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AZITHROMYCIN THERAPY REDUCES CARDIAC INFLAMMATION AND MITIGATES ADVERSE CARDIAC REMODELING AFTER MYOCARDIAL INFARCTION

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AZITHROMYCIN THERAPY REDUCES CARDIAC INFLAMMATION AND
MITIGATES ADVERSE CARDIAC REMODELING AFTER MYOCARDIAL
INFARCTION

DISSERTATION

A dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of
Philosophy in the College of Medicine at the
University of Kentucky

By

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2019

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ABSTRACT OF DISSERTATION

AZITHROMYCIN THERAPY REDUCES CARDIAC INFLAMMATION AND MITIGATES ADVERSE CARDIAC REMODELING AFTER MYOCARDIAL INFARCTION

Introduction: Myocardial infarction (MI) remains the leading cause of morbidity and mortality worldwide. Induced by cardiomyocyte death, MI initiates a prolonged and uncontrolled inflammatory response which impairs the healing process. Immune cells, such as macrophages, play a central role in organizing the early post-MI inflammatory response and the subsequent repair phase. Two activation states of macrophages have been identified with distinct and complementary functions (inflammatory vs. reparatory). This bimodal pattern of macrophage activation is an attractive therapeutic target to favorably resolve post-MI inflammation and enhance recovery. It has been demonstrated that azithromycin (AZM), a commonly used antibiotic with immunomodulatory effects, polarizes macrophages towards the reparatory phenotype. AZM has an excellent safety profile and has been approved for human use. We hypothesize that AZM reduces inflammation and improves heart function in MI.

Methods and results: In our initial studies, we demonstrated that oral free AZM (160 mg/kg daily for 7 days), initiated 3 days prior to MI, enhances post-MI cardiac recovery as a result of shifting macrophages to the reparatory state. We observed a significant reduction in mortality with AZM therapy. AZM-treated mice showed a significant decrease in pro-inflammatory and an increase in reparative macrophages, decreasing the pro-inflammatory/reparative macrophage ratio. Macrophage changes were associated with a significant decline in pro- and an increase in anti-inflammatory cytokines. Additionally, AZM treatment was correlated with a distinct decrease in neutrophil count due to apoptosis, a known signal for shifting macrophages towards the reparative phenotype. Finally, AZM treatment improved cardiac recovery, scar size, and angiogenesis. We designed this proof of concept study using pre-MI AZM therapy to achieve steady state levels prior to injury. Therefore, in our follow-up studies we targeted inflammatory macrophages using a non-Pegylated liposomal formulation of AZM (Lazm) which has been shown in multiple studies to promote drug efficacy and minimize off-target effects. To test the hypothesis that Lazm is more effective and safer than free AZM, low doses of free/liposomal AZM (10 or 40 mg/kg, administered intravenously) were initiated immediately after MI. We observed that Lazm induces early resolution of the post-MI inflammatory response as evidenced by switching

of the activation state of monocytes/macrophages towards the reparatory phenotype. Neutrophils were substantially decreased, particularly pro-inflammatory neutrophils. Cytokine profiles were also shifted to the anti-inflammatory status with Lazm therapy. Taken together, AZM treatment resulted in a significant shift in macrophage activation towards the reparatory state. The shift in inflammatory state was accompanied by a decrease in apoptosis and infarct size in the injured heart, as well as enhanced angiogenesis and LV functional recovery in our long-term studies. In addition, Lazm was protective against off-target effects of AZM on the heart.

Conclusion: This is the first evidence of a novel and clinically-relevant therapeutic strategy to modulate post-MI inflammation. We found that AZM reduces cardiac inflammation and improves adverse cardiac remodeling after infarction via promoting a shift of macrophage activation state. The overarching significance of this work is the modulation of sterile inflammation, which can be a viable therapeutic target in many conditions including stroke and heart attack. Additionally, this is the first study to demonstrate the immune modulation properties of liposomal AZM, which has wide potential therapeutic applications beyond the cardiovascular field. Importantly, liposomal formulation of AZM is protective from its cardiac off-target effects. Our findings strongly support clinical trials using AZM as a novel and clinically relevant therapeutic target to improve cardiac recovery and reduce heart failure post-MI in humans.

Keywords: Myocardial infarction, Post-myocardial infarction inflammation, Macrophage, Macrophage polarization, Azithromycin, liposomal azithromycin.

Ahmed Al-Darraj

02-25-2019

AZITHROMYCIN THERAPY REDUCES CARDIAC INFLAMMATION AND
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INFARCTION

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Chapter I. Introduction

1.1 Background

1.1.1 Myocardial infarction

Myocardial infarction (MI) and its common complication, heart failure, are leading causes of morbidity and mortality worldwide (Spatz, Beckman et al. 2016). Although the hospitalization rate for MI has significantly decreased in the last 15 years (~1238 vs ~ 750/ 100000 individual per year) (Spatz, Beckman et al. 2016), the five-year post-MI mortality rate remains high (~50%) (Lindsey, Saucerman et al. 2016). Cardiac death is mainly related to heart failure, induced by adverse post-infarction cardiac remodeling (Lindsey, Saucerman et al. 2016). Moreover, heart failure in the USA contributed to more than \$20 billion in medical expenses in 2012 (Writing Group, Mozaffarian et al. 2016). This cost is expected to increase to more than \$53 billion by 2030 due to increasing prevalence (Heidenreich, Albert et al. 2013).

MI refers to a clinical condition characterized by full or partial epicardial coronary artery occlusion due to rupture or erosion of vulnerable plaques (White and Chew 2008), which leads to myocardial injury and/or death from inadequate oxygen supply (Spatz, Beckman et al. 2016). Insufficient blood and nutrient supply may lead to coronary artery constriction and thrombus micro-embolisation, producing a more severe form of MI attack. When the occlusion persists for more than 30 minutes, irreversible injury occurs to the myocardium. Beyond 6 hours of arterial occlusion, the entire jeopardized myocardial region becomes necrotic and the infarct size massively increases. Necrotic areas expand from the central zone of the blocked artery, through the severely damaged endocardial layers, towards the borders, the less ischemic subepicardium, and eventually lead to transmural cardiac injury. Factors such as size and location of the injured zone supplied by the occluded artery, severity of the ischemia, temperature, and systemic hemodynamic status determine the ultimate infarct size (Skyschally, Schulz et al. 2008). Variability in resistance to ischemic injury and collateral circulation also

contribute to the progression of MI and, ultimately, its expansion (Heusch and Gersh 2017).

MI animal models show that the infarct size influences long-term survival and ventricular function (Pfeffer, Pfeffer et al. 1979, Pfeffer, Pfeffer et al. 1985). From a clinical standpoint, the likelihood of getting congestive heart failure, cardiac aneurysm formation, and/or myocardial rupture significantly increases if the patient experiences infarct expansion (Jugdutt and Michorowski 1987). During the resorption of dead tissue, the tensile strength of the injured area temporarily declines and the non-contractile wall becomes highly susceptible to structural deformation (Pfeffer 1995). Necrotic cardiomyocytes are replaced by fibrous connective tissue, leading to histological changes in the myocardium (Fishbein, Maclean et al. 1978). Fibroblast proliferation and collagen deposition progress simultaneously, leading to scar formation, leaving the living cardiomyocytes in the infarct zone to align to form a compressed collagen matrix. Heart dimensions begin to change and the damaged zone becomes thin and elongated (Pfeffer and Braunwald 1990). As a result, the ischemic insult induces serious and tangled modifications to the cellular and extracellular components of the heart muscle, producing alteration in cardiac capacity, architecture and function in a process called left ventricular (LV) remodeling. These changes begin immediately post-MI and progress for weeks to months at the site of injury, as well as in the surviving uninjured zones (Anversa, Olivetti et al. 1991, Beltrami, Finato et al. 1994).

