mouse using single cell RNA analysis (Delile et al., 2019).

Previous research also shows heterogeneity in cell types and existence of subsets of cells within the mouse heart (Skelly et al., 2018). Certain cell types including neutrophils and macrophages within the heart have their own subgroups that express genes differentially. Preliminary results based on single cell RNA-sequencing defined the differentially expressed genes in the circulating neutrophils and macrophages in the bone marrow, blood, and heart (D. Calcagno, personal communication). Further studies progress towards functionally characterizing neutrophils and macrophages and how different subgroups of both will participate in improving survival of mice post-MI. Besides the canonical two subgroups of M1 and M2 macrophages, there are other types of macrophages including proliferating macrophages and adipose tissue macrophages (ATMs). These ATMs are typically involved in obese mice that can be present in the bone marrow (Russo & Lumeng, 2018). These immune cell subgroups can provide valuable information on cell types that can be detrimental or beneficial in repairing and remodeling the heart post-MI.

Single cell characterization of interferon pathways *in vivo*

Using single cell techniques, new pathways and immune cell subsets present after MI can be analyzed for gene expression. By analyzing the single-cell gene expression of heart cells after

MI, our group unexpectedly discovered excessive Interferon Regulatory Factor 3 (IRF3) activation following damage to the heart, resulting in autoinflammatory conditions that pose a threat to patient health. IRF3 is a transcription factor that is activated by PAMPs such as DNA released by necrotic cells in the heart through the TLR pathway. Interestingly, mice deficient in IRF3 exhibited improved post-MI survival compared to WT mice, suggesting IRF3 signaling might be a promising target for reducing inflammation following heart damage. IRF3 acts as a cofactor to induce activation of interferon stimulated genes (ISGs) such as Cxcl10, Rsad2, and Isg20 (King et al., 2017). However, little is known of the origin of ISG activation and requires analysis of the single-cell expression of the immune cell transported from the bone marrow to the heart.

Single cell characterization of bone marrow *in vitro* vs. *in vivo*

The bone marrow provides the hematopoietic stem cells (hSCs) that differentiate into immune cells including monocytes, macrophages, and neutrophils, indicating a promising target for single cell analysis (Dutta et al., 2015). Bone marrow derived macrophages (BMDMs) are commonly used macrophages that are cultured *in vitro* and known to exhibit macrophage characteristics *in vivo*. To produce BMDMs in vitro, naïve bone marrow cells are differentiated by a growth factor, macrophage-colony stimulating factor (M-CSF). Research shows use of BMDMs to study the immune response in obese mice and macrophage polarization (Liu et al., 2015). We have previously stimulated these cells with interferon stimulants including interferon-alpha (IFN- α) and interferon-beta (IFN- β) to induce ISG expression, and we have taken naïve bone marrow cells from the bone marrow of MI-induced mice and control mice for culture.

It remains unclear whether ISG expression is induced in BMDMs cultured from MI-induced mice compared with BMDMs from control mice. Previous research has shown similarities in gene expression profiles of *in vitro* immune cells and *in vivo* immune cells after stimulation with IFN-α using quantitative PCR (Zimmerer et al., 2008). However, these differentially expressed genes present in macrophages *in vitro* and *in vivo* macrophages have not been studied using a single cell analysis approach. Novel distinct subpopulations of macrophages present *in vitro* could drive ISG expression to promote inflammation post-MI.

Anti-inflammatory pathways and immunometabolites

In contrast to the inflammatory pathways that detriment the heart, anti-inflammatory pathways exist to reduce the detrimental effects in the heart. In the IRF3 pathway, we understand that the inflammatory response involving immune cells including neutrophils and macrophages can exacerbate recovery post-MI. Interestingly, one notable ISG in the IRF3 pathway, Immunoresponsive gene 1 (Irg1), was found to activate the Nrf2 pathway, an anti-inflammatory response. This finding suggests a mechanism of how the heart ameliorates the detrimental effects of interferons. The Nrf2 pathway functionally inhibits NF-kB activation and subsequently eliminates reactive oxidants and electrophilic agents, whereas the IRF3 pathway promotes NF-kB activation inducing a pro-inflammatory response (Ma, 2013). Moreover, itaconate, an immunometabolite derived from molecules in the citric acid cycle, was found to increase Nrf2-related gene expression levels (Mills et al., 2018). Recently, our group found that the Nrf2 pathway is active after MI and expressed in cells not found in the IRF3 pathway (D. Calcagno, personal communication). Consequently, itaconate is an attractive therapeutic target for reducing detrimental interferon-stimulated inflammation post-MI.

Our group sought to study the effect of itaconate on ISG expression and the mechanism by which itaconate is transported. Exogenous itaconate was found to negatively regulate IFN-B, one of the key intermediates in the type I interferon response (Mills et al., 2018). However, the mechanism remains unclear by which itaconate attenuates ISG expression and whether we can activate the Nrf2 pathway without the use of exogenous itaconate. It is possible that other Nrf2 constituents may attenuate components of the IRF3 pathway.

Moreover, studies thus far have not focused on the mechanism of transporting endogenous itaconate between immune cells. Further investigation into the cell-to-cell communication of itaconate can elucidate information regarding itaconate's origin. Endogenous itaconate has been shown to modify proteins that limit glycolysis, suggesting attenuation of inflammation (O'Neill & Artyomov, 2019). Therefore, studying the mechanism by which endogenous itaconate is transported is a promising target for reducing inflammation after MI.

Potential anti-inflammatory therapeutics and neutrophils

A novel subgroup of neutrophils marked by a high expression of SiglecF appeared in elevated numbers post-MI and suggested potential anti-inflammatory antibody therapeutics (D. Calcagno, personal communication). Preliminary single cell analysis shows that circulating blood neutrophils post-MI not only induce ISGs but also SiglecF^{High} genes. Previously, these cancer-promoting neutrophils expressing high levels of the gene SiglecF have been found in lung tumors that drive an inflammatory reaction (Engblom et al., 2017). Recently, we found the presence of SiglecF neutrophils in the heart using single cell analysis (D. Calcagno, personal communication). It remains unknown the function and the temporal signature of these SiglecF neutrophils. Currently, it is unknown whether these SiglecF neutrophils can induce ISG

expression after MI and if so, what causes this ISG expression. Preliminary results of heart co-culture experiments show that co-culturing minced hearts, presumably dead tissue, can prolong the survival of neutrophils. If the viability of neutrophils can be further increased, potential SiglecF expression may be observed (D. Calcagno, personal communication). If SiglecF expression can be attenuated within the context of MI, there could be potential therapeutics by blocking SiglecF expression.

Project Aims

In summary, the immune system response to MI involves specialized immune cells including M1 and M2 macrophages and specialized neutrophils that orchestrate an inflammatory response to repair and remodel the heart. My overarching goal is to define the single cell transcriptomics of specialized macrophages and neutrophils and define the mechanisms of potential anti-inflammatory pathways with reference to the interferon pathway. My first aim is to identify the diversity of macrophages and neutrophils by single-cell RNA analysis. My second aim is to determine if bone marrow derived macrophages (BMDMs), a commonly used *in vitro* cell, is a good model for *in vivo* bone marrow macrophages after MI by comparing single-cell gene expression between the two. My last aim is determining if there is any crosstalk between Nrf2-stimulated macrophages, IRF-3-stimulated macrophages and SiglecF neutrophils using single-cell RNA analysis and quantitative PCR.

MATERIALS AND METHODS

Animals

Adult male C57BL/6J (WT, stock: 000664) and Nrf2-deficient mice (stock: 017009) mice were purchased from the Jackson Laboratory or obtained from Fitzgerald lab after derivation from cryopreserved embryos obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM). IRF3$^{-/-}$ mice were a generous gift from Tadatsugu Taniguchi and provided by Michael Diamond. Experiments were performed with 10-14-week-old animals and carried out using age and gender matched groups without randomization. All genotyping was performed either in-house using methods recommended by Jackson Laboratory or by Transnetyx. All mice were maintained in a pathogen-free environment at the UC San Diego Leichtag Research Building vivarium. All experiments with animals were approve by the Subcommittee on Animal Research Care and IACUC at UC San Diego.

Murine Tissue Processing and Cell Culture

Mice were anesthetized using isoflurane and death was confirmed by cervical dislocation. Bone marrow cells were extracted from the femur and tibia and flushed with ice-cold PBS. The flow-through solution was filtered through a 40 μm nylon mesh and treated with red blood cell (RBC) lysis buffer (BioLegend) to remove erythrocytes. Bone marrow cells were spun down and resuspended in DMEM (containing 10% FBS, 1% penicillin/streptomycin and 10ng/mL M-CSF) for 6 days. Unless stated, 1 x 10^6 BMDMs per milliliter were used in *in vitro* experiments.

