THE PROTECTIVE ROLE OF PERICARDIAL MACROPHAGES
AND THEIR EFFEROCYTOSIS POTENTIAL DURING
EXPERIMENTAL CVB3 MYOCARDITIS

by
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Abstract

Myocarditis is an inflammatory disease of the heart responsible for the development of acute and chronic heart failure. While the clinical manifestations of myocarditis vary, severe disease can cause arrhythmias, progression to dilated cardiomyopathy and sudden death. There are approximately 1.5 million cases of myocarditis annually, with an incidence of 22 out of 100,000 patients worldwide. Although different etiologies have been identified, in the United States, viral infections are a main cause of myocarditis development, mostly affecting children and young adults. The lack of biological therapies for the disease makes imperative the development of new strategies, which require deeper mechanistic understanding of the leukocyte subsets involved in the disease's immunopathogenesis.

This thesis attempts to advance the understanding of recently identified murine GATA6+ macrophages in the pericardial cavity and the role they play in limiting cardiac inflammation through myocardial migration and efferocytosis during CVB3 myocarditis. The first contribution of this thesis is the description of the gene expression and physical characterization of GAT6+ macrophages. In the analysis, four main efferocytosis receptors were identified to be expressed among GATA6+ pericardial macrophages: Tyro3, Axl, MERTK, and TIM4. Second, we assessed the functional role of phagocytosis by GATA6+ pericardial macrophages in vitro. Compared to GATA6+ peritoneal macrophages and bone marrow-derived macrophages, GATA6+ pericardial macrophages appear to have an equal or slightly higher ability to phagocytose opsonized cargo.
Third, we investigated the role of GATA6+ pericardial macrophages in clearing damaged cells by efferocytosis during experimental CVB3-induced murine myocarditis. Altogether, our data suggest that GATA6+ pericardial macrophages are a unique tissue-resident population with efferocytosis potential that can regulate inflammation and possibly decrease fibrosis development during CVB3 induced myocarditis.

Although the reparatory properties of GATA6+ peritoneal and myocardial macrophages have been previously described, there are multiple questions that remain unanswered for GATA6+ pericardial macrophage. This thesis attempts to describe the efferocytosis properties of GATA6+ pericardial macrophages, something new in the cardiac immunology field. Finally, it is proposed that GATA6+ reparative macrophages could be manipulated as a potential cellular therapy to induce enhanced efferocytosis and prevent the development of cardiac fibrosis.

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Sin sacrificio no hay victoria.
Dedication

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I. Introduction

Myocarditis

Definition and epidemiology
Myocarditis is an inflammatory disease of the heart that is responsible for the development of acute and chronic heart failure. Following acute inflammation, myocarditis can progress to subacute and chronic stages. Severe myocarditis can result in systolic dysfunction, cardiac tissue remodeling, fibrosis, loss of contractile function, arrhythmias, progression to dilated cardiomyopathy (DCM), heart failure and sudden death (1). While the clinical manifestations of myocarditis may vary from patient to patient, the Dallas criteria provides a histopathological definition for myocarditis characterized by inflammatory cellular infiltrates and the extent of damage and/or necrosis in the absence of an ischemic event (2). The immunohistochemical criteria for abnormal immune cell infiltrate are defined as “≥14 leucocytes/mm² including up to 4 monocytes/mm² with the presence of CD3-positive T lymphocytes of ≥7 cells/mm²” (3, 4).

Whereas the overall incidence of myocarditis cases is unknown, over half of the global myocarditis cases occur in patients below the age of 40 years (5). A recent global estimate of myocarditis indicated approximately 1.5 million cases annually with an incidence of 22 out of 100,000 patients worldwide (6, 7). Myocarditis that develops to DCM and heart failure is the leading cause of worldwide mortality due to cardiovascular disease (CVD) (8, 9). In 2016, there were 840,768 deaths attributed to heart disease alone in the United States (10). In 2019,
coronary events were expected to occur in 1,055,000 individuals, including 720,000 new and
335,000 recurrent events. In addition to the morbidity and mortality caused by CVD, these
diseases cause a major economic burden with an estimated cost of $351.2 billion in 2014-2015
in the United States (10).

Etiology and diagnostics

Different etiologies have been identified in the development of myocarditis, including a large
number of infections agents, systemic diseases, drugs, toxins, and autoantigens (Figure 1). In the
United States, viral infections have been identified as a main cause of myocarditis mostly
affecting children and young adults (1, 11-13). With a heterogenous range of clinical
presentations marked by severe inflammation and damage of the heart muscle, often times
myocarditis symptoms overlap with other signs of cardiovascular diseases, making it difficult to
diagnose (1). In other cases, when symptoms are subclinical, cases tend to go undetected. The
current gold standard for the definitive diagnosis of myocarditis is an endomyocardial biopsy
(EMB) that is analyzed by immunohistochemical and molecular biological procedures (4).
Although the gold standard for proper myocarditis diagnosis, EMB has limitations that restricts
its use in the clinical settings that include low sensitivity and the potential for complications when
this invasive procedure is used (14). As a result, there are indirect diagnostic methods used in
clinical practice such as electrocardiogram, echocardiogram, and magnetic resonance imaging
(CMR).
**Figure 1.** Infectious and noninfectious etiologies of myocarditis

Figure drawn by P. Delgado.
Viral myocarditis – CBV3 model

In the United States and other developed nations, one of the most common cause of myocarditis is viral infection (1, 11-13). The historical and clinical features are often nonspecific. The main viruses associated with the development of myocarditis include coxsackievirus (CVB3), adenovirus, influenza A, influenza B, parvovirus B19, cytomegalovirus, and Epstein-Barr virus (1).

Previous studies have reported that major viral infections target cardiomyocytes and lead to cardiac dysfunction and inflammation. To understand the progression of myocarditis to heart failure and/or DCM, one of the most widely used and studied murine models is the coxsackievirus B3 (CVB3) myocarditis model. CVB2 is a ssRNA, non-enveloped enterovirus in the Picornaviridae family (1). In the coxsackievirus murine model, the virus enters cardiomyocytes using the coxsackievirus-adenovirus receptor (CAR), replicates, and causes virus-mediated lysis (15).

During the first phase, the destruction of cardiomyocytes leads to the recruitment of innate immune cells including natural killer cells (NK cells) and macrophages to the site of infection (15, 16). The initial immune response is responsible for the release of inflammatory cytokines such as TNF, IL-1, IL-6, setting the stage for the adaptive immune system. The second phase is triggered by an initial immune dysregulation and cardiomyocyte destruction. Severe inflammation results in the activation of adaptive immune responses and virus-specific and anti-self responses (15). While it is still unclear if molecular mimicry is critical in the pathogenic process of myocarditis, it has been described that antibodies generated during disease development may cross-react with adrenergic receptors (16). In severe cases where there is continuous inflammation and fibrosis,
about 1/3 of myocarditis cases patients do not recover and go on to develop fibrosis and loss of cardiac function.

**Macrophages**

*Origin and classification*

Resident macrophages are essential components of the innate immune system and are present in every tissue. Macrophages are a heterogeneous population that forms an essential first line of defense against pathogens and respond to physiological changes. Macrophage classification was first introduced by Elie Metchnikoff in 1833 who described the phagocytic clearance of microorganisms (17). Due to their phagocytic cell activity and position in tissues, macrophages were initially classified as part of the reticuloendothelial system. However, in 1968, this classification was replaced by that of the mononuclear phagocyte system (MPS) based on their morphological and functional differences (18). Although the MPS was a valuable model to distinguish mononuclear cells from polymorphonuclear leukocytes, the system proposed that all tissue-associated macrophages were derived from monocytes that are released continuously from the bone marrow (19). While the mononuclear phagocyte system was important to establish the concept of mononuclear cells, the vision of the MPS and the macrophage classification has changed over time. Over the years, the MPS model has been challenged and thought to be an oversimplification for the origin of tissue-resident macrophages.
Heterogeneity of tissue-resident macrophages

More recently, substantial evidence has accumulated that demonstrates that tissue-resident macrophages have their origin during embryonic development prior to initiation of hematopoiesis in the bone marrow (17). Initial studies utilizing specific fate-mapping technologies provided evidence that tissue-resident macrophages developed from prenatal origin (yolk sac or fetal liver) and monocytes produced by post-natal hematopoiesis (20). The first mapping studies primarily focused on brain-resident macrophages (microglia) originated from the yolk-sac (17). The origins of differentiation thus begin during organogenesis where embryonic tissue-resident macrophages derived from the yolk-sac and fetal liver precursors are seeded to the different tissues. These tissue macrophages can persist in several tissues through adulthood as self-maintaining populations with organ-specific functions (18). On the other hand, for some tissues such as the intestine and the lungs, after birth and during inflammation, bone marrow-derived monocytes are recruited to the sites of injury where they develop into bone marrow-derived tissue macrophages (BMDM).