Of note, peri-infarct border of the injured myocardium is also affected by the ischemic insult but contains viable myocardium contiguous with evolving infarction. At this zone, viable cardiomyocytes experience remarkable changes in cell-cell and cell-extracellular matrix interactions due to the death of neighboring cardiomyocytes and formation of scar tissue (Matsushita, Oyamada et al. 1999). In the past, peri-infarct zone was denoted exclusively by location as a part of myocardium adjacent to the infarcted zone. However, recent research suggests that this region is distinguished by its location and function. An increment in wall stress is observed in the peri-infarct border, causing a decrease in cardiac muscle contractility (Bogen, Rabinowitz et al. 1980). Studies also show that the activity of

Gq α , a protein contributing to myocardial hypertrophy, and phospholipase C elevate in this zone compared to more remote areas (LaMorte, Thorburn et al. 1994). In addition, cardiomyocytes in the peri-infarct zone play an essential role in initiating post-MI inflammation through production of pro-inflammatory mediators such as IL-6 and TNF- α (Frangogiannis 2014).

Immediately after cardiomyocyte death, an over-exaggerated inflammatory response is activated in the infarct/peri-infarct zones (Frangogiannis, Smith et al. 2002). Post-MI inflammation is initiated by necrotic cardiomyocytes to facilitate cellular debris removal (Frangogiannis, Smith et al. 2002). However, this response, characterized by leukocyte penetration into the infarcted myocardium, leads to exacerbated cardiac damage and infarct expansion (Frangogiannis, Smith et al. 2002). In addition to central necrosis, the myocardial injury is further exacerbated by the recruitment of inflammatory cells and their products such as extracellular matrix metalloproteinases (MMPs). Multiple lines of evidence in animal and human studies suggest that a more controlled inflammatory response, and thus, a significantly reduced cardiac injury, can be achieved by limiting polymorphonuclear leukocyte (PMN) recruitment and activation at the site of injured tissue (Hill and Ward 1971, Crawford, Grover et al. 1988). Furthermore, post-MI inflammation is important for proper cardiac healing and scar formation (Entman and Smith 1994, Frangogiannis, Youker et al. 1998, Mehta and Li 1999) .

Reperfusion of the ischemic myocardium precipitates ischemia-reperfusion (I/R) injury, leading to further damage of the infarcted myocardium (Frangogiannis, Smith et al. 2002). I/R injury has a complicated multifaceted myocardial pathology and is mainly influenced by the magnitude/duration of the blockage and the following reperfusion injury. In ischemia, Intracellular pH and ATP concentrations plummet due to anaerobic metabolism and lactate accumulation. As a result, ATPase-dependent ion transport pathways become inactive, leading to increased intracellular and mitochondrial calcium concentrations (calcium overload), cell swelling and rupture, and cell death by necrosis, apoptosis, necroptosis, and autophagy (Kalogeris, Baines et al. 2012). In the reperfusion state, oxygen supply returns to normal levels, however; there is a massive production of Reactive

Oxygen Species (ROS) and intense recruitment of pro-inflammatory neutrophils to the site of injury, exacerbating ischemic damage. The most likely mechanisms driving I/R cell dysfunction, injury, and/or death include: a) resupply of molecular oxygen during the reperfusion phase induces large amounts of ROS, b) intense neutrophil infiltration induces a potent inflammatory response, c) calcium overload, d) deterioration of endothelial function, e) mitochondrial permeability transition (MPT) pore opening, and f) thrombosis (Yellon and Hausenloy 2007, Kalogeris, Baines et al. 2012).

Cardiomyocyte death and extracellular matrix (ECM) deterioration in the injured myocardium produce alarming molecules, known as danger-associated molecular patterns (DAMPs), which initiate and exaggerate the inflammatory response (Timmers, Pasterkamp et al. 2012). Additional initiation of inflammation occurs via the nuclear chromatin-binding protein, High-mobility group box-1 (HMGB1), through activated Toll-Like Receptor (TLR) and Receptor for Advanced Glycation End products (RAGE) (Andrassy, Volz et al. 2008). Molecules, generated from ECM fragments, such as low molecular weight hyaluronan and fibronectin fragments, ATP, and Heat Shock Proteins (HSPs) induce inflammation post-MI as well (Huebener, Abou-Khamis et al. 2008, Dobaczewski, Gonzalez-Quesada et al. 2010, Arslan, de Kleijn et al. 2011, Timmers, Pasterkamp et al. 2012). DAMPs stimulate the complement cascade system, leading to the recruitment and local infiltration of immune cells (Hill and Ward 1971). DAMPs, through TLRs, activate the immune response to remove dead tissues but they also start the healing process (Frangogiannis, Smith et al. 2002). The post-MI inflammatory response is a complicated process controlled by various inflammatory mediators. The most important players in this response are:

1.1.1.1 Complement activation

Necrotic cardiomyocytes release subcellular membrane components enriched in mitochondria, which activate the classical complement cascade components (C1, C2, C3, and C4) (Pinckard, Olson et al. 1975). These components trigger the inflammatory response in the injured myocardium through

a complement-mediated inflammatory response. Protein and mRNA levels of members of the classical complement pathway are elevated at the site of myocardial injury (Vakeva, Agah et al. 1998, Yasojima, Kilgore et al. 1998) . Complement pathway activation contributes to the recruitment of neutrophils and monocytes (Dreyer, Michael et al. 1992). Some studies show that monocyte chemotactic activity in the heart lymphatic drainage in the early phase of the I/R injury is mainly attributed to C5a, a product of complement cascade activation (Birdsall, Green et al. 1997). Research also shows that some agents like cobra venom factor, antibody-induced inhibition of individual complement components, and soluble form of complement receptor type 1, could have some benefits in reducing infarct size and myocardial necrosis through inhibition of the classical complement cascade activity (Weisman, Bartow et al. 1990, Kilgore, Friedrichs et al. 1994, Lucchesi and Kilgore 1997, Czermak, Lentsch et al. 1998, Griselli, Herbert et al. 1999).

1.1.1.2 Reactive oxygen species

Reactive oxygen species (ROS) damage myocardial and vascular tissues through stimulation of cytokine production in the injured myocardium (Dhalla, Elmoselhi et al. 2000, Lefer and Granger 2000). Studies show that ROS are key players in the chemotaxis of leukocytes (Granger 1988) through induction of complementary cascades, upregulation of P-selectin expression, pro-inflammatory chemokine levels increase and potentiation of the capacity of neutrophil interaction with the endothelial Intercellular Adhesion Molecule 1 (ICAM-1) (Patel, Zimmerman et al. 1991, Sellak, Franzini et al. 1994, Lakshminarayanan, Beno et al. 1997, Lakshminarayanan, Drab-Weiss et al. 1998, Akgur, Brown et al. 2000). ROS destroy ECM molecules, degrade phospholipids in the membrane, and thus, induce cell damage and death (Fridovich 1978, Del Maestro, Thaw et al. 1980). Free radical scavengers such as enzymes, superoxide dismutase and catalase, have proven beneficial in reducing scar size in an experimental canine MI model, through inhibition of ROS signaling (Jolly, Kane et al. 1984). Mice that overexpress copper, zinc superoxide dismutase (SOD1) show a significant protection against

ischemic cardiac injury (Wang, Chen et al. 1998). SOD1 is a cytosolic enzyme that efficiently limits intracellular O₂ levels during oxidative stress situations such as I/R, in which multiple cellular oxidases become active (Zweier 1988). Superoxide and the protonated form, $\cdot\text{OOH}$, denature cellular enzymes like aconitase and fumarase, causing a marked change in O₂ levels (Gardner and Fridovich 1992). O₂ interacts with the metal-catalyzed Haber Weiss reaction to produce ROS (Hess and Kukreja 1995). Moreover, O₂ interaction with nitric oxide produces oxidant peroxynitrite, which causes cellular damage via protein nitration (Xia, Dawson et al. 1996). However, some studies including human trials do not show significant recovery in LV function with antioxidants (Gallagher, Buda et al. 1986, Uraizee, Reimer et al. 1987, Richard, Murry et al. 1988, Maxwell and Lip 1997).