Flow cytometry and sorting

Isolated cells from bone marrow were stained at 4°C in FACS buffer with DAPI to remove dead cells. Ter119 (BioLegend, clone TER-119) was used to removed unlysed red blood

cells. Secondary staining of the leukocyte subsets was performed using CD45.2 (BioLegend, clone 104), CD11b (BioLegend Clone M1/70), F4/80 (BioLegend, clone BM8), Ly6G (BioLegend, clone 1A8). Monocytes were identified as (DAPI/Ter119/Ly6G)^{low} (CD45/CD11b)^{high}. Neutrophils were identified as (DAPI/Ter119)^{low} (CD45/CD11b/Ly6G)^{high}. Further sub-classification of neutrophils was identified as SiglecF^{High} (BioLegend, clone E50-2440 or S17007L) or SiglecF^{low}. Flow cytometry was performed on a Sony MA900 and analyzed with FlowJo software (Tree Star).

RNA Extraction and Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from cell culture plates or FACS-sorted cells using the RNeasy Plus Micro kit (Qiagen). First-strand cDNA was prepared by using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed on cDNA using SYBR Green probes. qPCR was performed on a Quantstudio 3 (Applied Biosystems) using 2x PCR Advanced Fast master mix (Applied Biosystems). Taqman gene expression assays (Applied Biosystems) were used to quantify target genes (*Gapdh*: Mm99999915_g1, *Tnf*: Mm00443258_m1, *Icam1*: Mm00516023_m1, *Retnlg*: Mm00731489_s1, *Lrg1*: Mm01278767_m1, *Siglecf*: Mm00523987_m1, *Ppia*: Mm02342430_g1, *Slpi*: Mm00441530_g1, *Ccl6*: Mm01302419_m1, *Gclm*: Mm00514996_m1, *Nqo1*: Mm01253561_m1, *Isg20*: Mm00469585_m1, *Cxcl10*: Mm00445235_m1, *Irg1*: Mm01224532_m1, *Irf7*: Mm00516793_g1, *Il1b*: Mm00434228_m1, *Tnfaip3*: Mm00437121_m1, *Il6*: Mm00446190_m1, *Ifnb1*: Mm00439552_s1) Fold changes in expression were calculated using the ΔΔCt method using mouse Gapdh as a housekeeping gene control for mRNA expression.

Single cell RNA Sequencing and Analysis

Single-cell RNA-Seq (scRNA-seq) was performed using microfluidic droplet-based encapsulation, barcoding and library preparation (inDrop and 10X Genomics) as previously described. Paired end sequencing was performed on an Illumina Hiseq 2500 and Hiseq 4000 instrument. Low level analysis, including demultiplexing, mapping to a reference transcriptome (Ensembl Release 85 – GRCm38.p5), and eliminating redundant unique molecular identifiers (UMIs), was performed with a custom inDrops software (URL: https://github.com/indrops/indrops, accessed April, 2017) or a 10X CellRanger pipeline. All scRNA-seq analyses were conducted using the Seurat R package (v3.1).

Single-cell RNA-Seq Quality Control, Normalization, and Integration

The total transcript count for each cell was scaled to 10,000 molecules, and mitochondrial and ribosomal reads were removed. Raw counts for each gene were normalized to cell-specific transcript count and then natural-log transformed. Cells between 200 and 4000 uniquely expressed genes and <5% mitochondrial counts were retained for further analysis. Any highly variable genes across datasets were identified with the *FindVariableFeatures* method from Seurat R Package (v3.1) by selecting 4,000 genes with the highest feature variance after variance-stabilizing transformation. Integration of multiple single-cell RNA-seq datasets was performed to enable harmonized clustering and comparative analyses across *in vitro* and *in vivo* conditions. Anchoring paired cells between datasets were done by Canonical Correlation Analysis (CCA) and Mutual Nearest Neighbors (MNN) using the Seurat *FindIntegrationAnchors* function.

Single-cell RNA-Seq Dimensional Reduction, Clustering and Sub-clustering

After scaling and entering expression values for each variable gene, linear dimensionality reduction was performed on integrated data using principal component analysis (PCA). Cells were clustered using the Shared Nearest Neighbor (SNN) clustering method with the Louvain method for modularity optimization, as executed through the *FindNeighbors* and *FindClusters* functions. Uniform Manifold Approximation and Projection (UMAP) was used to visualize data in a two-dimensional space. Differentially expressed genes (DEGs) between clusters were determined using a Wilcoxon Rank Sum test. Sub-clustering was performed by subsetting cell-type clusters, identifying a new set of DEGs within that subset, and reclustering the subset based on the newly determined DEGs. This was performed to examine cell-state heterogeneity within cell-types in an unbiased manner.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. All data are represented as mean values ± standard error of mean (SEM), unless indicated otherwise. A statistical method was not used to predetermine sample size. For comparisons of the qPCR data, a 2-tailed Mann Whitney *U* test was used to determine statistical significance. All analyses were unpaired. $P<0.05$ were considered significant and are indicated by asterisks as followed: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

RESULTS

Single cell transcriptomics of specialized macrophages in *in vivo* bone marrow and *in vitro* BMDMs

The standard workflow to process bone marrow derived macrophages into single-cell data starts with the extraction of the bone marrow cells from the femur and tibia of a mouse. The bone marrow cells are then sorted into a flow cytometer and then barcoded using Illumina's HiSeq system. The remaining output is then analyzed using Seurat, an R package. (**Figure 1A**). Using heatmaps **(Figure 1B)**, we can see the top 10 differentiated genes and classify these yellow groups (expressed genes) as subsets. Generally, these subsets are ill-defined in the *in vitro* BMDMs, but we noticed that there are still some distinct groups present. We found four distinct subpopulations labeled 0, 1, 2 and 6. Cluster 0 highly expressed proliferative genes such as *Top2a* and *Mki67*. Cluster 1 expressed high levels of adipose tissue macrophage related genes including *Fapb5* and *Fabp4*. Cluster 2 expressed high levels of *Cd74* and *Ifitm3*. Lastly, cluster 6 expressed high levels of E2F cell cycle regulated genes including *Mcm5* and *Mcm6*. In the *in vivo* bone marrow, there are ten distinct subpopulations. Using previously defined genes to identify specific cell types, we classified each subpopulation (Skelly et al., 2018). The clusters include granulocytes, neutrophils, B cells, Schwann cells, macrophages, NK cells and T cells. In the BMDMs, we noticed there are seven distinct clusters of macrophages that are spatially close **(Figure 1C)**. In *in vivo* bone marrow, we noted that there were ten distinct clusters, and these clusters were more spatially distant than clusters in BMDMs. We then performed supervised clustering on the two subsets of macrophages in the in vivo bone marrow and this resulted with two spatially distinct clusters. **(Figure 1D)**.

Integrated Analysis of *in vitro* BMDMs and *in vivo* bone marrow

Beyond taxonomic listing of clusters, we can integrate two datasets together by a recently discovered method in Seurat and compare them directly with each other (Stuart et al., 2019). Using this new method, we integrated the *in vivo* bone marrow sample with the BMDMs and created an integrated UMAP. This allowed us to investigate if BMDMs, a commonly used *in vitro* tool for studying macrophages, was representative of *in vivo* bone marrow macrophages. We reported 11 distinct clusters and a slight overlap in conserved clusters amongst the two samples **(Figure 2A)**. Additionally, we performed a dot-plot to investigate the conserved genes between the *in vivo* and *in vitro* samples. The genes that were distinctly present in only the *in vivo* sample were: *Ltf, Retnlg, Ebf1, Vpreb3, AA467197,* and *Ms4a4b*. There were no significant genes that were only present in the *in vitro* sample. Conserved genes that were present in both samples include: *Birc5, Cdca3, H2afz, Stmn1, Mcm6,* and *S100a11* **(Figure 2B)**.

We further investigated *Retnlg*, a highly expressed gene in neutrophils, and we found significant expression only in the *in vivo* sample. We also investigated *Top2a,* a gene we previously found abundant in the *in vitro* sample and found that there was only significant expression in the *in vitro* sample **(Figure 2C)**. To further investigate the genes *Top2a* and *Mki67*, we plotted the cell cycle phases onto the UMAP for BMDMs. Most of the cells expressing these two genes were in the G2-M phase while cells expressing *Mcm6,* a gene regulated by E2F, were mostly in the S phase **(Figure 2D)**. To further elucidate differentially expressed genes between the in vitro and in vivo sample, we focused on two clusters of genes distinctly expressed in either sample. *Top2a, Stmn1, Tuba1b, Smc2* and *Ube2c* were robustly expressed in the *in vitro* sample while *Ltf, Lcn2, Anxa1, Wfdc21* and *Ifitm6* were robustly expressed in the *in vivo* sample **(Figure 2E)**.