The distinct populations of tissue-resident macrophages share several common features such as the ability to phagocytize, pathogen recognition, cytokine and chemokine production, and the expression of common surface markers such as CD11b, F4/80, CD64 (21). The phenotypic heterogeneity among tissue-resident macrophages is the result of tissue-specific and microanatomical niche-specific functions that are regulated by the expression of lineage-determining transcriptional factors (TFs) (18). According to the niche hypothesis, niche-specific signals such as extracellular matrix interactions, cytokines and growth factors, initiate TFs that
are responsible for activating signature genes during tissue-resident macrophage development (21) (Figure 2).

![Figure 2. Origins and transcription factors of tissue-resident macrophages](image)

Figure drawn by P. Delgado.

Tissue-specific functions of resident macrophages are essential to fulfill functional demands of different tissues. A key function of tissue-resident macrophages is their contribution to physiological homeostasis by responding to internal and external changes (18). During erythropoiesis, tissue resident stromal macrophages and fetal liver macrophages have been shown to play a role of providing signals that induce differentiation and proliferation of erythroid
progenitors (18, 22). During infection, resident macrophages in the gastrointestinal tract contribute to mucosal immunity by interacting with gut microbial flora to induce inflammatory responses after encountering a microbial pathogen, (23). In addition, to defend from respiratory infections in the lungs and the respiratory tract, alveolar macrophages play a role in recognizing and clearing particles, microbes, and pollutants that may have compromise respiratory functions. In the peritoneal cavity, tissue-resident macrophages contribute to the protection of the serous cavity as they migrate to site of injury and promote repair after cell death.

**Efferocytosis properties during tissue damage**

An important functional characteristic of professional phagocytes during inflammation and tissue damage, including macrophages, is the removal of dead cells (24). There are different types of cell death that initiate different immune responses and elicit the activation of different innate immune cells. Apoptosis is an evolutionary conserved, non-inflammatory cell death pathway and one of the most studied in the field as it occurs during development and aging of cells among all tissues (25, 26). Caspase-mediated apoptosis results in DNA fragmentation, membrane blebbing, and the engulfment of the cells by professional phagocytes (27). The activation of such caspases can be initiated by the intrinsic or extrinsic apoptosis pathway. Intrinsic apoptosis results from internal stimuli due to, but not limited to, mitochondria outer membrane permeabilization (MOMP), endoplasmic reticulum (ER) stress, or mitogenic stimulation (25). On the other hand, extrinsic apoptosis results from perturbations of the extracellular microenvironment that are detected by plasma membrane death receptors (FAS, TNFR1, TRAIL) and by dependence receptors (NTN1, DCC, UNCSA) (28).
Efferocytosis, the phagocytosis of dead cells, has a substantial effect on tissue homeostasis and is a vital process to prevent further inflammation (29). In order to prevent secondary necrosis and further release of toxic materials, engulfment of apoptotic cells is necessary to maintain a healthy balance (30). Therefore, the process of efferocytosis is accomplished through a series of four regulated mechanisms: the recruiting “find me” signal by dead cells, the recognition of “eat me” signals from extracellular cargos, the phagocytosis of the dead cell (typically in the form of apoptotic bodies), and lastly the digestion and degradation of engulfed cellular material (Figure 3).

Macrophage migration towards apoptotic cells begins during the early stages of apoptosis and is guided through a series of “find me” signals that include chemotactic factors secreted or released by dying cells (31). There are four main “find me” signals that have been previously identified, including the classic chemokine CX3CL1, lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), and nucleotides (ATP and UTP) (26). These “find me” signals are received by macrophages through their cognate receptors including sphingosine-1-phosphate receptor (S1PR1), purinergic receptors (P2Y2), G-protein-coupled receptor (G2A), and CX3CR1 that allow macrophages to migrate towards the location of the dying cells (26).

After arriving at the site of cell death, macrophages recognize the “eat me” signals on the extracellular apoptotic debris through specific efferocytosis receptors. Different “eat me” signals displayed on the surface of dying cells have been previously identified including lipids, proteins or carbohydrates (27). One of the best studied and evolutionary conserved eat-me signal in apoptotic cells is lipid phosphatidylserine (PtdSer). Normally expressed on the inner leaflet in living cells, PtdSer translocates to the outer leaflet during apoptosis due to lipid scrambling (26).
Figure 3. Process of efferocytosis

Accomplished by the recruiting ATP signal by dead cells, the recognition of PtdSer signal from extracellular cargos, the phagocytosis and release of anti-inflammatory IL-10 cytokine.

Figure drawn by P. Delgado.

Once PtdSer is expressed on the surface of apoptotic cells, macrophages bind directly through stablin-1, stabling-2, or the T cell immunoglobulin and mucin domain family receptors TIM-1/TIM4 (31). Alternatively, macrophages may bind indirectly through Gas6 and Protein S that act as binding molecules to the TAM family receptors (Tyro 3, Axl, and MERTK) that have intracellular tyrosine kinase domains (30). While PtdSer is also expressed in low levels among living cells, the presence of “do not eat me” signals such as CD31, CD47, and CD61, actively suppresses efferocytosis from occurring (31).

The third step of the efferocytosis process is the ingestion and internalization of the dead cell. The binding of PtdSer with the multiple receptors expressed in macrophages leads to signaling
that promotes actin cytoskeletal reorganization and the formation of F-actin around apoptotic cellular debris (27, 31). In addition, TIM-4 along with TAM receptors, fibronectin 1 and integrins, aid in signaling that facilitates the internalization of the cargo. Once the apoptotic cell is internalized, the last step is to completely digest and degrade the dead cell to amino acids, nucleic acids and sugars. Autophagy proteins are recruited to form a process called LC3-associated phagocytosis (LAP) that promotes the phagolysosome fusion (31). To prevent inflammation during the degradation of apoptotic cell materials by the phagolysosome, macrophages release a number of anti-inflammatory cytokines such as PGE2, TGFβ and interleukin-10 (26, 32). Consequently, highlighting the substantial effect of efferocytosis on tissue repair, homeostasis and the prevention of further inflammation.
Serous cavity macrophages

Biology of serous cavities

The biology of the cells that reside in serous cavities, including the pleural, peritoneal, and pericardial cavities, has been studied extensively over the years (33, 34). In the late nineteenth century, it was demonstrated that the cells lining the serous membranes are derived from the mesoderm and provide a smooth barrier function (35). Each serous membrane is continuous and covers both the outer (parietal) and inner (visceral) components of the cavity (36). There are two pleural cavities that make an enclosed space between visceral and parietal pleura. The peritoneal cavity is a large body cavity filled with serous fluid surrounded by mesothelial layers that cover the visceral tissues and the parietal wall (37). The pleural cavity is the space that lies between the pleura, the two membranes that surround the lungs. The pericardial cavity is found within the mediastinum of the thoracic cavity and is enclosed within the fibrous sac that surrounds the heart (38).

Tissue-resident peritoneal macrophages

Serous cavities, including peritoneal, pleural, and pericardial cavities, are known to harbor a diverse number of mononuclear phagocytes. Because of their ease of harvest, ability to phagocytose, and respond to invading pathogens, resident macrophages in the peritoneal cavity have been studied extensively over the years. Recently, two main subsets of peritoneal macrophages have been identified based on origin, surface markers and gene expression. Under normal conditions, large peritoneal macrophages (LPMs) compose the major population and are
characterized as $F4/80^{\text{high}}\text{MHCII}^{\text{low}}$. Fate-mapping studies suggest that LPMs are derived from prenatal origin, have the ability to self-renew, and selectively express the transcription factor GATA6 (37, 39). On the other hand, small peritoneal macrophages (SPMs) are characterized as $F4/80^{\text{low}}\text{MHCII}^{\text{high}}\text{GATA6}^{\text{neg}}$, are bone marrow derived, and thought to be short lived, monocyte-derived cells (40).

Under homeostatic conditions, SPMs and LPMs frequencies represent about 30-35% of the resident cells of the peritoneal cavity (41). The CD11b$^+$GATA6$^+$ LPMs make up approximately 90% of the peritoneal macrophage population (42). However, during inflammatory conditions, there is a dramatic shift in population frequency that include the disappearance of LPMS and the increase frequency of SPM populations (41). The so-called “macrophage disappearance reaction” (MDR) in the peritoneal cavity, was first described in the 1960s as the migration of peritoneal macrophages to the omentum after inflammatory stimuli (43). Recent intravital imaging studies of the peritoneal cavity suggest that LPMs possess free floating ability and are in continuous motion in the peritoneal fluid during homeostasis, a distinct feature compared to other tissue-resident macrophages (44). However, in order to trap microorganisms during inflammatory conditions in a fast-flowing environment, GATA6$^+$ LPMs selectively express coagulation factor V (FV) and other clotting factors that allow for pathogen capture through the creation of clots. Thus, through the well-known cellular adhesion to the omentum and the generation of clots, peritoneal macrophages account for their distinct MDR phenotype during inflammatory stimuli (44, 45).