1.1.1.3 Extracellular matrix (ECM)

Besides their structural support of the injured myocardium, ECM proteins are important regulators of post-MI inflammation (Frangogiannis 2012). Dynamic changes in ECM components contribute to the inflammatory response through modulation of adjacent fibroblast, endothelial cell, and inflammatory cell activity (Frangogiannis 2012). ECM fragments induce cytokine and chemokine synthesis, plasma-derived matrix accumulation, immune cell extravasation, and initiate the pro-inflammatory response in the early phases post-MI (Frangogiannis 2012, Timmers, Pasterkamp et al. 2012). Indeed, clearance of ECM fragments from the ischemic area is important in resolving inflammation and initiating anti-inflammatory pathways. Moreover, hyaluronan fragments, which are generated via ECM network disruption, may also resolve inflammation via CD44 pathway signaling (Frangogiannis 2012). Additional ECM-related mechanisms controlling post-MI inflammation include a matricellular protein which is significantly increased in the damaged zone, Thrombospondin (TSP)-1, TSP-1 can regulate inflammation through stimulation of TGF- β and activation of anti-inflammatory pathways (Frangogiannis and Entman 2005, Frangogiannis 2012).

1.1.1.4 Pro-inflammatory Chemokines and cytokines

Expression of cytokines and adhesion molecules is simultaneously upregulated after MI injury, and is primarily induced by nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IKK β) (Lenardo and Baltimore 1989). Multiple mediators such as cytokines and ROS stimulate NF- κ B. NF- κ B induces expression of genes that contribute to inflammation, cell adhesion, and growth (Stancovski and Baltimore 1997). Additionally, activation of NF- κ B by TLRs, which are stimulated by complement cascade and ROS, induces production of adhesion molecules in the endothelial cells and pro-inflammatory cytokines and chemokines in fibroblasts, white blood cells, and vascular cells (Nian, Lee et al. 2004, Gordon, Shaw et al. 2011). Suppression of post-MI inflammation and reduction of infarct size are observed with *in vivo* transfer of NF- κ B decoy oligodeoxynucleotides via interference with the action of this transcriptional factor (Morishita, Sugimoto et al. 1997).

Various chemokines are quickly upregulated after infarction, leading to accumulation of inflammatory cells in the ischemic myocardium (Frangogiannis 2007). Neutrophils are attracted to the injured area through CC chemokines, while glutamic acid-leucine-arginine motif (ELR)-negative CXC chemokines attract lymphocytes and may play anti-fibrotic and angiostatic (stopping angiogenesis) roles (Frangogiannis 2014). Leukocytes bind with chemokines on the surface of activated endothelial cells and the matrix through adhesive film interaction (Frangogiannis 2014). Leukocytes express different chemokine receptors, so that certain chemokine/chemokine receptor pairs stimulate and interact to control infiltration of a specific leukocyte subpopulation (Proudfoot, Handel et al. 2003, Nahrendorf, Swirski et al. 2007, Dobaczewski, Xia et al. 2010).

Pro-inflammatory cytokines, such as interleukin 1-beta (IL-1 β), are upregulated and activated in the injured myocardium immediately after insult (Bujak and Frangogiannis 2009). IL-1 β production depends on the creation of multiprotein oligomers, known as inflammasomes (Schroder and Tschopp 2010). Activation of inflammasomes occurs in leukocytes and resident fibroblasts in the

infarcted area and in cardiomyocytes in the border zone (Kawaguchi, Takahashi et al. 2011, Mezzaroma, Toldo et al. 2011). IL-1 β promotes the expression of multiple inflammatory genes; including inflammatory cytokines, chemokines, adhesion molecules, inducible nitric oxide synthase (iNOS), type 2 cyclooxygenase, and type 2 phospholipase A2 (Bujak and Frangogiannis 2009). As a result, IL-1 β may induce more damage to myocardium via recruitment of additional immune cells to the heart (Bujak and Frangogiannis 2009). IL-1 β levels are elevated in MI patients in the first few hours after attack (Guillen, Blanes et al. 1995). Indeed, recent evidence in patients with chronic atherosclerotic disease suggests that targeting IL-1 β with the specific monoclonal antibody, canakinumab, significantly decreases recurrent cardiovascular events (Ridker, Everett et al. 2017).

Data from our lab and others documented the early expression of tumor necrosis factor-alpha (TNF- α) in the myocardium after ischemic injury (Gordon and Galli 1990). TNF- α acts upstream of IL-6, since neutralizing antibodies for TNF- α can block the production of IL-6 (Frangogiannis, Smith et al. 2002). TNF- α acts through multiple receptors with divergent actions (TNF-R1 and TNF-R2). TNFR1 activation mediates NF- κ B activation, cardiomyocyte apoptosis and inflammation in the infarct and peri-infarct zones (Hamid, Gu et al. 2009). On the contrary, TNF-R2 is crucial for the healing process via reduction of inflammation and NF- κ B activation and enhancement of cell survival (Hamid, Gu et al. 2009). TNF- α , mainly produced by macrophages, is a cornerstone to the initiation and maintenance of the inflammatory response after MI. Indeed, elevated levels of TNF- α in humans suffering MI are correlated with more rapid deterioration in heart function and higher risk of mortality (Dunlay, Weston et al. 2008). IL-6 family members play an important role in both cardiac inflammation and healing after infarction (Fischer and Hilfiker-Kleiner 2008). Studies employing an anti-IL-6 receptor antibody show significant reduction in cardiac inflammation, infarct size, and adverse cardiac remodeling (Kobara, Noda et al. 2010). However, the role of IL-6 in post-MI is multifaceted and studies utilizing genetic deletion of IL-6 do not yield improvement in infarct size, LV function, or chronic adverse cardiac remodeling, suggesting a

role for IL-6 in proper cardiac healing (Fuchs, Hilfiker et al. 2003). More information about targeting certain cytokine/chemokine and other signaling pathways is located in Table 1.1.

1.1.1.5 Cellular players in inflammation after ischemic cardiac injury:

Multiple populations of bone-marrow (BM) derived and cardiac cells are involved in the inflammatory response after MI. The most important players in post-MI inflammation include:

1.1.1.5.1 Cardiomyocytes

Cardiomyocyte death stimulates the inflammatory response post-MI through release of several alarming/danger signals. Additionally, cardiomyocytes in the border zone express the inflammasome, leading to the production of IL-1 β , which subsequently upregulates the inflammatory response (Frangogiannis 2014).

1.1.1.5.2 Platelets

Platelets actively participate in the regulation of inflammatory and reparatory phases following cardiac injury (von Hundelshausen and Weber 2007). They contribute to the production of chemokines, cytokines, and growth factors in the injured myocardium (Frangogiannis 2014). Activated platelets, through formation of a fibrin-based provisional matrix, enhance hematopoietic cell influx into the infarct /peri-infarct zones (Dobaczewski, Xia et al. 2010).

1.1.1.5.3 Mast cells

Unlike small animals, the heart of large animals is occupied by a large number of mast cells (Gersch, Dewald et al. 2002). A canine model of experimental MI observed that resident mast cells in the injured zones exhibit a quick and massive degranulation after infarction, leading to the release of high quantities of pre-synthesized pro-inflammatory mediators like TNF- α and histamine (Frangogiannis, Lindsey et al. 1998). Degranulation of mast cells was found exclusively in the ischemic and reperfused zones of the injury. TNF- α induces IL-

6 production, leading to additional recruitment of mononuclear cells to the injured myocardium. In addition, histamine is an important autacoid in I/R injury via promotion of IL-6 production and leukocyte movement. However, recent work investigating the role of mast cells after infarction in mice shows that mast cells protect cardiomyocytes from damage post-MI and promote contractility (Ngkelo, Richart et al. 2016).