Exogenous itaconate increases Nrf2-regulated gene expression and attenuates interferon-stimulated gene expression

Nevertheless, BMDMs have provided robust results regarding stimulation assays. Our group attempted to study itaconate, an immunometabolite and component of the Nrf2 pathway. The Nrf2 pathway inhibits inflammation by inactivating the NF-kB pathway while the IRF3 pathway promotes inflammation by promoting the NF-kB pathway. Our group wanted to study if there is crosstalk between IRF3 and Nrf2. Previously, itaconate was found to negatively regulate IFN-B, one of the key intermediates in the type I interferon response (Mills et al., 2018). Interestingly, one notable interferon-stimulated gene (ISG) in the IRF3 pathway, Immunoresponsive gene 1 (Irg1), was found to activate the Nrf2 pathway, an anti-inflammatory response. Our group sought to study the effects of itaconate on ISG expression.

When BMDM cells were stimulated with itaconate alone, ISGs were not as active, but when stimulated with cyclic di-GMP (cdGMP), ISGs were significantly more expressed **(Figure 3A)**. On the other hand, BMDM cells stimulated with itaconate greatly increased Nrf2-stimulated genes (NSGs), especially *Gclm* and *Nqo1* while cdGMP did not increase NSG expression appreciably **(Figure 3B)**. Expectedly, BMDMs stimulated with IFN-B showed increased ISG expression **(Figure 3C)**. Additionally, ISG expression is attenuated when BMDMs were treated with both itaconate and IFN-β, suggesting that itaconate can reduce IFN-β induction of ISGs **(Figure 3C)**. BMDMs stimulated with both itaconate and IFN-β increase NSG expression but do not increase ISG expression (**Figure 3C and 3D)**.

Co-cultured SiglecF LO neutrophils with BMDMs attenuate Cxcl10 expression after interferon stimulation and SiglecF HI and LO increase IL6 expression after LPS stimulation

Tumor-infiltrating neutrophils that have high expression of a marker, SiglecF^{high} (SigF HI), appear in elevated numbers after myocardial infarction (MI). This unique class of neutrophil has been shown to exhibit cancer-promoting properties and has been found to promote tumor growth in the lung (Engblom et al., 2017); however, the effects of SigF HI on inflammation in the heart have not been well characterized. Here, we compared SigF HI neutrophils to neutrophils not expressing SiglecF (SigF LO) to determine how SiglecF expression induces macrophages, and ultimately, produce an interferon (IFN) response. When macrophages are activated by endotoxins including lipopolysaccharides (LPS), type I IFNs are produced. These IFNs such as interferon beta (IFN-β) subsequently activate IFN-stimulating genes (ISGs) driving the IFN response and ultimately, inflammation in the heart (Sheikh et al., 2014). This inflammation can then deter the heart's recovery, which underscores the importance of understanding the expression of these inflammation markers post-MI (King et al., 2017). We chose to look at both stimulating the production of type I IFNs indirectly through the LPS pathway and directly by adding IFN-β. We emulated the infarcted heart in vitro by extracting SigF HI and SigF LO neutrophils from mice post-MI using flow cytometry and co-culturing these neutrophils with bone marrow derived macrophages (BMDMs). BMDMs were extracted from femur and tibia of WT mice and cultured for 6 days. SigF HI, SigF LO and Ctrl were cultured alone as a negative control. LPS or IFN-β was added to Ctrl as a positive control.

Single-cell results show that Retnlg expressing neutrophils are mostly present within the *in vivo* bone marrow sample and minimally present within the *in vitro* BMDMs **(Figure 4A)**. Using violin plots, we confirmed that there is an overall absence of SiglecF HI expressing macrophages within both *the in vitro* and *in vivo* sample **(Figure 4B)**. Using qPCR, we measured gene expression profiles of both SigF HI and SigF LO granulocytes. SigF HI granulocytes

expressed significantly higher levels of *Siglecf*, *Ppia* and *Icam1* while SigF LO cells expressed higher levels of *Retnlg*, *Ccl6*, *Slpi* and *Lrg1* **(Figure 4C)**. Thus, we measured the gene expression of co-cultured neutrophils and macrophages using quantitative PCR (qPCR) and compared IFN activation and LPS activation in SigF HI and SigF LO neutrophils **(Figure 4D and 4E).** The expression of ISGs: Cxcl10, Isg20, and Irf7, were studied to determine the activation of an IFN response. Interestingly, we found that when SigF HI and SigF LO neutrophils were co-cultured with BMDMs, they reduced levels of Cxcl10, indicating that they may be valuable to reduce inflammation in the heart **(Figure 4D).** There was no significant difference between SigF HI and SigF LO neutrophil gene expression across all tested ISGs, which shows that shows the SiglecF expression may not play a role in the interferon pathway. However, when macrophages were indirectly activated through the LPS pathway and co-cultured with SigF HI and LO neutrophils, the expression of interleukin 6 (iL6), a pro-inflammatory cytokine, was increased **(Figure 4E).** Other LPS-stimulating genes including Tnfaip3 and IL1b showed no significant difference across all cellular groups **(Figure 4E).** These results suggest that these neutrophils may in fact, increase inflammation in the heart through elevation of IL6, a different pathway than previously suggested. Therefore, further investigation into SigF HI neutrophils in the IL6 pathway post-MI is necessary.

DISCUSSION

The bone marrow houses the hematopoietic stem cells (hSCs) that differentiate into immune cells including monocytes, macrophages, and neutrophils and travel to the injured heart. This indicates that the bone marrow is a promising target for single cell analysis (Dutta et al., 2015). Bone marrow derived macrophages (BMDMs) are commonly used macrophages that are cultured *in vitro* and are known to exhibit macrophage characteristics *in vivo*. However, we do not know the heterogeneity of both BMDMs and *in vivo* bone marrow. We are not certain of any specialized macrophages present in BMDMs that may have an impact in modeling the *in vivo* bone marrow. Based on our results, we were able to identify a group of proliferative macrophages that function like canonical M2 macrophages and are characterized with proliferative and wound healing properties (Yap et. al., 2019). We suspect that these macrophages may have become induced by the abundance of macrophage-colony stimulating factor (m-csf) and this is what causes them to be proliferative as well. However, if this were the case, we would imagine that every cluster expresses a high expression of proliferative genes such as *Top2a* and *Mki67* which makes us to believe that this is a unique and interesting group of macrophages **(Figure 1B).** Further studies we could perform could be focused on isolating and functionally characterizing these macrophages without the presence of m-csf.

Another interesting subpopulation of macrophages we found are called adipose tissue macrophages (ATMs) and are marked by genes, *Fabp4* and *Fabp5* (Russo & Lumeng, 2018). *Fabp4* plays an important role in atherosclerosis and cardiovascular diseases (Furuhashi, 2019). Previous studies also showed that these macrophages are required for neutrophil recruitment in *Pseudomonas* (Liang et al., 2019). Another study linked *Fabp4* with the regulation of macrophage redox signaling and inflammasome activation (Steen et al., 2017). Further studies could investigate the isolation of Fabp4-expressing BMDMs as a therapeutic target for

cardiovascular diseases, especially myocardial infarction. Other clusters included a subpopulation of cells expressing high levels of E2F-regulated genes or *Cd74*. This could point to the fact that there are a portion of cells regulated by E2F and in the G1 and S portion of the cell cycle. The cells expressing high levels of *Cd74* tells us that this subpopulation of macrophages is responsible for cell survival, especially because Cd74 has been associated with tumor progression and lymphocytic survival (Gil-Yarom et al., 2017). Further studies could investigate the isolation of these cells and performing viability assays on these types of macrophages. Overall, there were seven distinct subpopulations of macrophages. However, only four subpopulations had robust results showing differentially expressed genes in the BMDMs **(Figure 1C)**. On the other hand, there were ten distinct gene clusters in the *in vivo* bone marrow. This was expected due to the heterogeneity of all cells in the bone marrow. To further analyze the differences between in vivo and in vitro bone marrow, we performed a supervised cluster of the populations of macrophages.