Inflammation in the absence of pathogens, commonly referred to as sterile inflammation, has been extensively studied over the years. The initial inflammation of sterile injury has been
characterized by leukocyte recruitment and trafficking through the activation of pattern recognition receptors (PRRs) that are activated by damage-associated molecular patters (DAMPs) released during tissue damage. PRRs such as Toll-like receptors (TLRs), cytoplasmic Nod-like receptors (NLRs) and scavenger receptors are key elements of the innate immune system (46). Upon PRR activation, multiple immune cell types are recruited and migrate from the bloodstream to site of injury (47). Nevertheless, recent studies suggest that the recruitment of peritoneal cavity macrophages occurs through a different and more rapid way (40, 48). In response to a sterile thermal injury in the liver, GATA6+ peritoneal macrophages infiltrate to the site of injury in response to the DAMP ATP released by apoptotic cells as a “find me signal”. However, instead of being recruited through the traditional intravascular pathway, GATA6+ peritoneal macrophages use CD44 to migrate across the mesothelial layer that covers the liver and bind to exposed hyaluronan at the injury site (40). Once at the site of injury, these GATA6+ peritoneal macrophages rapidly proliferate and adopt a tissue repair (M2) phenotype that is characterized by the dismantling of nuclei from necrotic hepatocytes (40). The M2 phenotype also participates in tissue revascularization to reestablish tissue integrity and function.

To examine the specific role of the transcription factor GATA6 in LPMs, previous studies showed that the LysmCreGATA6fl/fl murine model, which is deficient in Gata6 in the macrophage lineage, is a good model to study the role of LPMs during disease. In addition, Mac-Gata6 KO LPMs result in the downregulation of unique peritoneal macrophage-specific genes (37). Just like any other transcription factor, expression of GATA6 in peritoneal macrophages, is driven by niche-specific signals. A particular signal required for the expression of GATA6 is retinoic acid (RA) (49). Furthermore, peritoneal macrophages highly express retinoic acid nuclear receptor RARβ (50).
By depleting vitamin A, which is the precursor for retinoic acid, studies have shown that using a vitamin-A-depleted diet in murine models results in the downregulation of the transcription factor GATA6 and reduction of LPMs (37, 51). Therefore, highlighting the importance GATA6 induction by retinoic acid and its downstream effect on the control of peritoneum-resident macrophages.

**Tissue-resident pericardial macrophages**

Although for decades the peritoneal cavity has been a focus of study and received much attention, pericardial macrophages have not been studied in detailed until recently. Enclosed by the pericardial sac, the pericardial cavity contains serous fluid produced by the visceral pericardium contains a heterogenic population of mononuclear phagocytes. In recent years, cardiac immunology has seen major advances in the understanding of origin, phenotypes, and function of mononuclear phagocytes, including macrophages, in the cardiac tissue (52). Among the macrophage population, the expression of GATA6⁺MHCII⁻CD102⁺ was identified as the predominant immune cell population in the pericardial fluid during steady state (48).

A healthy adult heart contains mostly embryonically derived macrophages and a smaller pool of macrophages replenished from blood monocytes (53). Recent studies showed the GATA6⁺MHCII⁻CD102⁺ resident macrophage population in the pericardial cavity is involved in the protection of the heart from fibrosis and cardiac inflammation (48). In response to induced myocardial infarction (MI) using a modified (intact pericardium) coronary ligation model in mice, pericardial Gata6+ macrophages were recruited into the heart following cardiac injury (48). In the absence of this macrophage population, Lysm<sup>Cre</sup>GATA6<sup>fl/fl</sup> mice showed an increased cardiac fibrosis after
MI. Therefore, supporting the notion that GATA6+ serous cavity macrophages are distinct from other tissue-resident macrophages in neighboring organs and playing a reparative function after sterile injury (48).

**Role of macrophages in myocarditis**

*Immune response after cardiac injury*

Following cardiac tissue injury, the immune system responds by mobilizing innate immune cells through a highly orchestrated, multi-level healing process. As the adult mammalian heart is non-regenerative, innate immune cells and the downstream cellular cascade play crucial roles in tissue recovery (54). After trauma, resident immune cells such as resident macrophages, resident innate lymphoid cells (ILCs), and resident mast cells are rapidly recruited to the site of injury to contain tissue damage and promote repair through the removal of dying cells and cytokine production (55). This fine balance between recruitment and expansion of immune cells, determines the outcome of cardiac tissue regeneration and repair.

*Macrophages and viral myocarditis*

Macrophages are key players involved in the development and pathogenesis of myocarditis. Using single-cell RNA sequencing, previous studies have demonstrated that at a steady state, healthy adult myocardium contains four different macrophage populations including a subset of tissue-resident cardiac macrophages (TIMD4+LYVE1+MHC-IIloCCR2−) with self-renewing
properties and a macrophage subset (TIMD4-LYVE1-MHC-IIhiCCR2+) often replaced by circulating monocytes (56). During viral myocarditis, myocardial macrophages are rapidly recruited to the myocardium site of infection where they can engulf infected cells and thus reduce viral replication (57). Although migrating properties to the site of injury were known for myocardial macrophages, recent preliminary studies from the Čiháková laboratory demonstrated in coxsackievirus B3 (CVB3)-induced myocarditis that GATA6+ pericardial macrophages also migrate into myocardium. In addition, the preliminary studies show that by using clodronate liposomes locally injected into pericardial cavity, depletion of GATA6+ pericardial macrophages worsened CVB3-induced myocarditis (58). Taken together, the heterogenous macrophage population in the heart is frequently the result of changes in the cardiac microenvironment that highlights the complexity and plasticity of macrophages involved in wound healing, tissue remodeling, and fibrosis (59).

The role of macrophage-mediated efferocytosis in myocarditis

The clearance of apoptotic cells during cardiac tissue injury is an essential step for proper wound repair and tissue remodeling. In the heart, a key characteristic of professional phagocytes is the ability to remove dead cardiomyocytes and extracellular matrix to avoid adverse outcomes post-injury (60). Although there is scarce literature on the role of efferocytosis in myocarditis, recent studies have linked the importance of efferocytosis and expression of phagocytosis receptors on myeloid cells with the resolution of myocardium inflammation (61). For example, the suppression of MerTK in murine models has been shown to result in less efficient apoptotic cell clearance, a proinflammatory phenotype in the heart during myocarditis, and a possible association with the
subsequent development of cardiac fibrosis (6). Importantly, properties of murine macrophages and the reduction of MerTK has also been observed in human patients with viral myocarditis (6).
II. Study Aims

Hypothesis: GATA6+ pericardial macrophages have efferocytosis properties and play a protective role during myocarditis.

The overall goal of this study is to identify genes involved in efferocytosis that are selectively expressed by GATA6+ serous cavity macrophages. To achieve this goal, we first compared gene expression in GAT6+ macrophage subsets versus bone marrow-derived macrophages using Real-Time RT-PCR. Second, we explored the physical and chemical characteristics of efferocytosis receptors including Tyro3, Axl, MERTK, and TIM4 in GATA6+ pericardial macrophages through flow cytometry. Third, we assessed the functional role of phagocytosis by GATA6+ pericardial macrophages in vitro using IgG-FITC labeled beads. Finally, we investigated the role that efferocytosis by GATA6+ pericardial macrophages play during experimental CVB3-induced murine myocarditis.
III. Materials and Methods

Animals

6-8 weeks old male C57BL/6J were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed and maintained in the Johns Hopkins University School of Medicine specific pathogen-free vivarium under conventional 12:12 light/dark cycle and standard chow food. All experiments involving animals were in compliance with the Animal Welfare Act and strictly followed the Guide for Care and Use of Laboratory Animals. The Animal Care and Use Committee of The Johns Hopkins University has approved all procedures and protocols used in this study.

Isolation of murine peritoneal macrophages

Peritoneal macrophages were isolated after a 5 ml injection of sterile PBS with a 10:1 dilution of hyaluronidase into the peritoneal cavity. A gentle massage was performed in the abdominal skin every 10 minutes during the 30-minute incubation period. Fluid was aspirated carefully without puncturing any organ using a 25-gauge needle and dispensed into a 5 ml polystyrene culture tube. Samples were centrifuged at 300xg for 8 minutes and red blood cell lysis was performed using ACK lysing buffer (ThermoFisher). Cells were washed, centrifuged and counted using a manual hemocytometer. Anti-Ly6G MicroBead Kit (Miltenyi Biotech) was used to deplete Ly6G+ cells using MS MACS Columns. CD11b MicroBead Kit (Miltenyi Biotech) was then used in Ly6G-
cells to positively enrich CD11b+ cells. Cell number was determined and the isolated Ly6G-CD11b+ peritoneal macrophages were determined by flow cytometric analysis, which suggested that >50% of the cells were macrophages. Although the non-macrophage population was not further characterized by flow cytometry, it is a heterogenous mix of CD11b+ myeloid cells including monocytes and eosinophils.