1.1.1.5.4 Dendritic cells

Although myocardial damage activates dendritic cells, their exact role remains unclear. Findings are contradictory; some studies show beneficial effects via modulation of post-MI inflammation and production of IL-10 (anti-inflammatory cytokine). Other studies show that these cells activate and prime CD+4 T cells in the setting of MI (cardiac-specific autoreactive cells), leading to cardiac autoimmune attack and damage (Anzai, Anzai et al. 2012, Van der Borgh, Scott et al. 2017).

1.1.1.5.5 Fibroblasts

The second most abundant population of cells in the heart is fibroblasts (Souders, Bowers et al. 2009). After MI they are activated, stimulating the inflammasome platform (Kawaguchi, Takahashi et al. 2011), which leads to an increase in IL-1 β production. Cardiac fibroblasts are also capable of producing chemokines and cytokines and, therefore; they are directly involved in modulation of the inflammatory response after MI (Frangogiannis 2014).

1.1.1.5.6 Endothelial cells

Endothelial cells are key players in inflammation after MI via production of pro-inflammatory chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interferon gamma-induced protein 10 (IP-10), in the affected territory. Additionally, production of cytokines in the infarcted area stimulates the expression of adhesion molecules on the surface of endothelial cells, which causes a profound adhesion of circulating inflammatory cells and initiates their migration into the

injured myocardium (Frangogiannis, Mendoza et al. 2001, Bujak, Dobaczewski et al. 2009). Hence, production of pro-inflammatory chemokines and adhesion molecules by endothelial cells at local sites of myocardial injury is crucial to the initiation and maintenance of the inflammatory response post-MI.

1.1.1.5.7 Vascular cells and pericytes

Angiogenesis is an essential element of the healing process (Frangogiannis 2014). Wound healing of the injured myocardium is highly correlated with the intensity of angiogenesis since more nutrients and oxygen will be available through a plentiful vascular network. Enhanced production of angiogenic growth factors facilitates the formation of a hyperpermeable microvascular network (Frangogiannis 2014). Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and other angiogenic growth factors are generated and released in the first hours after cardiac insult (Li, Brown et al. 1996, Lee, Wolf et al. 2000, Frangogiannis 2014). Inhibiting the formation of granulation tissue, enhancing the resolution of inflammation, and stabilization of scar tissue are processes regulated by the maturation of new blood vessels at the infarct/peri-infarct zones (Zymek, Bujak et al. 2006, Frangogiannis 2014). Platelet-derived growth factor (PDGF) is important in myocardial healing post-MI. PDGF, through activation of the PDGF receptor, stimulates mural cell recruitment through the vasculature into the infarcted myocardium, leading to accelerated acquisition of a mural coat by these newly formed blood vessels (Zymek, Bujak et al. 2006).

1.1.1.5.8 Lymphocytes

Multiple lymphocyte subpopulations (CD4⁺ and CD8⁺ T-cells, Foxp3⁺regulatory cells (Tregs), invariant natural killer (iNK) T-cells, and $\gamma\delta$ T-cells) infiltrate the injured heart. Regulatory T-cells (Tregs) play a key role in the immunomodulatory changes after MI (Frangogiannis, Mendoza et al. 2000, Hofmann and Frantz 2015). Recent evidence suggests that Tregs are involved in the suppression of post-MI inflammation through modulation of the macrophage and fibroblast response (Dobaczewski, Xia et al. 2010, Saxena, Dobaczewski et

al. 2014, Weirather, Hofmann et al. 2014). Upregulation of INF-gamma by Tregs induces anti-fibrotic changes in the heart through overexpression of IP-10 (anti-fibrotic chemokine CXCL10/IFN- γ) (Bujak, Dobaczewski et al. 2009, Ramjee, Li et al. 2017). In addition, it has been observed that CD4⁺ T-cell-deficient mice have higher levels of leukocytes and pro-inflammatory monocytes with more deteriorated cardiac remodeling during follow-up (Liu, Wang et al. 2016).

Apart from Tregs, circulating cytotoxic T (CD8) lymphocytes are elevated in the first week post-MI, suggesting a potential role in post-MI inflammation. In a rat model of experimental MI, harvesting these cells from the heart after infarction and culturing them with healthy neonatal cardiomyocytes induces a cytotoxic effect on cardiomyocytes (Varda-Bloom, Leor et al. 2000). However, whether their recruitment to the injured myocardium exacerbates injury has not been established yet (Ong, Hernandez-Resendiz et al. 2018). Finally, B lymphocytes are also crucial players in the pathology of MI and adverse cardiac remodeling. Mature B lymphocytes secrete large amounts of CCL7 and stimulate Ly6C^{hi} monocyte migration and infiltration to the injured myocardium, leading to progression of cardiac injury and poor recovery (Zouggari, Ait-Oufella et al. 2013).

1.1.1.5.9 Neutrophils

The first population of inflammatory cells that infiltrates the injured myocardium is neutrophils (Figure 1.1) (Reimer, Murry et al. 1989). This influx is dependent on the complement cascade, ROS synthesis, and ELR/CXC chemokines (Frangogiannis 2014). Neutrophils are attracted to the MI zone through complement C5a, which is an important mediator in the stimulation and chemoattraction of neutrophils (Reimer, Murry et al. 1989). Research shows that depletion of complement system using cobra venom factor blocks polymorphonuclear neutrophil (PMN) recruitment and activation and reduces myocardial damage after I/R injury (Hill and Ward 1971, Crawford, Grover et al. 1988). These outcomes are likely related to the effects of C3a, C4a, and C5a on vessels, to the increased vascular permeability, to the altered vessels tone, and to the production of cytolytic membrane attack complex (C5b-9). C5b-9 induces

formation of transmembrane channels, promotes calcium influx to the cell, and interferes with cardiomyocyte function.

Conversely, other studies show that endothelial cells may be a primary source for certain neutrophil chemotactic factors (Strieter, Kunkel et al. 1988). For instance, TNF- α and/or IL-1 β treatment activates endothelial cells to produce a neutrophil chemotactic factor, with a similar structure to the human monocyte-derived neutrophil activating factor (NAF) (Thelen, Peveri et al. 1988). Hypoxia/reperfusion challenge to endothelial cells stimulates neutrophil attachment to the surface of endothelial cells (Reimer, Murry et al. 1989), via an interaction between neutrophil CD18 and endothelial intracellular adhesion molecule-1 (ICAM-1) (Smith, Rothlein et al. 1988). In addition, endothelial leukocyte adhesion molecule-1 (ELAM-1) can also facilitate adhesion between neutrophils and endothelial cells (Bevilacqua, Pober et al. 1987, Smith, Marlin et al. 1989). Chemoattraction of neutrophils to the injured myocardium is further enhanced in the first 48 hours by the upregulated activity of myocardial myeloperoxidase (Mullane, Kraemer et al. 1985).