Visually, the differences in gene expression and cluster of cell types vary greatly between BMDMs and the *in vivo* bone marrow. As seen on the UMAP of the integrated sample, only one cluster within the in vivo sample showed appreciable overlap, marked by genes *Birc5, Cdca3, H2afz* and *Stmn1* **(Figures 2A, 2B)**. *H2afz* is a histone variant responsible for regulation of heterochromatin and DNA modification by H2A occurs in the cell nucleus (Giaimo et al., 2019). Naturally, we would expect this gene to be expressed ubiquitously considering this gene can be found in the nucleus of all macrophages. Previously, researchers found that down-regulation of *Stmn1* is required for the classical activation of macrophages (Xu et al., 2015). This leads us to believe that these macrophages are not yet activated, and this is expected for both samples that have not induced an immune response. Further studies could measure the level of *Stmn1* between

non-MI and post-MI samples to determine the validity of *Stmn1* as a biomarker for an immune response. Both *Cdca3* and *Birc5* are highly regulated by the cell cycle during the G2-M phase (Phan et al., 2018). This is further confirmed by being in the same cluster with proliferation-related genes such as *Top2a*, *Mki67,* and *Stmn1* **(Figure 2E)**. Interestingly, the gene *Top2a* was not significantly present in the *in vivo* bone marrow. This confirms our previous hypothesis that proliferative macrophages marked by genes such as *Top2a* are unique in BMDMs. In addition, *Lcn2*, a gene primarily found in the in vivo sample, was previously found to promote M1 polarization, suggesting that most BMDMs are mostly M2 polarized (Cheng et al., 2015).

Unexpectedly, we found that *Retnlg*, a gene highly expressed in neutrophils, was only expressed significantly in the *in vivo* sample (Paris et al., 2016). This could be caused by incidental clustering of neutrophils into the macrophage cluster we sub-clustered. The absence of *Retnlg* in the BMDMs further confirms that the differentiation into macrophages was successful and there is no contamination in the sample and little to no presence of neutrophils **(Figure 2C)**. Previously, researchers have studied three proliferation-related genes, *Mki67*, *Top2a* and *E2F1* (Malhotra et al., 2011). Our results show that cells expressing *Top2a* and *Mki67* are typically in the G2-M cell cycle phase **(Figure 2D)**. This confirms previous findings that *Top2a* is majorly expressed during the G2-M phase (Chen et al., 2015). In addition, this confirms that *Mki67* is implicated during the G2-M phase as well (Sun et al., 2018). The presence of most cells expressing *mcm6* in the S phase was surprising considering that this gene is most active in the G1 and S phase. In fact, previous research findings indicate that high mcm6 expression indicated early G1 phase arrest (Schrader et al., 2005). Other findings showed that mcm6 is mediated by the transcription factor E2F and studies have shown that mRNA levels peaked at the G1/S phase (Ohtani et al., 1999). Our results support that *mcm6* expressing cells are more likely in the S

phase than the G1 phase. This indicates that there may be an abundance of the transcription factor, E2F, causing cells to transition from G1 to S. Further studies could measure levels of *E2F1* using quantitative PCR. Taken together, BMDMs, a commonly used *in vitro* cell, is not a good model for *in vivo* bone marrow macrophages.

Nevertheless, our group succeeded in using BMDMs to study the interactions between the Nrf2 pathway and IRF3 pathway. In our experiment, we added cdGMP to BMDMs to activate ISGs and itaconate to activate NSGs. Activating ISGs with cdGMP is expected because stimulator of interferon genes (STING) is known to directly bind cdGMP and subsequently activate an interferon response (Yin et al., 2012). Itaconate was also expected to increase NSG expression, considering this immunometabolite was previously found to alkylate KEAP1 and activate the Nrf2 pathway (Mills et al., 2018). Our results also confirmed previous findings that itaconate was found to negatively regulate IFN-B. Despite IFN-B present, itaconate strongly reduced expression of *Ifnb1* and *Cxcl10*, while the reductions in *Irg1* and *Isg20* were present but less impressive **(Figure 3C)**. Previous findings have suggested that Nrf2 negatively regulates STING directly by decreasing STING mRNA stability (Olagnier et al., 2018). Our findings lead us to believe that itaconate's inhibition of the IRF3 pathway may be positioned downstream of STING. Furthermore, our findings show that BMDMs stimulated with both itaconate and IFN-B can rescue cells that were determined to become ISGs. Further studies could focus on studying the interactions of itaconate directly with STING.

Neutrophils, a class of immune cell, recruit macrophages to clear necrotic cells from the infarcted heart. In addition to macrophages, we decided to study neutrophils as well considering its integral role in inflammation and great presence in the bone marrow. This study identifies gene expression differences between cancer promoting SiglecF HI neutrophils and interferon

promoting SiglecF LO neutrophils when co-cultured with macrophages. These results will elucidate to which inflammatory pathway neutrophils recruit macrophages post-MI. Our single-cell results confirm that Retnlg-expressing neutrophils or macrophages are mostly present within the in vivo bone marrow. This leads us to believe that there are little to no presence of Retnlg or SiglecF LO neutrophils present within BMDMs to begin with **(Figure 4A)**. SiglecF HI neutrophils are also Retnlg LO, suggesting that SiglecF HI neutrophils are distinct from other subsets of neutrophils. Overall, there are no significant differences in ISG expression and IL6 related gene expression between SiglecF HI and SiglecF LO (**Figure 4D and 4E**). Previous single-cell gene expression analysis suggested that SiglecF LO neutrophils participate in the interferon pathway while SiglecF HI do not participate (D. Calcagno, personal communication). Our results confirm that SigF HI neutrophils do not stimulate ISG expression: Cxc10, Isg20, Irf7 (**Figure 4D**). However, these results contradict previous findings that SigF LO neutrophils may drive the IFN response. A possible explanation may be that the SigF LO neutrophil viability was low or that there was not clear cell-to-cell communication between neutrophils and macrophages. Both SiglecF HI and LO neutrophils participate in the IL6 pathway based on increased IL6 expression (**Figure 4E**). LPS is known to stimulate the IL-6 pathway and recently has been associated with pathways including the inflammatory JAK-STAT pathway (Beurel et al., 2009). SigF LO neutrophils should not stimulate IL6 expression, but LPS may have a dose dependent response, and overstimulating with LPS could increase IL6 expression.

We hypothesize that both SiglecF HI and SiglecF LO neutrophils drive inflammation in the infarcted heart through the IL6 pathway based on our LPS stimulated results (**Figure 4E**). We are limited to the low sample size of the study so increasing our sample size may prove a stronger significance. To further test this hypothesis and the relationship to MI, we will perform

SiglecF antibody treatments between control and post-MI mice to examine any therapeutic benefits of blocking both SiglecF HI and SiglecF LO expression. To address the issue pertaining to neutrophil and macrophage communication, we will test the cell-to-cell communication using contactless co-culture systems to study whether this interaction is either contact-dependent or paracrine mediated. We are also limited to treatments in vitro so further in vivo studies will portray a more accurate measure of SiglecF treatment in the heart. Due to the vascular inflammation that occurs post-MI with mice, we suggest the IL6 pathway is also associated with the inflammatory NF-KB pathway. Previously, NF-KB has been shown to control monocyte activation via the IL-6 pathway (Brasier et al., 2010). It would be interesting to investigate SiglecF neutrophils and their effect on the NF-KB pathway.

This study highlights the importance of investigating specialized neutrophils, SiglecF HI and SiglecF LO neutrophils in their role in improving or exacerbating the inflammatory response of the infarcted heart. Specifically, we should consider the potential therapeutic benefit of attenuating SiglecF HI neutrophil recruitment in the heart to alleviate inflammation post-MI. This can be achieved by potential SiglecF antibody treatments to reduce overall eosinophil counts during inflammation of the heart.