**Isolation of murine bone marrow-derived macrophages**

Bone marrow was isolated from murine femurs. DMEM (Gibco; 10 ml) was flushed into the bone marrow cavity of the femur using a 27 G needle. Medium was collected, filtered using 70 μm cell strainer and centrifuged at 300g for 8 minutes. Red blood cell lysis was performed using ACK lysing buffer (ThermoFisher). Cells were seeded at a concentration of 2 × 10⁶ cells/ml in a 6-well plate in 4 ml of DMEM complete media. To differentiate cells, 10 ng/ml of recombinant M-CSF (416-ML-010, R&D Systems) were added and replenished every 2 days. Media was removed on day 5 and fresh DMEM media was added to the culture plates. Cells used for Real-Time RT-PCR were harvested on day 8 by removing media and adding 500 μl of Trizol reagent (Invitrogen) directly into each well. To confirm the differentiation of bone-marrow derived macrophages, the remaining cells were harvested on day 8 using CellStripper (Corning) and analyzed by flow cytometric analysis.

**Isolation of pericardial and myocardial macrophages**

Pericardial and myocardial murine macrophages were isolated after aortic cannulation for 2 minutes. The main descending artery was cut to allow full perfusion using 1X PBS and heart was
harvested after cutting at the root of the aorta. To isolate the pericardial macrophages, the pericardial cavity was carefully flushed under a microscope using an insulin syringe with 500 μl 1X PBS with a 10:1 dilution of heparin. Flush was repeated four times and the petri dish was washed with 1 ml of 1X PBS. Samples were centrifuged at 300xg for 8 minutes and red blood cell lysis was performed using ACK lysing buffer. Cell number was determined, and samples underwent a Ly6G⁺CD11b⁺ MACS sorting as outlined above. Pericardial macrophages were determined by flow cytometric analysis, which suggested that >50% of the cells were macrophages.

To harvest myocardial macrophages, following the pericardial flush, hearts were placed in MACS tubes containing 5 ml of HBSS, 5000 U of Collagenase II and 500 U DNase I. Samples were placed in shaker at 37°C 200 RPM for 30 minutes. After incubation, samples were processed using gentleMACS program m_heart_01 followed by program m_heart_02. Cells were filtered using a 40 μm cell strainer into 50 ml tubes and washed with 5 ml of PBS. Samples were centrifuged at 300g for 8 minutes and red blood cell lysis was performed using ACK lysing buffer. Cell number was determined, and myocardial samples underwent a CD45⁺ MACS sorting. A small sample was used for flow cytometry analysis and the remaining samples were analyzed by Real-Time PCR.

**Real-time RT-PCR**

RNA isolation from samples was conducted under RNAse free conditions. Samples were centrifuged at 300xg for 8 minutes and supernatant was discarded. TRIzol™ Reagent (Invitrogen; 1.0 ml) was added to each sample and mixed with 200 μl of chloroform. After a 3-minute incubation period, samples were centrifuged for 15 minutes at 12,000xg at 4°C. The aqueous
phase was transferred to a new tube and 1 μl of RNase-free glycogen was mixed with 500 μl of isopropanol into each sample. Followed by an incubation period of 10 minutes, samples were centrifuged for 10 minutes at 12,000xg at 4°C. Pellets were then resuspended in 1 ml of 75% ethanol and centrifuged for 5 minutes at 7500xg at 4°C. RNA pellet was air dried and each sample was mixed with 18 μl DEPC treated water. RNA concentrations were quantified using a Nanodrop. cDNA synthesis was carried out using the RT-PCR program run using the stabilized concentrated RNA samples and 4 μl of iScript mastermix (BioRad) for a total of 20 μl volume for each sample.

The cDNA library obtained was used as template to test the transcript levels of primers designed by Primer-Blast (NCBI): GAPDH, Tyro3, Axl, MERTK, TIM4, CD163, MARCO, CCR1, TLR4, ICAM2, FN1, Lgals9, Pros1, Gas6, Del-1, CD14, Lamp2, CD164, TPP1, Dram1, Lgmn, IL-10, TGF-β, IL-1β, TNF, and IL-6 (Table 2).

The RT-PCR reactions were done using of 10 μl of Sybr Green 2x, 1 μl forward primer, 1 μl reverse primer, and 4 μl diH2O per 4 μl of sample into a 96-well PCR plate. Results were then analyzed using the 2−ΔΔCt method for fold change differences in Excel and graphed using Prism.

Table 1. Murine prime sequences for RT-PCR

<table>
<thead>
<tr>
<th>GENE</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GTTGTCCTCTGCGACTTCA</td>
<td>GGTGGTCCAGGGTTTCTTA</td>
</tr>
<tr>
<td>TYRO3</td>
<td>GTGAAGCCCGCAACATAAAAG</td>
<td>GGTGCTTGAGGCGAACAAT</td>
</tr>
<tr>
<td>AXL</td>
<td>GGAGGAGCCTGAGGACAAGGC</td>
<td>TACAGCATCTTGAGCCAAGAGTC</td>
</tr>
<tr>
<td>MERTK</td>
<td>ACCAAGTAGAGTTTCATGGCCG</td>
<td>GGCACGAGCTCTCAAGAC</td>
</tr>
<tr>
<td>TIM4</td>
<td>TCCGGAACCTTGATCTCCAA</td>
<td>GGCTGGGTTAAGATCGAGCT</td>
</tr>
<tr>
<td>CD163</td>
<td>GCAATCGCTGGGAACCTGAAG</td>
<td>CATGGGTCTTGTGCCATCGTA</td>
</tr>
</tbody>
</table>
Flow Cytometry

A 20 minute-long Fcγ receptor blocking step (CD16/CD32 (14-0161-82) with serum of the host primary antibody, preceded all antibody staining. Cells were then stained with respective anti-mouse monoclonal antibodies for 30 minutes at 4°C, washed, and resuspend in FACS buffer. Data was acquired on LSR II (BD Biosciences) and analyzed using FlowJo software V10 (FlowJo LLC, Ashland, Oregon, USA).

Antibodies and Reagents

Antibodies against CD45.2 (109814), CD11b (101262), Ly6G (127612), Ly6C (128012), F4/80 (123146), CD64 (139311), and MERTK (151506) were obtained from Biolegend, antibodies
against CD16/CD32 (14-0161-82), LIVE/DEAD (L34957), MHC II (16-5321-81), and AXL (25-1084-82) were obtained from Invitrogen. Antibodies against CD102 (740227-BD), TIM4 (745206) were obtained from BD Bioscience, antibody against TYRO3 (28635F) was obtained from Novus Biologicals.

**Phagocytosis Assay**

To test phagocytic activity, cells were plated at a concentration of 5x10⁴ in a 96-Well Polystyrene Round Bottom Microwell Plate (ThermoFisher) at an 80% confluency. Latex Beads-Rabbit IgG-FITC Complex (Cayman) were added directly to the culture medium to a final dilution of 1:100. Cells were incubated at 37°C for 4 hours. CellStripper (Corning) was used to remove the adherent macrophages followed by a wash with the Cell-Based Assay Buffer (Cayman). Samples were then transferred to FACS tubes for flow cytometry staining. To distinguish phagocytosis from simple surface binding, samples were incubated with trypan blue for 2 minutes and then washed with Cell-Based Assay Buffer to quench surface FITC fluorescence. Cells were stained with the appropriate anti-mouse monoclonal antibodies prior to proceed with flow cytometry analysis.

**TUNEL Assay**

To test for DNA fragmentation and apoptosis in LysmCreGATA6fl/fl, TUNEL assay was conducted in the paraffin-embedded heart tissue slides using the DeadEnd™ Fluorometric TUNEL System Kit (Promega). For pretreatment of tissue, slides were washed twice in xylene and once in 100% ethanol for 5 minutes. Samples were rehydrated in decreasing concentrations of 95% ethanol,
85% ethanol, 70% ethanol, and 50% ethanol for 3 minutes each wash. Lastly, slides were washed in 0.85% NaCl and PBS for 5 minutes each wash.

Cells were fixed in 4% formaldehyde for 15 minutes and washed twice in PBS for 5 minutes. Slides were incubated with 100 μl of 20 μg/ml Proteinase K solution at room temperature for 8 minutes followed by a 5-minute PBS wash. The second fixation was done by immersing the slides in cells in 4% formaldehyde for 5 minutes and a 5-minute PBS wash. Cells were covered with 100 μl of Equilibration Buffer and incubated for 8 minutes. Liquid was removed by tapping the slides and then stained with 45 μl equilibration buffer, 5 μl nucleotide mix, 1 μl rTdT enzyme. Slides were covered with plastic coverslips and incubated for 60 minutes in a humidified chamber. To stop the staining reaction, plastic coverslips were removed after the 60-minute incubation and samples were washed in 2X SSC for 15 minutes. Slides were washed three times in PBS for 5 minutes each wash and immersed for 2 minutes in 300 nM DAPI staining solution. Samples were washed 3 times in PBS and excess liquid was removed by tapping the slides. A cover slip was applied using 1-2 drops of mounting media. A fluorescent microscope was utilized to detect the localized green fluorescence of apoptotic tissue. Negative control was obtained by not including the rTdT enzyme in the incubation staining. TUNEL assay quantification was calculated with an Image Analysis MATLAB (Mathlabs) code using the Image Processing Toolbox to select for green staining.