In acute inflammation, neutrophils are essential in removing dead cells and debris, as well as in resolving inflammation and maintaining homeostasis (Ortega-Gomez, Perretti et al. 2013). Recruited neutrophils secrete products to facilitate inflammatory cell infiltration to the infarcted zone (Frangogiannis 2012, Ma, Yabluchanskiy et al. 2013). However, these products are toxic and may lead to more damage to the local cardiomyocytes and endothelium (Reimer, Murry et al. 1989). Neutrophils release a wide range of products such as proteolytic enzymes (producing liquefaction of cells), platelet-activating factor, arachidonic acid metabolites, and ROS (Reimer, Murry et al. 1989). ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and hypochlorous anion (OCL^-) (Reimer, Murry et al. 1989), are mainly produced by neutrophils after infarction. ROS destroy extracellular matrix molecules, degrade phospholipids in the membrane, and thus, induce cell damage and death (Fridovich 1978, Del Maestro, Thaw et al. 1980). Activated neutrophils produce a positive feedback mechanism by converting phospholipids in the cell membrane into arachidonic

acid, which then transforms into a strong chemotactic molecule, hydroxy-eicosatetraenoic acid (HETE), by the effect of certain lipoxygenases. HETEs are important chemoattractants responsible for further neutrophil infiltration into the injured myocardium (Snyderman and Goetzl 1981). In addition, neutrophils generate an array of pro-inflammatory cytokines which further recruit mononuclear cells (Frangogiannis 2014).

Prolonged neutrophil accumulation in the heart post-MI is deleterious (Chia, Nagurney et al. 2009, Guasti, Dentali et al. 2011). Some studies show that there is a beneficial “time window” for neutrophil infiltration into the infarcted myocardium, beyond which persistent neutrophil accumulation leads to infarct expansion and exacerbated heart failure (Simpson, Fantone et al. 1988). Studies show that inhibiting neutrophil stimulation and infiltration into the injured myocardium is effective in preventing infarct expansion and irreversible myocardial damage (Reimer, Murry et al. 1989). Systemically, high neutrophil counts after MI are associated with progression of atherosclerosis and comorbid complications in murine models (Doring, Drechsler et al. 2015). Clinical data suggests that any further delay in neutrophil apoptosis after MI contributes to this exaggerated inflammatory response (Garlischs, Eskafi et al. 2004).

While controversial, research shows neutrophil products released post-MI can influence the recruitment of different subsets of monocytes (Ly6C^{hi} and Ly6C^{lo}) in the first week after MI in mice (Nahrendorf, Swirski et al. 2007). Neutrophils may play a role in the migration and local activation of the predominant cardiac monocytes, Ly6C^{hi} (classical, pro-inflammatory), 4 days after injury (Soehnlein, Zernecke et al. 2008, Frodermann and Nahrendorf 2017). Additionally, animal data suggests that neutrophils negatively affect myocardial healing by selectively recruiting pro- rather than anti-inflammatory monocytes (Nahrendorf, Swirski et al. 2007, Soehnlein, Zernecke et al. 2008). Indeed, treatment with monoclonal antibodies against either α or β subunits of surface membrane adhesion glycoprotein (Mo1), which facilitates neutrophil aggregation and monocyte/neutrophil interaction, is protective against myocardial damage and reduces myocardial risk area (Simpson, Todd et al. 1990).

Although there is wide acceptance that neutrophils are important mediators of cardiac injury after ischemia, aggressive neutrophil depletion studies following MI have shown contradictory results (Horckmans, Ring et al. 2017). Some studies show reduced scar size with neutrophil depletion, while others fail to demonstrate any beneficial effects (Romson, Hook et al. 1983, Chatelain, Latour et al. 1987). The migrated and accumulated neutrophils in the cardiac tissue may cause more tissue damage due to dysregulated repair processes. A recent study shows that depletion of neutrophils using anti-Ly6G antibody in a murine experimental MI model reduces Ly6C^{hi} monocyte (pro-inflammatory) release from the spleen and accumulation in the injured myocardium. Moreover, there was an increase in the proliferation of macrophages in the anti-inflammatory activation state in the injured myocardium, and impaired heart healing (Horckmans, Ring et al. 2017). These changes improve with resupply of the neutrophil product, neutrophil-derived lipocalin (NGAL), which acts via myeloid-epithelial-reproductive tyrosine-protein kinase (merck) to enhance cardiomyocyte efferocytosis, suggesting an essential role for neutrophils in the healing process after infarction. (Horckmans, Ring et al. 2017). Aggressive depletion of neutrophils, the first immune cells infiltrating the injured myocardium responsible for inflammation regulation, may not be the most practical method examining the role of these cells in healing since this strategy may disturb the entire inflammatory response. To our knowledge, this is the first study showing that M2 macrophages may have detrimental effects on cardiac recovery. They identified M2 macrophages using 3 markers (arginase, YM1, and IL-4). Further research shows that this is not sufficient analysis to determine specific macrophage phenotype. Their view of the macrophage-neutrophil interaction as merely dependent on activation of merck by NGAL to resolve post-MI inflammation does not take into consideration that cardiomyocytes can directly activate merck in macrophages through efferocytosis (Wan, Yeap et al. 2013). Using more specific strategies, like NGAL^{-/-} specific knock out in neutrophils or inhibition of neutrophil adhesion, are probably better in identifying the effects of neutrophils in the healing process.

These contradictory results targeting neutrophils may be due to a dual role of neutrophils in cardiac inflammation and healing. While the initial wave of inflammatory/primed neutrophils can lead to exaggerated cardiac damage if not cleared in a timely fashion, the activity of neutrophil clearing can reduce inflammation and initiate the healing process. Clinical data suggests that delayed neutrophil apoptosis after MI contributes to an exaggerated inflammatory response (Garlachs, Eskafi et al. 2004). Additionally, a recent report shows that the neutrophil/lymphocyte ratio positively correlates to myocardial necrosis and C-reactive protein (CRP) elevation (Tahto, Jadric et al. 2017). Apoptotic neutrophils have anti-inflammatory and angiogenic effects through production of apoptotic factors (annexin A1 and lactoferrin) and polarization of macrophage activation states to the anti-inflammatory phenotype which results in production of inflammatory resolving mediators, such as IL-10 and TGF- β (Soehnlein and Lindbom 2010).

Resolution of cardiac inflammation after MI occurs 3-7 days after the acute event (Dewald, Ren et al. 2004). It is likely that apoptotic cell removal from the injured myocardium is the most influential cellular mechanism in resolution of inflammation due to production of inhibitory mediators by phagocytes (Huynh, Fadok et al. 2002). The life-span of neutrophils is somehow increased in the presence of TNF- α and IL-1 β , in the early pro-inflammatory microenvironment (Colotta, Re et al. 1992). Monocytes and differentiated macrophages sterilize the site of injury through phagocytosis of dead cardiomyocytes and apoptotic neutrophils (Frodermann and Nahrendorf 2017). The mechanisms of inflammation resolution attributed to engulfment of apoptotic granulocytes include: a) neutrophil death generates resolving mediators which inhibit inflammatory cell recruitment to the heart (Bournazou, Pound et al. 2009), b) macrophage engulfment of apoptotic neutrophils stimulates the anti-inflammatory machinery to produce inflammation resolving cytokines (IL-10 and TGF- β) and lipid mediators and reduce pro-inflammatory cytokine production (Soehnlein and Lindbom 2010).

The disparate contributions of neutrophils to MI pathology may relate to multiple activation states of a single cell type. Recent work investigating the

temporal activation of neutrophils reports that they have two distinct activation states in ischemic heart: pro-inflammatory (N1) (CD11b⁺/Ly6G⁺/CD206⁻) and anti-inflammatory (N2) (CD11b⁺/Ly6G⁺/CD206⁺) (Ma, Yabluchanskiy et al. 2016). The N1 phenotype is associated with LV thinning and dominates the heart after infarct. The N2 phenotype increases in abundance over time and correlates with proper healing (Ma, Yabluchanskiy et al. 2016). Interestingly, post-MI migrating neutrophils are CD206⁻ in the bloodstream, then become CD206⁺ in the heart, which could suggest that the activation state is changed by microenvironment (Ma, Yabluchanskiy et al. 2016). Accordingly, this may explain the disparate outcomes of strategies targeting neutrophils, and at the same time, could represent an attractive therapeutic avenue in MI treatment via reprogramming of neutrophils to the anti-inflammatory phenotype.