FIGURES

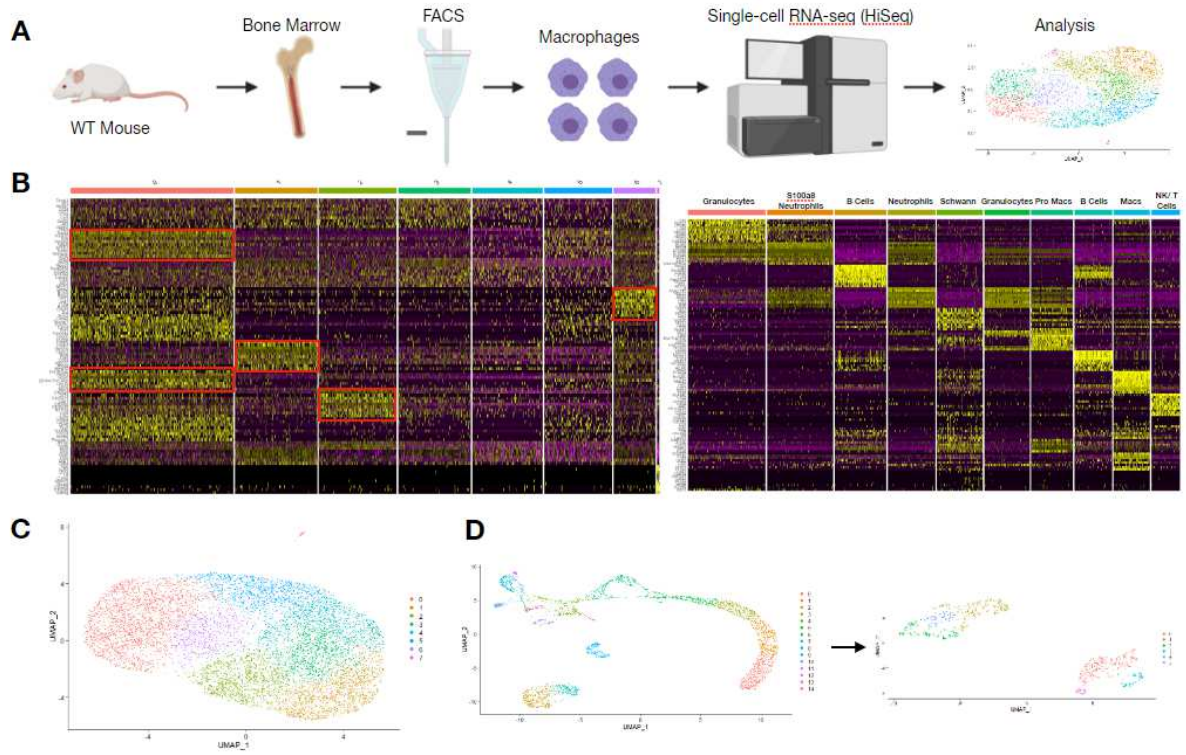

Figure 1: Single-cell transcriptomics of *in vitro* bone marrow derived macrophages (BMDMs) and *in vivo* bone marrow. **A.** Schematic showing standard workflow for single-cell processing of BMDM cells. **B.** Heatmap showing differentially expressed genes and clusters in *in vitro* BMDMs (left) and *in vivo* bone marrow (right). **C.** Uniform Manifold Approximation and Projection (UMAP) of specialized macrophage clusters in BMDMs **D**. Clustering strategy and UMAP of sub-clustered macrophages in *in vivo* bone marrow.

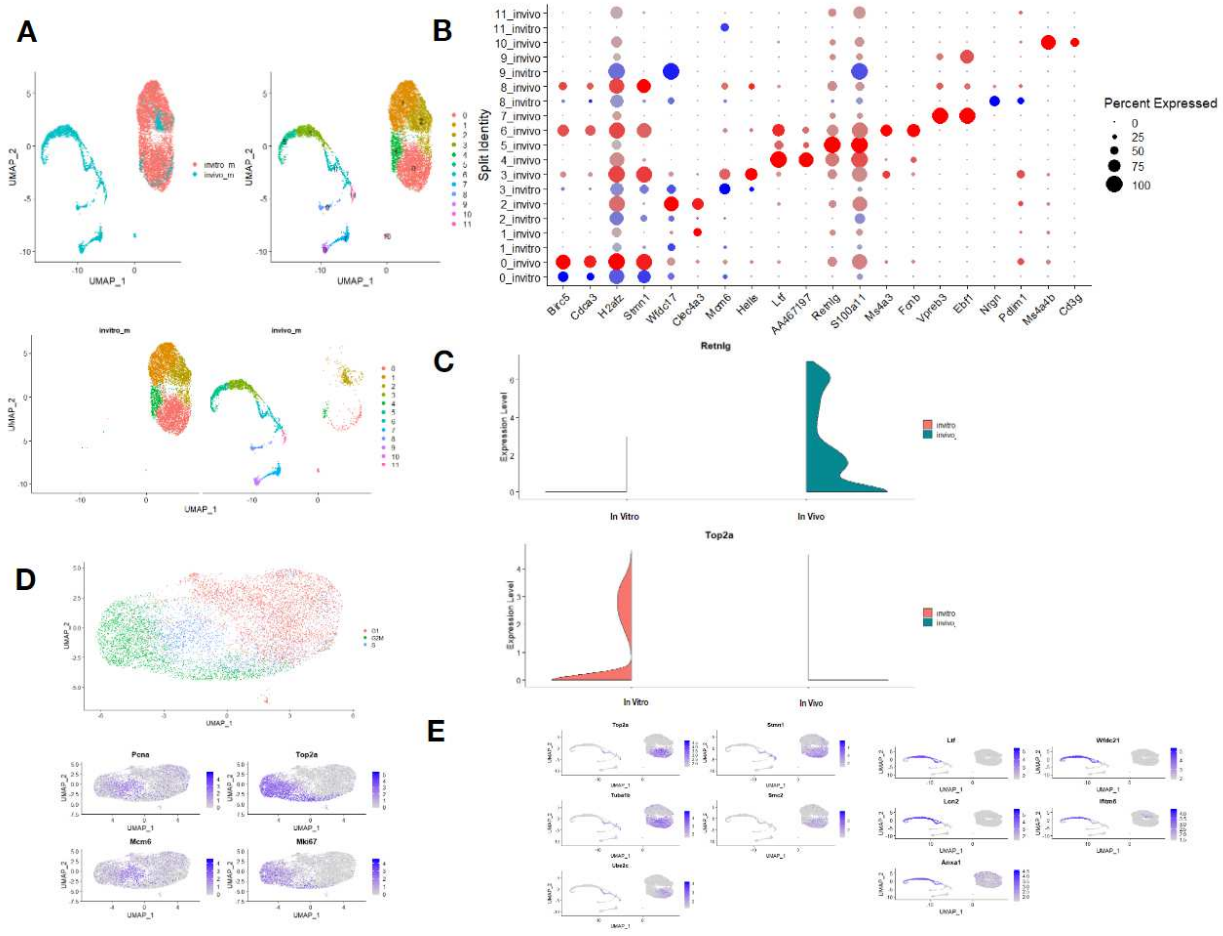

Figure 2: Integrated Analysis of *in vitro* BMDMs and *in vivo* bone marrow. A. Integrated UMAP of BMDMs and *in vivo* bone marrow. **B.** Dot-plot of conserved genes across both *in vitro* and *in vivo* clusters. **C.** Violin plot of *Retnlg*, a canonical neutrophil marker, and *Top2a*, a proliferative macrophage marker. **D.** Dimensional reduction plot showing cell cycle phases of clustered macrophages in BMDMs (top) and Feature plot of *Top2a*, *Mki67*, *Mcm6* and *Pcna* (bottom). **E.** Feature plots of differentially expressed clusters of genes and cell types between *in vitro* and *in vivo* bone marrow.