**Statistical Analysis**

Statistical significance was determined using GraphPad Prism 7 by using unpaired Student’s two-tailed t-test or one-way ANOVA. (⁎P < 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.001 were considered significant).
IV. Results

**GATA6+ Peritoneal macrophages show upregulated tissue-repair gene expression compared to bone marrow-derived macrophages**

With the exception of a few genes identified from in the literature review, to properly analyze the gene-expression profiles of GATA6+ macrophages, genes were selected from those up-regulated in F4/80<sup>high</sup> peritoneal macrophages previously by a study that employed a whole-mouse genome microarray (39). A total of 192 genes were analyzed through the Database for Annotation, Visualization, and Integrated Discovery (DAVID) to assess their functional annotation and 186 genes were converted yielding clusters based on enrichment score and gene count. Given our interest in tissue repair properties of macrophages, 21 genes that showed enriched expression by F4/80<sup>high</sup> peritoneal macrophages were selected in the functional categories of scavenger receptors, efferocytosis, secreted extracellular matrix molecules, immune regulation, phagosome, and cytokines (Table 2).

Comparative analysis of gene expression between tissue-resident peritoneal macrophages and bone-marrow-derived macrophages was accomplished through real time RT-PCR. Bone marrow-derived macrophages were selected as controls since they lack GATA6 expression (62). To confirm the differentiation of BMDM, samples were analyzed by flow cytometric after 7 days of culture (Figure 4). Bone marrow-derived cells were >50% differentiated into CD115<sup>+</sup>F4/80<sup>+</sup> macrophages.
The cells isolated from the peritoneal cavity were enriched for large peritoneal macrophages (LPM) as outlined in the Methods section and the surface phenotype of the LPM was analyzed with flow cytometry (Figure 5) and were found to be CD45+CD11b+Ly6g-Ly6C-MHC-F4/80+.

After the surface phenotype of BMDM and peritoneal macrophages were established, Real-time RT-PCR was conducted on both macrophage populations. Peritoneal macrophages were harvested from naïve mice and then MACS sorted using Ly6G CD11b+ microbeads to further undergo qPCR. BMDM were harvested from culture plates and entire population underwent qPCR to determine the levels of expression of the 21 genes outlined in Table 2.

Of the 21 genes of interest, 15 were up-regulated in peritoneal macrophages compared to bone marrow-derived macrophages (Figure 6). The up-regulation of ICAM2 (CD102) in peritoneal macrophages, supports the unique phenotype of GATA6+ LPM phenotype (F4/80*ICAM2+) (40). Among the immune regulation genes, the upregulation of Gas6 and ProS1 in LPMs were of particular interest as they interact with the TAM family receptors. Gas6 functions as a ligand for all three TAM receptors with a direct activation of Axl, while ProS1 results in the activation of Tyro3/MERTK (63). Although IL-10 and IL-6 gene expression was highly up-regulated, testing expression of these two cytokine genes on biological replicates did not yield statistical significance. The discordant expression of these two cytokines in biological replicates might reflect the activation status and plasticity of LPMs. From the existing literature on efferocytosis receptors (26-32), Tyro3, Axl, MERTK, and TIM4 were four of the main genes of interest. Although up-regulation among all four genes was hypothesized, data obtained from RT-PCR gene expression showed up-regulation of only Tyro3 and TIM4 in the LPMs.
Table 2. The functional categories and the enrichment scores of the 21 genes used to characterize gene expression in peritoneal macrophages.

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>ENRICHMENT SCORE</th>
<th>GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagosome</td>
<td>17.02</td>
<td>CD164</td>
</tr>
<tr>
<td>Receptors</td>
<td>7.72</td>
<td>ICAM2, TIM1, CD163, MARCO, CCR1</td>
</tr>
<tr>
<td>Efferocytosis</td>
<td>7.72</td>
<td>Tyro3, Axl, MERTK, TIM4</td>
</tr>
<tr>
<td>Immune regulation</td>
<td>5.41</td>
<td>Gas6, Pros1, Lgals9, Lamp2, Del-1</td>
</tr>
<tr>
<td>Extracellular Matrix</td>
<td>5.55</td>
<td>FN1</td>
</tr>
<tr>
<td>Secretion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokines</td>
<td>2.3</td>
<td>IL-10, TGF- β, IL-1 β, IL-6, TNF-α</td>
</tr>
</tbody>
</table>

Genes selected from upregulated genes in F4/80$^{\text{high}}$ peritoneal macrophages previously identified by whole-mouse genome microarray. Analysis of enrichment score was obtained by using the Database for Annotation, Visualization, and Integrated Discovery (DAVID).
Figure 4. Bone marrow-derived macrophages after 7 days of culture.

(A) Bone marrow-derived cells were cultured for 7 days. 10 ng/ml of recombinant M-CSF was added and replenished every 2 days. Representative gating strategy used to identify differentiated bone marrow-derived macrophages as CD45⁺Ly6G⁻CD115⁺F4/80⁺.
Figure 5. Phenotype of peritoneal macrophages and bone marrow-derived macrophages.

Figure 6. Gene expression in peritoneal macrophages and BMDM as measured by Real-Time PCR. Data were analyzed by two-tailed Student’s t-test.
Tissue-resident macrophages and BMDM were harvested as previously described. TRIzol™ Reagent protocol was utilized for RNA isolation and cDNA isolation. (A) RT-PCR of receptor genes ICAM2, TIM1, CD163, MARCO, CCR1. (B) RT-PCR of immune regulation genes Gas6, Pros1, Lgals9, Lamp2 Del-1. (C) RT-PCR of cytokine genes IL-10, TGF-β, IL-1β, IL-6, TNF. (D) RT-PCR of efferocytosis genes Tyro3, Axl, MERTK, TIM4. (E) RT-PCR of phagosome genes Lamp2 and CD164. (F) RT-PCR of FN1 extracellular matrix gene.
GATA6+ Pericardial macrophages phenotype support efferocytosis properties

Flow cytometric analysis was used to characterization tissue-resident macrophages from the heart. Three different macrophage populations were studied: GATA6+ peritoneal LPM (F4/80\textsuperscript{high}MHCII\textsuperscript{low}CD102\textsuperscript{+}), GATA6+ pericardial macrophages (F4/80\textsuperscript{high}MHCII\textsuperscript{low}CD102\textsuperscript{+}) and cardiac resident macrophages (myocardial macrophages) (CD64\textsuperscript{+}). Because of our specific interest in studying efferocytosis properties in serous cavity pericardial macrophages, LPMs and myocardial macrophages were used as control groups. To confirm GATA6 expression in peritoneal and pericardial macrophages, intracellular GATA6 staining was analyzed with flow cytometry. After gating on Ly6G CD11b\textsuperscript{+}, peritoneal cells demonstrate that >89% of the population were GATA6+ and pericardial cells show 69% of GATA6+ macrophages (Figure 7). The gating strategy was consistent among all three macrophage populations as it is shown for pericardial macrophages (Figure 8) and fluorescence minus one (FMOs) controls were used to properly interpret positive receptor expression. Initially there was an attempt to keep the cell numbers consistent between the three groups of cells, but the number of cells obtained in the pericardial cavity and the myocardium after CD45\textsuperscript{+} selection, remained lower than peritoneal macrophages across multiple experiments. Therefore, there were significant differences in the cell count number between groups.

Four receptors were characterized using flow cytometry that included the TAM family receptors (Tyro3, Axl, MERTK) and TIM4. Compared to FMOs, there is a right shift in the positive direction among all macrophage subsets for all four receptors (Figure 9). However, there was difference in the percentage of cells that expressed these surface receptors depending on the macrophage
group. (Figure 10). Through the analysis of positive cell frequency among GATA6+ macrophages, there was a trend for the Tyro3, Axl and MERTK receptors to have higher expression levels in pericardial macrophages. On the other hand, TIM4 trended to be higher in peritoneal macrophages compared to both pericardial and myocardial macrophages. Overall, expression of all four efferocytosis receptors in myocardial macrophages appears to be lower than serous cavity macrophages. A possible explanation is due to their higher background expression observed in the FMO controls in the histogram plots (Figure 9). Thus, background noise reduction is essential for future experiments. The data suggests that, based on the surface-associated levels of Tyro3, Axl, MERTK, and TIM4, pericardial, peritoneal, and myocardial macrophages have different potentials for efferocytosis.
Figure 7. GATA6 expression in tissue-resident macrophages

Figure 8. Phenotype of tissue-resident pericardial macrophages

(A) Pericardial macrophages isolated after aortic cannulation and full perfusion of the heart. Pericardial cavity was carefully flushed with a 10:1 dilution of heparin. Representative gating strategy used to characterize pericardial macrophages as CD45+CD11b+Ly6G-Ly6C-F4/80+MHCII-CD102+.
Figure 9. Tyro3, Axl, MERTK and TIM4 receptors expression of tissue-resident macrophages

Flow cytometry analysis for TAM and TIM4 receptor expression of macrophage subsets. Black, FMO; red pericardial, green peritoneal, orange myocardial macrophages. (A) Pericardial macrophage subsets. Cells were gated on size, viability, CD45+ F4/80+ MHCI-CD102+. Plots are representative of all experiments. (B) Peritoneal macrophage subsets. were gated on size, viability, CD45+ F4/80+ MHCI-CD102+. (C) Myocardial macrophages macrophage subsets. Cells were gated on size, viability, CD45+ F4/80-CD64+. Data are representative of three independent experiments. n=2. Representative plots from one experiment are shown.
Figure 10. Frequency of positive receptor expression in macrophage subsets. Data were analyzed by one-way ANOVA.