1.1.1.5.10 Monocytes

Monocytes and macrophages, as members of the innate immunity system, play crucial roles in the heart at steady state, as well as after injury (Hulsmans, Sam et al. 2016). They are the second most prevalent immune cells in the injured myocardium, after the early neutrophil accumulation (Zlatanova, Pinto et al. 2016). In mice, there are two subsets of monocytes: a) Ly6C^{hi} chemokine (C-C motif) receptor-2 (CCR2)^{hi} chemokine (C-X3-C motif) receptor-1 (CX3CR1)^{lo} (classical/pro-inflammatory), which infiltrate the heart early, immediately after neutrophils (day1), and disappear during the resolution phase of inflammation, b) Ly-6C^{lo}CCR2^{lo}CX3CR1^{hi} monocytes (nonclassical/anti-inflammatory), which are recruited to the heart beginning on the third day after injury (Nahrendorf, Swirski et al. 2007, Nahrendorf and Swirski 2013, Hulsmans, Sam et al. 2016). Monocytes migrate to the heart from the bone marrow (BM) and spleen in two sequential periods of monocyte movement (Nahrendorf, Swirski et al. 2007, Nahrendorf and Swirski 2013). In humans, research has identified three subsets of monocytes based on the expression of CD14 and CD16: a) classical/pro-inflammatory (CD14⁺⁺CD16⁻), b) intermediate (CD14⁺⁺CD16⁺), and c) nonclassical/anti-inflammatory (CD14⁺CD16⁺⁺) monocytes (Hulsmans, Sam et al. 2016). It is likely

there are very few monocytes in the heart during steady state (Hulsmans, Sam et al. 2016). Information about the classification and surface markers of immune cells in the heart after MI is found in Table 1.2.

In BM, hematopoietic stem cells (HSCs) give rise to common myeloid progenitors. These progenitors differentiate into granulocyte myeloid progenitors that give rise to Ly6C^{hi} monocytes (Hettinger, Richards et al. 2013). Ly6C^{hi} monocytes migrate from the BM to the peripheral blood, a process controlled by the monocyte chemoattractant protein-1 (MCP1)/CCR2 signaling axis (Serbina and Pamer 2006, Hulsmans, Sam et al. 2016). Studies show that the Ly6C^{lo} monocytes derive from Ly6C^{hi} under the control of a nuclear receptor subfamily-4-dependent transcriptional process (Hanna, Carlin et al. 2011). Although it is rare for this to happen in the steady state condition, HSCs may also migrate to extramedullary hematopoietic sites such as the spleen and initiate monocytopoiesis after tissue injury (Leuschner, Rauch et al. 2012, Robbins, Chudnovskiy et al. 2012). The spleen may also host monocytes, providing an important source of rapid response to tissue injury and inflammation, as seen in the first 24 hours after MI (Swirski, Nahrendorf et al. 2009). Under the control of adrenergic stimuli, bone marrow HSCs move towards the spleen and reside there. In the first 24 hours after MI, Ly6C^{hi} monocyte recruitment to the heart occurs, mainly from the spleen (40%) (Leuschner, Rauch et al. 2012, Honold and Nahrendorf 2018).

Ly6C^{hi} monocytes differentiate into pro-inflammatory macrophages under the effect of M-CSF. These two populations are involved in the removal of dead cells and tissue debris via phagocytosis, resulting in production of proteolytic enzymes, which may lead to increased tissue damage. On the other hand, Ly6C^{lo} monocytes participate in tissue healing via induction of angiogenesis, fibroblast proliferation, and collagen accumulation (Dutta and Nahrendorf 2015). Ly6C^{lo} monocytes are the primary sources of fibronectin, which is essential in stabilizing the infarct region and preventing cardiac rupture (Frangogiannis, Smith et al. 2002). Similar to neutrophilia, profound monocytosis disturbs healing post-MI, while inhibition of pro-inflammatory monocyte recruitment improves cardiac

recovery (Dutta and Nahrendorf 2015). Studies show that MCP-1 deletion, an important regulator of monocyte recruitment to the injured myocardium, results in a substantial decrease in monocyte infiltration into the heart. Although MCP-1 deficient mice did not differ in scar size from wild type mice, they had significant improvement in LV function, implying the importance of monocyte repair processes in the infarcted myocardium (Dewald, Zymek et al. 2005). In human studies, an increased number of pro-inflammatory monocytes after MI correlates with higher incidence of future clinical events (Han, Jing et al. 2014). Moreover, clinical studies reveal that elevated levels of inflammatory blood monocytes CD14⁺CD16⁻ in MI patients increase the risk of poor myocardial salvage and heart failure during long term follow-up (Maekawa, Anzai et al. 2002, Tsujioka, Imanishi et al. 2009).

1.1.1.5.11 Macrophages

At steady state, macrophages are the primary immune cells in organs such as the liver, brain, and heart (Hulsmans, Sam et al. 2016). In the murine heart, ~10% of non-cardiomyocyte cells are macrophages with a comparable percentage in the human heart (Pinto, Paolicelli et al. 2012, Heidt, Courties et al. 2014). Macrophages have a spindle-like shape and typically exist in the interstitial space or near endothelial cells (Nahrendorf, Swirski et al. 2007, Pinto, Paolicelli et al. 2012, Heidt, Courties et al. 2014). Macrophage numbers significantly increase post-MI due to infiltration of monocytes and the enhanced self-renewal capacity of resident macrophages (Heidt, Courties et al. 2014). As illustrated in lineage tracing studies, there are two major macrophage populations in the adult heart, classified according to their origin. The first population is embryonically-derived cardiac resident macrophages; these cells are present at birth and are maintained largely by local proliferation. The second subset is Ly6C^{hi}-differentiated bone BM-derived macrophages (Ginhoux, Greter et al. 2010, Schulz, Gomez Perdiguero et al. 2012). Of note, intestinal and dermal macrophages derive mostly from Ly6C^{hi} cells in peripheral blood, originated from the BM, due to the fast-turnover rate (Zigmond, Varol et al. 2012, Tamoutounour, Guilliams et al. 2013). A recent study shows that the self-renewal capacity of embryonically derived macrophages substantially

decreases with age and BM-derived monocytes become the major source of macrophages, even under physiological conditions (Molawi, Wolf et al. 2014).

Under normal physiological status, most cardiac macrophages are embryonically derived. These cells classify into multiple subsets based on the expression of histocompatibility-2 (MHC-II) and CCR2 (Epelman, Lavine et al. 2014). Embryonically derived macrophages primarily originate from embryonic yolk-sac progenitors and from local proliferation of cardiac macrophages, without major contribution from BM-derived monocytes. Most cardiac resident macrophages are MHC-II^{lo} CCR2⁻ (Epelman, Lavine et al. 2014, Leid, Carrelha et al. 2016). The second largest population of macrophages is MHC-II^{hi} CCR2⁻, arising from MHC-II^{lo} CCR2⁻ macrophages (Epelman, Lavine et al. 2014). The third and most rare population is CCR2⁺ cells, which generate from hematopoiesis in the fetal liver and migrate during embryonic development to the heart (Epelman, Lavine et al. 2014, Leid, Carrelha et al. 2016, Honold and Nahrendorf 2018).

Embryonically-derived CCR2⁻ macrophages are primarily located in the myocardial wall and play crucial developmental roles in the formation of blood vessels and development of the heart conduction system (adult atrioventricular and sinus nodes) (Honold and Nahrendorf 2018). Research shows that macrophages communicate with cardiomyocytes through Cx43 (connexin 43) gap junctions and depolarize simultaneously with attached cardiomyocytes (Pinto, Paolicelli et al. 2012, Honold and Nahrendorf 2018). Conversely, CCR2⁺ macrophages reside in the trabecular projections of the endocardium and have a limited role in heart development (Honold and Nahrendorf 2018). Cardiac resident macrophages can also contribute to early neutrophil extravasation after infarction by production of CXCL (C-X-C motif chemokine ligand) 2 and CXCL5, facilitating transendothelial leukocyte migration into ischemic myocardium (Hulsmans, Clauss et al. 2017, Honold and Nahrendorf 2018).