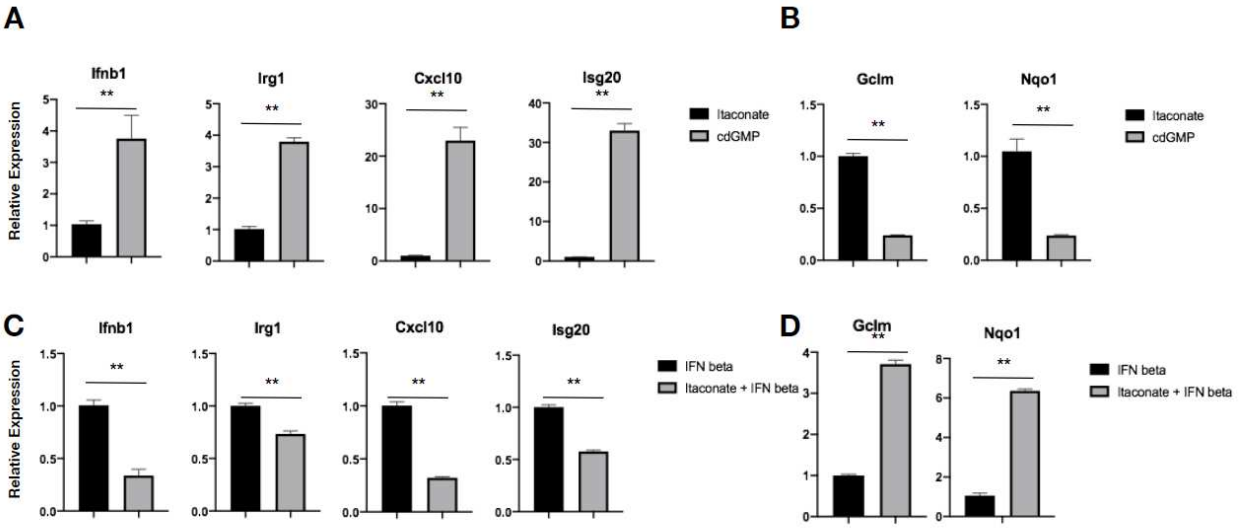

Figure 3: Exogenous itaconate increases Nrf2-stimulated gene (NSG) expression and attenuates interferon-stimulated gene (ISG) expression. A. Gene expression of ISGs (*Ifnb1, Irg1, Cxcl10, Isg20*) in BMDM cells stimulated with Itaconate (black) or cdGMP (grey). B. Gene expression of NSGs (*Gclm, Nqo1*) in BMDM cells stimulated with Itaconate or cyclic di-GMP (cdGMP). C. Gene expression of ISGs in BMDM cells stimulated with IFN-β or Itaconate + IFN-β. D. Gene expression of NSGs in BMDM cells stimulated with IFN-β or Itaconate + IFN-β. Data are shown as mean ± s.e.m. (n = 6), p-values calculated using two-tailed Mann Whitney test. * P<.05, ** P<.01, *** P<.001

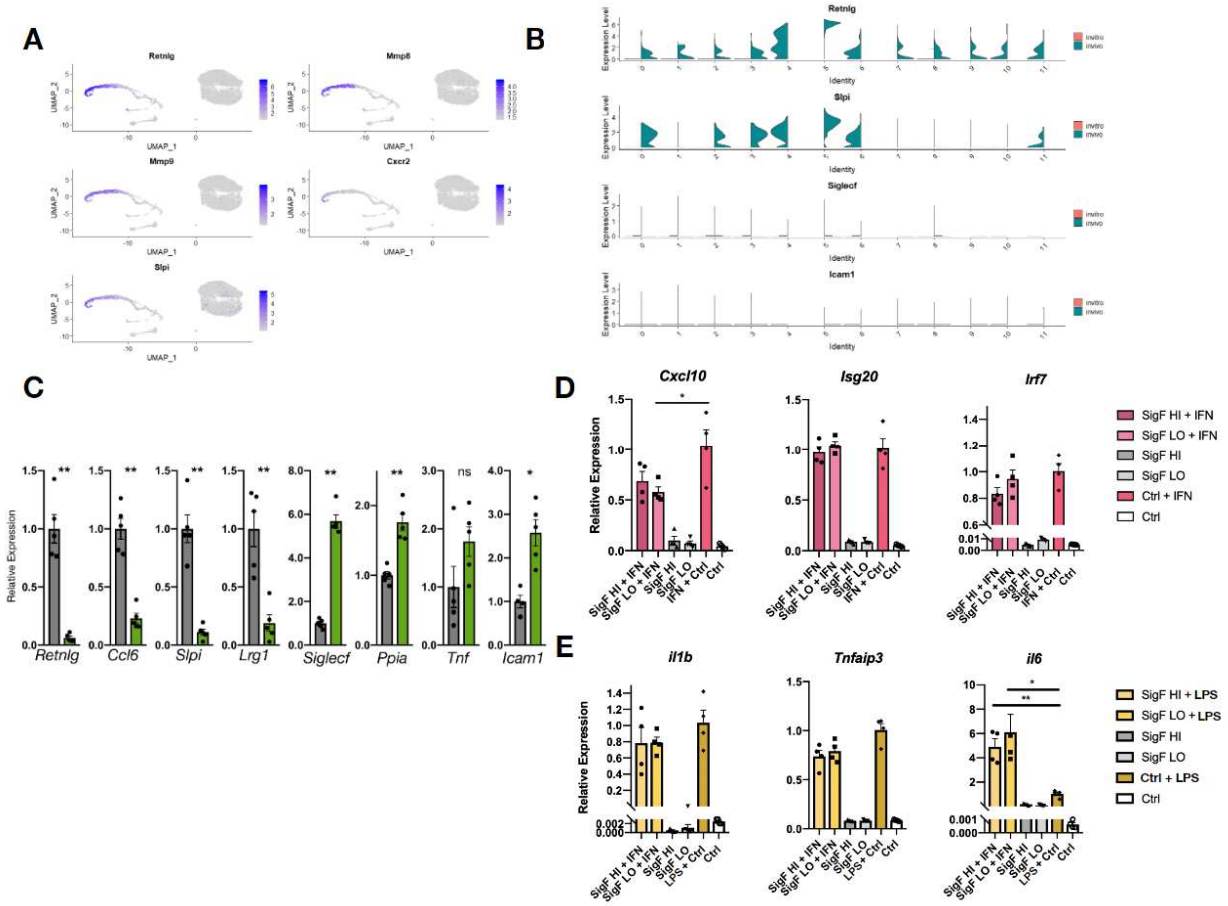

Figure 4: Co-cultured SiglecF LO neutrophils with BMDMs attenuate Cxcl10 expression after interferon stimulation and co-cultured SiglecF HI and LO neutrophils increase IL6 expression after LPS stimulation A. Feature plot of Retnlg and associated genes shown in a cluster within the *in vivo* bone marrow (*Retnlg, Mmp8, Mmp9, Cxcr2, Slpi*). B. Violin plots of *Retnlg, Slpi, SiglecF* and *Icam1*. C. Relative gene expression of SiglecF LO (grey) and SiglecF HI (green) granulocytes (n=5) D. Relative gene expression of interferon beta (IFN- β) stimulated BMDMs co-cultured with cancer promoting SiglecF HI and SiglecF LO neutrophils from post-myocardial infarction (post-MI) mice. E. Relative gene expression of Lipopolysachharide (LPS) stimulated BMDMs co-cultured with SiglecF HI and SiglecF LO neutrophils. Ctrl is only BMDMs. SiglecF HI and LO neutrophils were sorted from post-MI mice using flow cytometry and cultured with mature BMDMs. SigF HI neutrophils have high expression of a cancer promoting marker and potential inflammatory properties. Cxcl10, Isg20 and Irf7 are interferon-stimulated genes (ISGs) and IL6, Tnfaip3, and IL1b are LPS-stimulated genes. (n =4), individual points are individual co-culture wells, bars are shown as mean ± s.e.m of 4 co-cultures. $p < 0.05$ *, $p < 0.01$ **, Student *t* test).

Material from this thesis appears in “Calcagno, D. M., Ng, R. P., Jr, Toomu, A., Zhang, C., Huang, K., Aguirre, A. D., Weissleder, R., Daniels, L. B., Fu, Z., & King, K. R. (2020). The myeloid type I interferon response to myocardial infarction begins in bone marrow and is regulated by Nrf2-activated macrophages. Science immunology, 5(51), eaaz1974.” and “Calcagno, D. M., Zhang, C., Toomu, A., Huang, K., Ninh, V. K., Miyamoto, S., Aguirre, A. D., Fu, Z., Heller Brown, J., & King, K. R. (2021). SiglecF(HI) Marks Late-Stage Neutrophils of the Infarcted Heart: A Single-Cell Transcriptomic Analysis of Neutrophil Diversification. Journal of the American Heart Association, 10(4), e019019.” The thesis author was the sole author of the subsets of these publications that appear in the thesis.