(A) Tyro3 expression on CD102⁺ pericardial and peritoneal macrophages and CD64⁺ myocardial macrophages. (B) Axl expression on CD102⁺ pericardial and peritoneal macrophages and CD64⁺ myocardial macrophages. (C) MERTK expression on CD102⁺ pericardial and peritoneal macrophages and CD64⁺ myocardial macrophages. (D) TIM4 expression on CD102⁺ pericardial and peritoneal macrophages and CD64⁺ myocardial macrophages. Data are representative of two independent experiments. n=3. Cell number was limited in some replicates, yet, representative plots from one experiment are shown.
GATA6+ Pericardial macrophages possess functional phagocytosis properties in vitro

The expression of Tyro3, Axl, MERTK and TIM4 receptors in GATA6+ pericardial macrophages allowed us to hypothesize that these serous cavity-derived macrophages are superior phagocytic macrophages. We tested this hypothesis by examining their functional properties in vitro. As in the previous experiments, three different macrophage populations were studied. LPMs and myocardial macrophages were used as control groups to compare them with pericardial macrophages.

To study phagocytosis properties in vitro, tissue-resident macrophages were cultured with latex beads coated with rabbit IgG-FITC complexes. Different titrations of the latex beads were tested and optimized to a final 1/100 dilution of the stock suspension. To distinguish phagocytosis from simple surface binding, samples were incubated with trypan blue to quench the fluorescent signal associated with the outside of the cells. Cells were analyzed by flow cytometry where the pericardial and peritoneal macrophages were gated on CD45+ F4/80+ MHCII+ CD102+ and the myocardial macrophages were gated on CD45+ F4/80+ CD64+.

Compared to controls that received no treatment, pericardial, peritoneal and myocardial macrophage samples incubated with the IgG-FITC beads showed an increase FITC signal, suggesting their uptake of beads, supporting their phagocytosis properties in vitro (Figure 11). To better visualize the data, controls and experimental groups are shown as histogram plots to detect signal intensity on the FITC channel (Figure 12). Through the analysis, the frequency of the uptake of the opsonized beads trends greater in pericardial macrophages (Figure 12). Nonetheless, statistical significance was not found among the groups. As observed with the
irregular expression of Tyro3, Axl, MERTK, and TIM4, variability in phagocytic capacity among the macrophage populations is found across all three subpopulations.

Although the phagocytic properties of peritoneal and myocardial macrophages have been previously described, research on pericardial macrophage phagocytosis/efferocytosis properties is new to the field. Compared to the other two macrophage subsets, and consistent with our hypothesis, pericardial macrophages demonstrate an equal or slightly higher ability to phagocytose opsonized cargo.
Figure 11. IgG-FITC beads uptake by macrophages

Pericardial, peritoneal and myocardial murine macrophages were cultured on a 96 well-plate at 5x10⁴ cells/ml. Latex beads-rabbit IgG-FITC complex was added directly to the culture medium at a 1:100 dilution and incubated at 37°C for four hours. Cells were washed and stained for flow cytometry. Macrophage populations gating strategy was defined as previously shown. Figure shows the uptake of FITC beads on experimental groups incubated with the latex beads-rabbit IgG-FITC complex. Control groups were cultured with plain media. Data are representative of two independent experiments. n=3. Representative plots from one experiment are shown.
Figure 12. Murine macrophage populations phagocytose opsonized IgG-FITC beads

Flow cytometry analysis for functional phagocytosis assay of macrophage subsets. (A) Black line FMO; red pericardial macrophages, green peritoneal macrophages, orange myocardial macrophages. Using FlowJo software, tissue-resident macrophages are gated and shown for FITC fluorescence. Data are representative of two independent experiments. n=3. Representative plots from one experiment are shown. (B) Positive uptake of IgG-FITC beads by macrophage subsets gated on CD102⁺ pericardial and peritoneal macrophages and CD64⁺ myocardial macrophages. Triplicates were used to validate data. Data are representative of two independent experiments. n=3. Representative plots from one experiment are shown.
GATA6+ Pericardial macrophages play a role in efferocytosis/phagocytosis during CVB3 myocarditis

Although the role of efferocytosis of pericardial macrophages has not been previously described in the literature, their tissue-repair properties have been described during cardiac injury (48). Recent preliminary studies from the Čiháková’s laboratory demonstrate that murine GATA6+ macrophages located in the pericardial cavity outside of the ventricles can limit cardiac inflammation through myocardial migration during CVB3 myocarditis. By inducing myocarditis in Gata6venus reporter mice, invasion of GATA6+ pericardial macrophages were observed in myocardium during disease (Figure 13). In addition, expression of multiple efferocytosis surface receptors in human GATA6+ pericardial macrophages from patients with ischemic cardiomyopathy (ICM) was identified through scRNA-seq. In the following experiments the goal was to study the efferocytosis role of pericardial macrophages during CVB3 myocarditis.

To gain an insight into the role that GATA6+ pericardial macrophages play in removing dead cells caused by virus infection in the CVB3-myocarditis mouse model, the number of apoptotic cells were measured in Lyz2creGata6fl/fl mice compared to infected Lyz2cre controls. Gata6 deletion in myeloid cells using Lyz2creGata6fl/fl mice, has been previously validated in multiple research studies that aimed to study the role of Gata6 in serous cavity macrophages (40, 48, 64). From the samples obtained, TUNEL assay was used to quantify apoptosis in both experimental groups. We hypothesized that the mice deficient in GATA6+ pericardial macrophages (Lyz2creGata6fl/fl mice) would be unable to efficiently clear apoptotic cells and thus show a higher level of TUNEL+ cells in the heart. Using confocal fluorescence microscopy, samples show DAPI-stained nuclei
with blue and localized green FITC fluorescence of apoptotic cells. A naïve Gata6\(^{fl/fl}\) mouse used as the control shows little to no apoptosis compared to a Lyz2\(^{cre}\)Gata6\(^{fl/fl}\) mouse that is undergoing CVB3-induced myocarditis (Figure 14). Further, the number of cells undergoing apoptotic death in the heart of CVB3-infected Lyz2\(^{cre}\)Gata6\(^{fl/fl}\) animals was much higher that the number of apoptotic cells in the heart of the infected control Lyz2\(^{cre}\) animals (Figure 15).

To measure the difference between the three groups, a quantitative assay was performed to investigate the protective role of GATA6+ pericardial macrophages during CVB3 disease in one individual per group (Figure 16). The % number of cells calculated with an Image Analysis MATLAB code that measures green fluoresce suggest that the hearts of infected Lyz2\(^{cre}\)Gata6\(^{fl/fl}\) mice undergoing myocarditis show greater level of apoptosis. However, experiments will need to be further validate this observation with a larger number of animals.

Based on the original hypothesis on the protective role of GATA6+ pericardial macrophages, our data suggests that this is a unique tissue-resident macrophage population that can migrate into myocardium and can regulate inflammation during myocarditis – possibly through its ability to efficiently efferocytosis apoptotic cells and cellular debris. Although the mechanism needs to be further studied, our results demonstrate that lack of GATA6+ pericardial macrophages during CVB3 induced myocarditis results in decreased efferocytosis by Lyz2\(^{cre}\)Gata6\(^{fl/fl}\) mice. Thus, supporting the original hypothesis on the efferocytosis properties of GATA6+ pericardial macrophages and their potential to reduce inflammation and fibrosis development.
Figure 13. GATA6+ macrophages exist in naïve mouse pericardial cavity and migrate into myocardium during myocarditis.

Experiment done in collaboration with David H. (A) Macrophages (CD11b+Ly6G- F4/80hi) in naïve pericardial cavity expressed CX3CR1 and Gata6. In contrast, myocardial macrophages were negative for Gata6. (B) Invasion of GATA6+ pericardial macrophages (Venus+CD11b+Ly6G-F4/80+) were observed in myocardium during myocarditis. Gata6Venus mice were infected with CVB3 to induce myocarditis.
Figure 14. TUNEL assay in control WT GATA6^{fl/fl} mouse and Lyz2^{cre}Gata6^{fl/fl}

To test apoptosis and cell death clearance in L Lysm^{cre}GATA6^{fl/fl}, TUNEL assay was conducted in the paraffin-embedded heart tissue slides. A fluorescent microscope was utilized to detect the localized green fluorescence of apoptotic tissue. (A) GATA6^{fl/fl} mouse 100x. (B) Lysm^{cre}GATA6^{fl/fl} with CVB3 induced myocarditis 100x.
**Figure 15.** TUNEL assay in CVB3 induced myocarditis.