Although it remains highly controversial, the belief is that Ly6C^{hi} monocytes differentiate into Ly6C^{lo} macrophages shortly after migration to the heart. Research shows that Ly6C^{lo} macrophages infiltrate the injured myocardium directly from the blood in the reparative phase. They reside in the heart for many weeks in low

numbers with unclear function and limited capacity to renew locally (Leuschner, Rauch et al. 2012, Courties, Heidt et al. 2014, Hilgendorf, Gerhardt et al. 2014). Another controversial finding is that cardiac macrophages with embryonic origin die locally soon after infarction, therefore; they are not involved in post-MI inflammatory events (Leuschner, Rauch et al. 2012).

Macrophage function is primarily dependent on the surrounding environment. Adoptive studies show that lung transplantation of peritoneal macrophages leads to significant changes in the gene expression profiles and function of macrophages, which were compatible in new environments (Lavin, Winter et al. 2014). Steady state macrophage function is not fully understood in the heart, however; gene-profiling studies suggest they are vital for angiogenesis (Pinto, Paolicelli et al. 2012, Honold and Nahrendorf 2018). In addition, macrophages perform immunological cardiac functions such as regulation of infection defense and removal of dead cells (Ma, Mouton et al. 2018).

Following MI, infiltrated monocytes/macrophages phagocytize dead cardiomyocytes and neutrophils, thereby removing cellular and tissue debris from the injured myocardium (Figure 1.1). These cells produce inflammatory cytokines, proteases, and ROS (Frodermann and Nahrendorf 2017). It has been observed that macrophage depletion strategies in an experimental MI model cause progressive impairments in post-MI inflammation and, ultimately, cardiac recovery (van Amerongen, Harmsen et al. 2007, Leblond, Klinkert et al. 2015). Besides that, a more severe form of cardiac remodeling occurs with increased macrophage numbers, as reported in ApoE^{-/-} (apolipoprotein E)-deficient or chemokine Decoy Receptor D6 (resolving CC chemokine-driven inflammatory responses)-deficient mice (Panizzi, Swirski et al. 2010, Cochain, Auvynet et al. 2012). While these observations may appear contradictory at face value, careful teasing apart of the used strategies in these studies can elucidate. Macrophages are very heterogeneous and plastic populations of cells with various and rather complementary functions. Interventions that specifically target a certain category or function of macrophages could be

more useful for better understanding the immunopathology of MI, unlike general suppression or enhancement of post-MI inflammation.

In addition to their primary function (phagocytosis and efferocytosis), macrophages produce large amounts of chemokines/cytokines/lipid signaling molecules after injury (Swirski and Nahrendorf 2013, Ma, Mouton et al. 2018), which enable them to communicate with other cells in the heart and other tissues (Honold and Nahrendorf 2018). Macrophages secrete MMPs and proteolytic enzymes, which degrade ECM and accelerate tissue remodeling and scar formation (Honold and Nahrendorf 2018). Additionally, macrophages produce copious amounts of anti-inflammatory cytokines, such as TGF- β , an essential player in cardiac healing and scar stabilization (Frangogiannis, Smith et al. 2002, Honold and Nahrendorf 2018). Macrophages induce angiogenesis through secretion of Vascular Endothelial Growth Factor (VEGF) (Nahrendorf, Swirski et al. 2007). The multiple polarization and activation states of macrophages govern their diversity in function (Ma, Mouton et al. 2018). Macrophage activation states are reflected by different gene expression profiles and functions, and are influenced by different microenvironments, time after injury, macrophage origin, and age (Nahrendorf and Swirski 2016, Ma, Mouton et al. 2018). Post-MI, macrophages are generally classified into pro-inflammatory/classically activated/M1-like or anti-inflammatory/alternatively activated/reparative/M2-like based on genetic and cytokine profiles and functions (Nahrendorf and Swirski 2016, Ma, Mouton et al. 2018).

Of note, macrophage classification into pro- and anti-inflammatory phenotypes is oversimplified and is mainly derived from *in vitro* systems and artificial stimulation strategies (Nahrendorf and Swirski 2016). *In vivo*, the activation state of macrophages more realistically represents a spectrum between pro- and anti-inflammatory phenotypes, which is highly influenced by surrounding microenvironment. Removing macrophages from these highly complex conditions may affect their activation state, as seen in microglia after 7 days in culture (Gosselin, Link et al. 2014, Nahrendorf and Swirski 2016). Secondly, M1 and M2 may represent two extremes of activation state, leading one to ignore important

transitionally activated macrophages (Gosselin, Link et al. 2014). Activation of human monocytes with M-CSF or G-CSF followed by various stimuli generates macrophages with distinctly different profiles from the traditional M1/M2 classifications, which may be relevant to cardiovascular disease (Xue, Schmidt et al. 2014, Nahrendorf and Swirski 2016). Despite the limitations in the M1/M2 paradigm, it remains a helpful tool in examining changes in macrophage phenotype and respective function in response to various stimuli, environmental changes, therapeutic interventions, etc. Below, we summarize the main characteristics of pro- and anti-inflammatory macrophages.

1.1.1.5.11.1 Classically activated macrophages (M1; pro-inflammatory)

Activated T-helper 1 lymphocytes (Th-1), which secrete large amounts of IFN- γ and other pro-inflammatory mediators like lipopolysaccharide (LPS) and cytokines (TNF- α , GM-CSF) interact with and classically activate macrophages (Mantovani, Sica et al. 2004, Gombozhapova, Rogovskaya et al. 2017). Further classification of M1 macrophages subdivides them into M1a if they are activated through TLRs or M1b if they are activated through high mobility box group protein 1 (HMBG1), with lower phagocytic capability than M1a macrophages (Ben-Mordechai, Palevski et al. 2015, Ma, Mouton et al. 2018). M1 macrophages are the dominant myocardial macrophages in the first 3 days after MI (Ma, Mouton et al. 2018). Activated M1 macrophages phagocytize cellular debris, inducing changes in MHCII configuration and other cell surface markers (Bauersachs, Galuppo et al. 2001, Murray, Allen et al. 2014). This process also stimulates production of pro-inflammatory cytokines (IL12, IL-23, IL-27, TNF) and chemokines (CXCL9, CXCL10, CXCL11) and expression of cell surface markers (CD40, CD80, CD86) (Bauersachs, Galuppo et al. 2001). In addition, classically activated macrophages release high quantities of nitric oxide, ROS, and proteolytic enzymes (MMP-1, MMP-2, MMP-7, MMP-9, MMP-12), which break down fibronectin, collagen, elastin and other extracellular components (Mantovani, Biswas et al. 2013, Mulder, Banete et al. 2014, Ma, Mouton et al. 2018). While these mechanisms are essential for clearing damaged cardiac tissue after MI, if

left unchecked they can exacerbate the ischemic damage, expand the infarct region, and delay inflammation resolution (Ma, Mouton et al. 2018). More information about pro-inflammatory macrophages is found in Table 1.3. More information about markers of different subsets of immune cells is present in Table 1.4.

1.1.1.5.11.2 Alternatively activated macrophages (M2; reparative)

Macrophages alternatively activate in response to anti-inflammatory mediators such as IL-4 and IL-13, produced by T-helper lymphocytes (Th-2) (Gombozhapova, Rogovskaya et al. 2017). Additional mediators, such as IL-12, IL-33, and IL-34, also cause M2 activation (Martinez, Gordon et al. 2006, Pesce, Kaviratne et al. 2006, Hazlett, McClellan et al. 2010). Moreover, M1 macrophages may be repolarized into M2 through treatment with pro-M2 factors, as detailed here (Pelegriin and Surprenant 2009).