REFERENCES

Aday, A. W., & Ridker, P. M. (2018). Antiinflammatory Therapy in Clinical Care: The CANTOS Trial and Beyond. *Front Cardiovasc Med, 5*, 62. doi:10.3389/fcvm.2018.00062

Barron, H. V., Cannon, C. P., Murphy, S. A., Braunwald, E., & Gibson, C. M. (2000). Association between white blood cell count, epicardial blood flow, myocardial perfusion, and clinical outcomes in the setting of acute myocardial infarction: a thrombolysis in myocardial infarction 10 substudy. *Circulation, 102*(19), 2329-2334. doi:10.1161/01.cir.102.19.2329

Beurel, E., & Jope, R. S. (2009). Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *Journal of neuroinflammation*, 6, 9. https://doi.org/10.1186/1742-2094-6-9

Brasier A. R. (2010). The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovascular research*, 86(2), 211–218. https://doi.org/10.1093/cvr/cvq076

Chen, T., Sun, Y., Ji, P., Kopetz, S., & Zhang, W. (2015). Topoisomerase IIα in chromosome instability and personalized cancer therapy. *Oncogene*, 34(31), 4019–4031. https://doi.org/10.1038/onc.2014.332

Cheng, L., Xing, H., Mao, X., Li, L., Li, X., & Li, Q. (2015). Lipocalin-2 promotes m1 macrophages polarization in a mouse cardiac ischaemia-reperfusion injury model. *Scandinavian journal of immunology*, 81(1), 31–38. https://doi.org/10.1111/sji.12245

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature*, *420*(6917), 860–867. https://doi.org/10.1038/nature01322

de Haan, J. J., Smeets, M. B., Pasterkamp, G., & Arslan, F. (2013). Danger signals in the initiation of the inflammatory response after myocardial infarction. *Mediators Inflamm, 2013*, 206039. doi:10.1155/2013/206039

Delile, J., Rayon, T., Melchionda, M., Edwards, A., Briscoe, J., & Sagner, A. (2019). Single cell transcriptomics reveals spatial and temporal dynamics of gene expression in the developing mouse spinal cord. *Development, 146*(12). doi:10.1242/dev.173807

Dutta, P, Sager, H. B., Stengel, K. R., Naxerova, K., Courties, G, Saez, B, Silberstein, L., Heidt, T., Sebas, M., Sun, Y., Wojtkiewicz, G., Feruglio, P. F., King, K., Baker, J. N., van der Laan, A. M., Borodovsky, A., Fitzgerald, K., Hulsmans, M., Hoyer, F., Iwamoto, Y., Vinegoni, C., Brown, D., Di Carli, M., Libby, P., Hiebert, S. W., Scadden, D. T., Swirski, F. K., Weissleder, R., Nahrendorf, M. (2015). Myocardial Infarction Activates CCR2(+) Hematopoietic Stem and Progenitor Cells. *Cell Stem Cell, 16*(5), 477-487. doi:10.1016/j.stem.2015.04.008

Engblom, C., Pfirschke, C., Zilionis, R., Da Silva Martins, J., Bos, S. A., Courties, G., Rickelt, S., Severe, N., Baryawno, N., Faget, J., Savova, V., Zemmour, D., Kline, J., Siwicki, M., Garris, C., Pucci, F., Liao, H. W., Lin, Y.J. , Newton, A., Yaghi, O. K., Iwamoto, Y., Tricot, B., Wojtkiewicz, G. R., Nahrendorf, M., Cortez-Retamozo, V., Meylan, E., Hynes, R. O., Demay, M., Klein, A., Bredella, M. A., Scadden, D. T., Weissleder, R., Pittet, M. J. (2017). Osteoblasts remotely supply lung tumors with cancer-promoting SiglecF(high) neutrophils. *Science, 358*(6367). doi:10.1126/science.aal5081

Ford, E. S., & Capewell, S. (2007). Coronary heart disease mortality among young adults in the U.S. from 1980 through 2002: concealed leveling of mortality rates. *J Am Coll Cardiol, 50*(22), 2128-2132. doi:10.1016/j.jacc.2007.05.056

Frangogiannis, N. G. (2014). The immune system and the remodeling infarcted heart: cell biological insights and therapeutic opportunities. *J Cardiovasc Pharmacol, 63*(3), 185-195. doi:10.1097/FJC.0000000000000003

Frangogiannis, N. G. (2015). Pathophysiology of Myocardial Infarction. *Compr Physiol, 5*(4), 1841-1875. doi:10.1002/cphy.c150006

Frantz, S., Hofmann, U., Fraccarollo, D., Schäfer, A., Kranepuhl, S., Hagedorn, I., Nieswandt, B., Nahrendorf, M., Wagner, H., Bayer, B., Pachel, C., Schön, M. P., Kneitz, S., Bobinger, T., Weidemann, F., Ertl, G., & Bauersachs, J. (2013). Monocytes/macrophages prevent healing defects and left ventricular thrombus formation after myocardial infarction. *FASEB J, 27*(3), 871-881. doi:10.1096/fj.12-214049

Furuhashi M. (2019). Fatty Acid-Binding Protein 4 in Cardiovascular and Metabolic Diseases. *Journal of atherosclerosis and thrombosis*, 26(3), 216–232. https://doi.org/10.5551/jat.48710

Giaimo, B. D., Ferrante, F., Herchenröther, A., Hake, S. B, Borggrefe, T. (2019) The histone variant H2A.Z in gene regulation. *Epigenetics & Chromatin* 12, 37. https://doi.org/10.1186/s13072-019-0274-9

Gil-Yarom, N., Radomir, L., Sever, L., Kramer, M. P., Lewinsky, H., Bornstein, C., Blecher-Gonen, R., Barnett-Itzhaki, Z., Mirkin, V., Friedlander, G., Shvidel, L., Herishanu, Y., Lolis, E. J., Becker-Herman, S., Amit, I., & Shachar, I. (2017). CD74 is a novel transcription regulator. Proceedings of the National Academy of Sciences of the United States of America, 114(3), 562–567. https://doi.org/10.1073/pnas.1612195114

Hwang, B., Lee, J. H., & Bang, D. (2018). Single-cell RNA sequencing technologies and bioinformatics pipelines. *Exp Mol Med, 50*(8), 96. doi:10.1038/s12276-018-0071-8

King, K. R., Aguirre, A. D., Ye, Y. X., Sun, Y., Roh, J. D., Ng, R. P., Jr, Kohler, R. H., Arlauckas, S. P., Iwamoto, Y., Savol, A., Sadreyev, R. I., Kelly, M., Fitzgibbons, T. P., Fitzgerald, K. A., Mitchison, T., Libby, P., Nahrendorf, M., & Weissleder, R. (2017). IRF3 and type I interferons fuel a fatal response to myocardial infarction. *Nat Med, 23*(12), 1481-1487. doi:10.1038/nm.4428

Liang, X., Gupta, K., Quintero, J. R., Cernadas, M., Kobzik, L., Christou, H., Pier, G. B., Owen, C. A., & Çataltepe, S. (2019). Macrophage FABP4 is required for neutrophil recruitment and bacterial clearance in Pseudomonas aeruginosa pneumonia. *FASEB* journal : official publication of the Federation of American Societies for Experimental Biology, 33(3), 3562–3574. https://doi.org/10.1096/fj.201802002R

Liu, K., Zhao, E., Ilyas, G., Lalazar, G., Lin, Y., Haseeb, M., Tanaka, K. E., & Czaja, M. J. (2015). Impaired macrophage autophagy increases the immune response in obese mice by promoting proinflammatory macrophage polarization. *Autophagy, 11*(2), 271-284. doi:10.1080/15548627.2015.1009787

Lu, L., Liu, M., Sun, R., Zheng, Y., & Zhang, P. (2015). Myocardial Infarction: Symptoms and Treatments. *Cell Biochem Biophys, 72*(3), 865-867. doi:10.1007/s12013-015-0553-4

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol, 53*, 401-426. doi:10.1146/annurev-pharmtox-011112-140320

Malhotra, S., Lapointe, J., Salari, K., Higgins, J. P., Ferrari, M., Montgomery, K., van de Rijn, M., Brooks, J. D., & Pollack, J. R. (2011). A tri-marker proliferation index predicts biochemical recurrence after surgery for prostate cancer. *PloS one*, 6(5), e20293. https://doi.org/10.1371/journal.pone.0020293

Mann, D. L. (2011). The emerging role of innate immunity in the heart and vascular system: for whom the cell tolls. *Circ Res, 108*(9), 1133-1145. doi:10.1161/CIRCRESAHA.110.226936

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep, 6*, 13. doi:10.12703/P6-13

Mills, E. L., Ryan, D. G., Prag, H. A., Dikovskaya, D., Menon, D., Zaslona, Z., Jedrychowski, M. P., Costa, A. S. H., Higgins, M., Hams, E., Szpyt, J., Runtsch, M. C., King, M. S., McGouran, J. F., Fischer, R., Kessler, B. M., McGettrick, A. F., Hughes, M. M., Carroll, R. G., Booty, L. M., Knatko, E. V., Meakin, P. J., Ashford, M. L. J., Modis, L. K., Brunori, G., Sévin, D. C., Fallon, P. G., Caldwell, S. T., Kunji, E. R. S., Chouchani, E. T., Frezza, C., Dinkova-Kostova, A. T., Hartley, R. C., Murphy, M. P., O'Neill, L. A. (2018). Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature, 556*(7699), 113-117. doi:10.1038/nature25986

Mogensen T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical microbiology reviews*, *22*(2), 240–273. https://doi.org/10.1128/CMR.00046-08

Murray, C. J., & Lopez, A. D. (1997). Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet, 349*(9063), 1436-1442. doi:10.1016/S0140-6736(96)07495-8