To investigate the protective role of GATA6+ pericardial macrophages during disease, Lyz2^{cre}Gata6^{fl/fl} mice were compared to Lyz2^{cre} both with CVB3-induced myocarditis. TUNEL assay was conducted on experimental and control groups to detect clearance of apoptotic cells. (A) Lyz2^{cre} undergoing CVB3 myocarditis 160x. (B) Lyz2^{cre}Gata6^{fl/fl} undergoing CVB3 myocarditis 160x.
Figure 16. TUNEL assay quantification in CVB3 induced myocarditis.

Quantitative analysis of apoptotic cell death by TUNEL assay. TUNEL staining as a quantitative assay was performed to investigate the protective role of GATA6+ pericardial macrophages at day 8 post-CVB3 infection. The number of TUNEL+ cells was calculated with an Image Analysis MATLAB code using the Image Processing Toolbox to select for green staining. Experiments need to be validated with larger groups.
V. Discussion

The biology of serous cavities, including pleural, peritoneal, and pericardial, has been studied extensively over the years (33, 34). Nonetheless, the peritoneal cavity has served as a source to study serous cavity macrophages and their properties. Because of their ease of harvest, ability to phagocytose, and their respond to invading pathogens, it has been established that large peritoneal macrophages (LPMs) have functions that are shared among other tissue-resident macrophage populations. However, there are signature genes and functions that are defined by niche-specific signals that suggest distinctive function of serous cavity-resident macrophages. Among LPMs, the transcription factor \textit{Gata6} has been found to be important for their development and tissue-repair properties.

An important tissue-repair characteristic of macrophages during inflammation and tissue damage is the removal of dead cells and cellular debris. The process of efferocytosis is accomplished through a series of regulated mechanisms that includes the detection of the “find me” signal released by dead cells, the recognition of “eat me” signal from extracellular cargos, the phagocytosis of the dead cells, and lastly the digestion and degradation of engulfed cellular debris. Efferocytosis and phagocytosis properties of GATA6+ peritoneal macrophages have been extensively researched. In response to a sterile thermal injury in the liver, GATA6+ peritoneal macrophages infiltrate to the site of injury in response to DAMP molecule ATP released by apoptotic cells as a “find me signal”. Once at the site of injury, GATA6+ peritoneal macrophages
rapidly proliferate and adopt a tissue repair phenotype that is characterized by the dismantle of nuclei from necrotic hepatocytes (40). While the origin, phenotype and function of serous cavity peritoneal macrophages are well defined, those of the pericardial cavity are not well understood. We used three different techniques to evaluate the phenotype and efferocytosis properties of GATA6+ macrophages: gene-expression, phenotype-associated receptor expression, phagocytic capacity in the context of CVB3 myocarditis.

In recent years, cardiac immunology has seen major advances in understanding the immune cell populations of the heart. The pericardial cavity contains serous fluid with a true heterogeneity of mononuclear phagocytes including different macrophage subsets. Recent studies suggest that during steady state, expression of GATA6+MHCII-CD102+ is the predominant immune cell population (48). In response to induced myocardial infarction (MI) and in the absence of this macrophage population, LysmCreGATA6fl/fl mice result in increased cardiac fibrosis and cardiac inflammation. Thus, suggesting their involvement in the protection of the heart from fibrosis and cardiac tissue damage (48).

Although it has been previously suggested, the ability of GATA6+ pericardial macrophages to remove apoptotic cells has not been described in the literature. Therefore, we hypothesized that GATA6+ pericardial macrophages play a significant role during tissue repair by assisting in dead cell clearance. Due to the low cell yield of GATA6+ pericardial macrophages in the heart, in this study we initially compared the gene expression of GATA6+ peritoneal macrophages to bone marrow-derived macrophages. Real-Time RT-PCR was conducted on LPMs and BMDM because of their ease to harvest and sufficient cell number isolation to conduct RT-PCR. Genes were selected based on the functional categories of scavenger receptors, efferocytosis, extracellular...
matrix secretion, immune regulation, phagosome, and cytokines. The enhanced gene expression among most of the genes, suggested that GATA6+ peritoneal macrophages play a tissue-repair role compared to GATA6- BMDM. The Gas6, Pros1, TAM family receptors, and TIM4 were of particular interest in this study to confirm the previously described efferocytosis gene expression in LPMs. After studying gene expression between LPMs and BMDM, the RT-PCR results supported the existing data and our original hypothesis on the efferocytosis properties of LPMs based on Tyro3, Axl, MERTK, TIM4, Gas6 and Pros1 expression. With flow cytometric analysis, we compared the Tyro3, Axl, MERTK, TIM4, receptor expression on GATA6+ pericardial macrophages, GATA6+ peritoneal macrophages, and myocardial macrophages. Surprisingly, with the exception of TIM4, GATA6+ pericardial macrophages expressed the efferocytosis-associated receptors including Tyro3, Axl, and MERTK at a higher frequency than LPMs or myocardial macrophages. The results of this study further support preliminary data from the Čiháková’s laboratory that demonstrate the expression of multiple efferocytosis surface receptors in human GATA6+ pericardial macrophages using scRNA-seq. To our knowledge, the results presented in this thesis represents the first studies comparing Tyro3 Axl, MERTK, and TIM4 receptor expression of two different GATA6+ macrophage populations: pericardial and peritoneal macrophages.

Next, we compared the efferocytosis potential of GATA6+ pericardial macrophages, GATA6+ LPMs and myocardial macrophages using a functional phagocytosis assay. All three macrophage subsets demonstrated the ability to phagocytose opsonized beads. However, GATA6+ pericardial macrophages once again appeared to have higher potential to carry out efferocytosis compared to the other macrophage subsets as measured by their higher frequency of uptake of the
opsonized latex beads. Thus, our data suggest that GATA6+ pericardial macrophages possess
greater ability to ingest and engulf other cells or particles compared to GATA6+ peritoneal
macrophages in vitro.

Although it was previously demonstrated that Lysm$^{Cre}$GATA6$^{fl/fl}$ mice developed an increased
cardiac fibrosis and cardiac inflammation after MI, there was no study that used the
coxsackievirus B3 (CVB3)-induced myocarditis model to describe cardiac pathogenesis in
Lysm$^{Cre}$GATA6$^{fl/fl}$ mice. Preliminary studies by the Čiháková’s laboratory demonstrate that
GATA6+ pericardial macrophages migrate from the pericardial cavity to the myocardium in CVB3-
0induced myocarditis. Therefore, we wanted to test the efferocytosis potential of GATA6+
pericardial macrophages during CVB3 induced myocarditis.

Compared to the naïve Gata6$^{fl/fl}$ mouse used as the control, both Lyz2$^{cre}$ and Lyz2$^{cre}$Gata6$^{fl/fl}$ mice
undergoing CVB3-induced myocarditis showed positive staining for cell apoptosis quantified by
TUNEL assay. However, after quantifying the frequency of TUNEL-positive cells using an Image
Analysis MATLAB code that selects for green staining, we found a greater number of TUNEL-
stained cells in the Lyz2$^{cre}$Gata6$^{fl/fl}$ mice. While experiments must be replicated and expanded for
validation, the results suggest that the lack of clearance of the apoptotic cells in the
Lyz2$^{cre}$Gata6$^{fl/fl}$ mice may be associated with a greater development of fibrosis, possibly resulting
in adverse cardiac injury, increase inflammatory responses and affected tissue regeneration. Yet,
the protective role towards cardiomyocytes needs to be further studied as TUNEL assay staining
alone is not sufficient to differentiate between apoptotic cardiomyocytes and apoptotic immune
infiltrating cells.
Conclusion and Future Directions

The heart is an organ that cannot regenerate after undergoing cardiac tissue damage. Every year, myocarditis is responsible for a significant number of acute and chronic heart failure cases worldwide especially among children and younger adults. While most patients with myocarditis recover, about 1/3 progress to cardiac fibrosis, loss of cardiac function and develop dilated cardiomyopathy. There is a lack of therapies for the disease and the development of interventions requires deeper mechanistic understanding of the leukocyte subsets involved in the immunopathogenesis of the myocarditis. Recently identified, GATA6+ macrophages located in the pericardial cavity have been shown to possess tissue-repair properties that can play a role in limiting cardiac inflammation during MI.

Because the GATA6+ pericardial macrophage population was identified only within the past year, there are multiple questions that remain unanswered. In this study we explore the previously unknown efferocytosis properties of GATA6+ pericardial macrophages. Whereas GATA6+ peritoneal macrophages and myocardial macrophages do play a role in the clearance of dead cells as it has been previously described in the literature, GATA6+ pericardial macrophages express efferocytosis receptors and phagocytose in vitro at a higher frequency. In addition, through an experimental viral myocarditis murine mode in LysmCreGATA6fl/fl which lack GATA6+ cells, we were able to quantify and compare the impact on the loss of GATA6+ pericardial macrophages during disease. Therefore, supporting our original hypothesis of their efferocytosis role during cardiac tissue damage and their dead cell clearance properties.
Moving forward, it is important to further explore their tissue-repair properties including the expression of other efferocytosis receptors, the gene expression of Gas6 and Pros1, and efferocytosis properties \textit{in vitro} with cardiomyocytes undergoing apoptosis. In addition, studies to identify possible therapies that could induce efferocytosis to prevent the development of fibrosis in the heart would greatly enhance the clinical outcomes of patients. Lastly, it is imperative to study the translational impact in human pericardial GATA6+ macrophages and their efferocytosis role during myocarditis.
References


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PROFILE
Master of Science candidate in molecular microbiology and immunology. Research focus includes cardiac immunology, community outreach, and racial health disparities. Professional experience includes assisting with the implementation of graduate courses, working with student services and student organizations. Fluent in Spanish and English.

EDUCATION
Master of Science, Molecular Microbiology and Immunology (MMI) Expected May 2020
Johns Hopkins Bloomberg School of Public Health (JHSPH), Baltimore, MD
• Thesis: Serous Cavities Macrophages and Their Efferocytosis Role During Disease

Health Disparities and Health Inequality Certificate Expected May 2020
Johns Hopkins Bloomberg School of Public Health
• Relevant Coursework: Seminar in Health Disparities, Health and Homelessness, Latino Health: Measures and Predictors, Urban Health in Developing Countries.

Tropical Medicine Certificate May 2019
Johns Hopkins Bloomberg School of Public Health
• Relevant Coursework: Fundamental Virology, Biology of Parasitism, Immunology Infection and Disease, Chronic Disease in Low- and Middle-Income Countries.

 Bachelor of Science, Biology May 2018
University of Oklahoma, Norman, OK
• Minor: Spanish

RESEARCH EXPERIENCE
Master of Science Candidate - JHSPH/MMI Nov 2018 – Present
Member of the Dr. Daniela Čiháková Myocarditis Laboratory
• Studied the phenotypic and functional properties of resident pericardial macrophages and their efferocytosis role during myocarditis disease.
• Designed and performed animal experiments in collaboration with other laboratory members.
• Developed assays to study cell death and measure phagocytosis in vitro and in vivo.
• Optimized numerous immunological techniques.
• Presented experimental results orally or in a written report monthly, assisted in grant writing proposal and abstract submission.
Research Assistant - Prime Time Sister Circle (PTSC) project Dec 2018 – Present
Johns Hopkins Center for Health Disparities Solutions (HCHDS)

The HCHDS strives to eradicate health disparities among racial groups, socioeconomic groups, and geopolitical categories such as urban, rural, and suburban populations.

• Coordinated implementation of focus groups and in-depth, semi-structured interviews of mid-life African American women with hypertension.
• Assisted in data collection on blood pressure, health status, health behaviors, social support, social networks, and health care utilization of participants.
• Assessed participants experiences and behaviors after intervention.

Summer Intern - Diversity Summer Internship Program May 2017 - Aug 2017
Johns Hopkins Bloomberg School of Public Health

• Evaluated data from the 2015-2016 influenza season in Baltimore under the mentorship of Andrew Pekosz Ph.D. in the Department of Molecular Microbiology and Immunology.
• Isolated influenza viruses for detection and identification in tissue cultures.
• Conducted a retrospective evaluation from passive samples that captured detailed assessment of severe disease, clinical, and laboratory characteristics using multiple databases.

Ronald E. McNair Scholar Aug 2016 - May 2017
The University of Oklahoma

• Designed a research protocol to address diabetes health issues in the Oklahoma City Latino community under Institutional Review Board (IRB) approval.
• Conducted a series of classes in Spanish with a focus group for a period of two months.
• Assessed participants perspectives, knowledge, and evaluated surveys using pre-post survey analysis.
• Maintained record of research project, data, and study progress

TEACHING EXPERIENCE

Teaching Assistant, The Robert Wood Johnson Foundation Jan 2020-present
Health Policy Research Scholars Program (HPRS)

• Course title: HPRS:
  Facilitated online live talks, assisted with attendance, communication bridge between students and instructors; 2 terms.

Teaching Assistant, Johns Hopkins Bloomberg School of Public Health Sept 2019-present
Department of Health Behavior and Society

• Course title: Fundamental Tools for Promoting Health Equity:
  Moderated student discussion forum, communicated with students, assisted professors in grading assignments including final projects; 1 term.
• Course title: Critical Issues in Health Disparities:
  Facilitated student-run seminar, assisted professor in topic selection, communicated with students through in-person and virtual class format; 4 terms.
Teaching Assistant, Johns Hopkins Bloomberg School of Public Health Oct 2019-Dec 2019
Department of Molecular Microbiology and Immunology
• Course title: Immunology, Infection and Disease:
  Assisted professor in developing course materials, held review sessions, graded quizzes and exams, communicated with students; 1 term.

STUDENT SERVICES/MENTORSHIP EXPERIENCE
Graduate Assistant Aug 2019-Present
JHSPH Office of Student Life, Baltimore, MD
• Provided support to graduate students at the Johns Hopkins Bloomberg School of Public Health.
• Created bi-weekly blog posts focused on helping students navigate academic, personal, and professional environments.
• Held in-person and virtual events covering topics such as Impostor Phenomenon and Navigating Graduate School as First-Generation Students.
• Assisted with the implementation of social media strategies including Instagram and LinkedIn.

Diversity Summer Internship Program Assistant May 2019 – Sept 2018
JHSPH Office of Student Life, Baltimore, MD
• Directed the residential portion of the Diversity Summer Internship Program
• Conducted sessions on topics pertaining to personal development.
• Offered mentorship and held 1-1 meetings throughout the summer with interns.
• Reviewed assignment submissions including the final research paper.

Mentor Service Specialist May 2019 - Sept 2018
University of Oklahoma, Information Technology, Norman, OK
• Identified and assisted in the resolution of incidents by providing technical support in person, in chat, and over the phone.
• Guided the services team part-time employees through professional development in a healthy environment.

OU SACNAS Chapter Co-Founder and President Aug 2017- May 2018
University of Oklahoma, Norman, OK
• Assisted in the establishment of a local SACNAS chapter at OU to advocate for underrepresented minority students in STEM.
• Created bylaws, assisted advisor with national paperwork requirements, recruited members, established initial budget analysis.

PRESENTATIONS
Delgado P. A., Cihakova, D. (October 2019). *Regulatory Role and Therapeutic Implications of Serous Cavities’ Macrophages in Autoimmune Myocarditis*. Accepted at the National Diversity in STEM Conference, Honolulu, Hawai‘i.

Delgado P. A., Mata, S, Undiano, R. (March 2018). *A Perspective on Diabetes in the Oklahoma City Latino Community*. Presented at the 23rd Annual Research Day at the Capitol, Oklahoma City, OK.


Delgado P. A., Mata, S, Undiano, R. (April 2017). *A Perspective on Diabetes in the Oklahoma City Latino Community*. Presented at the 2017 Health Disparities, Social Science and Humanities of Health Symposium at University of Oklahoma Health Science Center, Oklahoma City, OK.

**PROFESSIONAL DEVELOPMENT**

**Laboratory Techniques:** Plaque assay, TCID$_{50}$, sample staining, use of selection of markers, isolation of viruses, flowcytometry, phagocytosis assay, TUNEL assay, immunohistochemistry, qPCR.

**Computer Skills:** Microsoft Office Suite, STATA, Autodesk, SolidWorks, FlowJo, GraphPad prism

**Languages:** Spanish (Fluent), English (Fluent)

**Memberships:** American Association for the Advancement of Science (2019-present), Society for Advancement of Chicanos/Hispanics and Native Americans in Science (2018-present)

**Travel Abroad:** Canada, Mexico, France

**HONORS AND AWARDS**

- The National Diversity in STEM Conference Travel Award
  Oct 2018
- Diversity Summer Internship Program Scholar
  Sept 2018
- ABRCMS 2017 Public Health Presentation Awardee
  Nov 2017
- Federation of American Societies for Experimental Biology DREAM Travel Award
  Oct 2017
- Oklahoma Louis Stokes Alliance for Minority Participation Scholar
  2017 - 2018
- Ronald E. McNair Scholar
  2016 - 2018
- William Jr. & Jane Longmire Academic Scholar
  2015 - 2018
- College of Arts and Sciences Withrow Leadership Scholars
  2015 – 2018

**LEADERSHIP AND VOLUNTEER**

- Biomedical Scholars Association JHSPH representative
  Aug 2019 – present
- Hopkins-UMB SACNAS Chapter Secretary
  2018 - 2019
- Sigma Lambda Gamma, Vice President of Program Development
  2015 - 2018
- Fundación Manos Juntas Lead Volunteer
  2015 - 2018