Nagaev, I., Bokarewa, M., Tarkowski, A., & Smith, U. (2006). Human resistin is a systemic immune-derived proinflammatory cytokine targeting both leukocytes and adipocytes. *PloS one*, 1(1), e31. https://doi.org/10.1371/journal.pone.0000031

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J. L., Libby, P., Weissleder, R., & Pittet, M. J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med, 204*(12), 3037-3047. doi:10.1084/jem.20070885

Naidoo, J., Page, D. B., & Wolchok, J. D. (2014). Immune modulation for cancer therapy. *Br J Cancer, 111*(12), 2214-2219. doi:10.1038/bjc.2014.348

Ohtani, K., Iwanaga, R., Nakamura, M., Ikeda, M., Yabuta, N., Tsuruga, H., & Nojima, H. (1999). Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. *Oncogene*, 18(14), 2299–2309. https://doi.org/10.1038/sj.onc.1202544

Olagnier, D., Brandtoft, A. M., Gunderstofte, C., Villadsen, N. L., Krapp, C., Thielke, A. L., Laustsen, A., Peri, S., Hansen, A. L., Bonefeld, L., Thyrsted, J., Bruun, V., Iversen, M. B., Lin, L.,

Artegoitia, V. M., Su, C., Yang, L., Lin, R., Balachandran, S., Luo, Y., Nyegaard, M., Marrero, B., Goldbach-Mansky, R., Motwani, M., Ryan, D. G., Fitzgerald, K. A., O'Neill, L. A., Hollensen, A. K., Damgaard, C. K., de Paoli, F. V., Bertram, H. C., Jakobsen, M. R., Poulsen, T. B., Holm, C. K. (2018). Nrf2 negatively regulates STING indicating a link between antiviral sensing and metabolic reprogramming. *Nature communications*, 9(1), 3506. https://doi.org/10.1038/s41467-018-05861-7

O'Neill, L. A. J., & Artyomov, M. N. (2019). Itaconate: the poster child of metabolic reprogramming in macrophage function. *Nat Rev Immunol, 19*(5), 273-281. doi:10.1038/s41577-019-0128-5

Paris, A. J., Liu, Y., Mei, J., Dai, N., Guo, L., Spruce, L. A., Hudock, K. M., Brenner, J. S., Zacharias, W. J., Mei, H. D., Slamowitz, A. R., Bhamidipati, K., Beers, M. F., Seeholzer, S. H., Morrisey, E. E., & Worthen, G. S. (2016). Neutrophils promote alveolar epithelial regeneration by enhancing type II pneumocyte proliferation in a model of acid-induced acute lung injury. *American journal of physiology. Lung cellular and molecular physiology*, 311(6), L1062–L1075. https://doi.org/10.1152/ajplung.00327.2016

Phan, N. N., Wang, C. Y., Li, K. L., Chen, C. F., Chiao, C. C., Yu, H. G., Huang, P. L., & Lin, Y. C. (2018). Distinct expression of CDCA3, CDCA5, and CDCA8 leads to shorter relapse free survival in breast cancer patient. *Oncotarget*, 9(6), 6977–6992. https://doi.org/10.18632/oncotarget.24059

Pockley, A. G., Foulds, G. A., Oughton, J. A., Kerkvliet, N. I., & Multhoff, G. (2015). Immune Cell Phenotyping Using Flow Cytometry. *Curr Protoc Toxicol, 66*, 18 18 11-34. doi:10.1002/0471140856.tx1808s66

Prame Kumar, K., Nicholls, A. J., & Wong, C. H. Y. (2018). Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell Tissue Res, 371*(3), 551-565. doi:10.1007/s00441-017-2753-2

Rowland, S. L., Riggs, J. M., Gilfillan, S., Bugatti, M., Vermi, W., Kolbeck, R., Unanue, E. R., Sanjuan, M. A., & Colonna, M. (2014). Early, transient depletion of plasmacytoid dendritic cells ameliorates autoimmunity in a lupus model. *J Exp Med, 211*(10), 1977-1991. doi:10.1084/jem.20132620

Russo, L., & Lumeng, C. N. (2018). Properties and functions of adipose tissue macrophages in obesity. *Immunology, 155*(4), 407-417. doi:10.1111/imm.13002

Schrader, C., Janssen, D., Klapper, W., Siebmann, J. U., Meusers, P., Brittinger, G., Kneba, M., Tiemann, M., & Parwaresch, R. (2005). Minichromosome maintenance protein 6, a proliferation marker superior to Ki-67 and independent predictor of survival in patients with mantle cell lymphoma. *British journal of cancer*, 93(8), 939–945. https://doi.org/10.1038/sj.bjc.6602795

Sheikh, F., Dickensheets, H., Gamero, A. M., Vogel, S. N., & Donnelly, R. P. (2014). An essential role for IFN-β in the induction of IFN-stimulated gene expression by LPS in macrophages. *Journal of leukocyte biology*, 96(4), 591–600. https://doi.org/10.1189/jlb.2A0414-191R

Skelly, D. A., Squiers, G. T., McLellan, M. A., Bolisetty, M. T., Robson, P., Rosenthal, N. A., & Pinto, A. R. (2018). Single-Cell Transcriptional Profiling Reveals Cellular Diversity and Intercommunication in the Mouse Heart. *Cell Rep, 22*(3), 600-610. doi:10.1016/j.celrep.2017.12.072

Steen, K. A., Xu, H., & Bernlohr, D. A. (2017). FABP4/aP2 Regulates Macrophage Redox Signaling and Inflammasome Activation via Control of UCP2. *Molecular and cellular biology*, 37(2), e00282-16. https://doi.org/10.1128/MCB.00282-16

Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., 3rd, Hao, Y., Stoeckius, M., Smibert, P., & Satija, R. (2019). Comprehensive Integration of Single-Cell Data. *Cell*, 177(7), 1888–1902.e21. https://doi.org/10.1016/j.cell.2019.05.031

Sun, X., & Kaufman, P. D. (2018). Ki-67: more than a proliferation marker. *Chromosoma*, 127(2), 175–186. https://doi.org/10.1007/s00412-018-0659-8

Suthahar, N., Meijers, W. C., Sillje, H. H. W., & de Boer, R. A. (2017). From Inflammation to Fibrosis-Molecular and Cellular Mechanisms of Myocardial Tissue Remodelling and Perspectives on Differential Treatment Opportunities. *Curr Heart Fail Rep, 14*(4), 235-250. doi:10.1007/s11897-017-0343-y

Swirski, F. K., & Nahrendorf, M. (2018). Cardioimmunology: the immune system in cardiac homeostasis and disease. *Nat Rev Immunol, 18*(12), 733-744. doi:10.1038/s41577-018-0065-8

Tang, D., Kang, R., Coyne, C. B., Zeh, H. J., & Lotze, M. T. (2012). PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunological reviews*, *249*(1), 158–175. https://doi.org/10.1111/j.1600-065X.2012.01146.x

Timmers, L., Pasterkamp, G., de Hoog, V. C., Arslan, F., Appelman, Y., & de Kleijn, D. P. (2012). The innate immune response in reperfused myocardium. *Cardiovasc Res, 94*(2), 276-283. doi:10.1093/cvr/cvs018

Xu, K., & Harrison, R. E. (2015). Down-regulation of Stathmin Is Required for the Phenotypic Changes and Classical Activation of Macrophages. *The Journal of biological chemistry*, 290(31), 19245–19260. https://doi.org/10.1074/jbc.M115.639625

Yap, J., Cabrera-Fuentes, H. A., Irei, J., Hausenloy, D. J., & Boisvert, W. A. (2019). Role of Macrophages in Cardioprotection. *International journal of molecular sciences*, 20(10), 2474. https://doi.org/10.3390/ijms20102474

Yin, Q., Tian, Y., Kabaleeswaran, V., Jiang, X., Tu, D., Eck, M. J., Chen, Z. J., & Wu, H. (2012). Cyclic di-GMP sensing via the innate immune signaling protein STING. *Molecular cell*, 46(6), 735–745. https://doi.org/10.1016/j.molcel.2012.05.029

Zimmerer, J. M., Lesinski, G. B., Ruppert, A. S., Radmacher, M. D., Noble, C., Kendra, K., Walker, M. J., & Carson, W. E., 3rd (2008). Gene expression profiling reveals similarities between the in vitro and in vivo responses of immune effector cells to IFN-alpha. *Clin Cancer Res, 14*(18), 5900-5906. doi:10.1158/1078-0432.CCR-08-0846