M2 macrophages are further subdivided into M2a, M2b, and M2c subsets (Nahrendorf and Swirski 2013) based on mechanism of activation, function, and gene profile. For example, M2a and M2c macrophages are important players in the adaptive immune response, while M2b macrophages are crucial for regulation and suppression of inflammation (Lindsey, Saucerman et al. 2016, Gombozhapova, Rogovskaya et al. 2017). Phenotypes can shift from one state to another as shown in both *in vivo* and *in vitro* experiments (Gombozhapova, Rogovskaya et al. 2017, Ma, Mouton et al. 2018). Recent evidence indicates a new M2 macrophage subset, M4, identified in response to monocyte activation with CXCL4 (Gleissner, Shaked et al. 2010). This phenotype expresses both M1 (CD86 and major histocompatibility complex II) and M2 (mannose receptor and fibrinogen-like protein 2) genes and their corresponding cytokines, while phagocytic function is almost entirely lost (Gleissner, Shaked et al. 2010).

M2 is the most dominant macrophage phenotype in the myocardium 5-7 days after injury (Yan, Anzai et al. 2013, Ma, Mouton et al. 2018). These cells phagocytize necrotic cells to accelerate inflammation resolution, forming scar tissue (Ma, Mouton et al. 2018). These macrophages characteristically produce

large amounts of IL-10, IL-1ra, and decoy type II receptors. Receptors such as mannose (e.g. CD206), scavenger, galactose-type, and CD163 are also highly expressed by this phenotype (Bauersachs, Galuppo et al. 2001, Dinarello 2005, Gombozhapova, Rogovskaya et al. 2017). Furthermore, M2 macrophages secrete CCL17, CCL22, CCL24 and TGF- β , which mediate inflammation resolution and demonstrate anti-inflammatory effects (Kzhyshkowska, Gratchev et al. 2006, Mantovani 2008, Medzhitov and Horng 2009). TGF- β stimulates fibroblast generate of extracellular matrix components, as well as induction of myofibroblast differentiation (Gombozhapova, Rogovskaya et al. 2017). M2 macrophages secrete TGF- β , insulin like growth factor, and platelet-derived growth factor, which and promote angiogenesis and cell proliferation (Gombozhapova, Rogovskaya et al. 2017).

After infarct, the macrophage life-span is approximately 20 hours. They are continuously replenished by infiltrating monocytes and the phenotypic changes of infiltrated macrophages (Lindsey, Saucerman et al. 2016). In humans, macrophages are recruited to the heart in a similar pattern but with a delayed kinetic due to age-related changes in the immune system (Ma, Mouton et al. 2018). Reperfusion strategies inhibit cardiac immune cell infiltration, resulting in an accelerated timeline of innate immune activation and reduced adaptive immune response (Yan, Anzai et al. 2013, Ma, Mouton et al. 2018). More information about anti-inflammatory macrophages is found in Table 1.3. More information about markers of different subsets of immune cells is present in Table 1.4.

1.1.1.6 Inflammation resolution after cardiac ischemia

Timely resolution of post-MI inflammation is important to prevent adverse cardiac remodeling and subsequent heart failure (Ma, Mouton et al. 2018). This response peaks in the first five days and decreases gradually (Ma, Mouton et al. 2018). This process is related to neutrophil recruitment inhibition, apoptotic cell (neutrophils, myocytes) and matrix debris removal, and stimulation of fibroblast formation and collagen deposition (Frangogiannis 2012). Resolution of post-MI inflammation is primarily dependent on engulfment of apoptotic cardiomyocytes

and neutrophils which release high quantities of inhibitory mediators (Huynh, Fadok et al. 2002). Interruptions to this process negatively affect the following healing process (Wan, Yeap et al. 2013). More information about interfering with the immune response post-MI is found in Table 1.5.

Macrophages are key players in the post-MI healing phase (Ma, Mouton et al. 2018), as evidenced by a decrease in recovery and exaggeration of adverse cardiac remodeling with total macrophage depletion and by the significant improvement in cardiac functional recovery with adoptive transfer of activated macrophage strategies (Leor, Rozen et al. 2006, van Amerongen, Harmsen et al. 2007). Macrophages phagocytize apoptotic cells, inducing potent anti-inflammatory and immune resolution activities. This process induces production of anti-inflammatory cytokines such as TGF- β and IL-10, actively participating in resolution of post-MI inflammation (will discuss later in Cytokines responsible for resolution of inflammation post-MI). Moreover, macrophages primarily contribute to fibrotic and angiogenic events post-MI.

In addition, certain pathways may resolve post-MI inflammation. Expression of Interleukin Receptor Associated Kinase (IRAK)-M, an endogenous suppressor of innate immune response, has been shown to upregulate in macrophages and fibroblasts in the injured myocardium. Accordingly, this results in suppression of macrophage-derived cytokine expression and enhancement of a matrix-preserving myofibroblast phenotype in the heart (Chen, Saxena et al. 2012). Activation of mertk in macrophages is crucial for efficient removal of dead cardiomyocytes and initiation of the anti-inflammatory phase. Mertk deficiency is associated with more accumulation of apoptotic cardiomyocytes and unresolved inflammation, leading to decreased myocardial salvage (Wan, Yeap et al. 2013). Lipid pro-resolving mediators (resolvins, lipoxins, protectins, and maresins), potent anti-inflammatory compounds activated during phagocytosis, may have an essential role in resolving post-MI inflammation (Prabhu and Frangogiannis 2016). Studies show that exogenous administration of resolvin E1 or resolvin D1 enhances inflammation resolution and prevents adverse cardiac remodeling after infarction (Keyes, Ye et al. 2010, Kain, Ingle et al. 2015). There may be other pathways (TLR signaling,

Janus activated kinases (JAKs), and Pentraxin-3) that are potential mediators in resolution of post-MI inflammation, however their contribution remains unclear and needs further investigation (Frangogiannis 2012).

1.1.1.6.1 Cytokines involve in inflammation resolution post-MI

1.1.1.6.1.1 IL-10

IL-10 is a potent anti-inflammatory cytokine, mainly expressed by stimulated Th2 lymphocytes and alternatively activated macrophages (Mosmann 1994, Frangogiannis 2012). IL-10 modifies monocyte/macrophage function, morphology, and phenotype. Secretion of IL-1 α , IL-1 β , TNF- α , IL-6, and IL-8 is inhibited by IL-10 in macrophages (Frangogiannis 2012). Furthermore, IL-10 may participate in matrix remodeling via enhancement of TIMP-1 synthesis, leading to stabilization of ECM and scar, and thus, reduction of cardiac rupture risk (Lacraz, Nicod et al. 1995, Frangogiannis 2012). IL-10 mRNA and protein levels are both increased in experimental I/R injury models (Frangogiannis 2012). On one hand, some studies show that depletion of IL-10 in mice is associated with exaggerated inflammation, excessive neutrophil recruitment, more advanced cardiac remodeling, and increased mortality (Yang, Zingarelli et al. 2000). Conversely, other studies reveal that IL-10^{-/-} mice have comparable mortality rate and resolution of inflammation to wild type mice, but higher levels of TNF- α and MCP-1 in the damaged myocardium (Zymek, Nah et al. 2007). In addition, these studies show that deposition of fibrous tissue and scar formation are not affected by IL-10 deletion (Aoki and Kao 1999). These contradictory results may be explained by redundant and overlapping pathways responsible for inflammation resolution that are simultaneously activated and rescued in IL-10^{-/-} mice after MI.

1.1.1.6.1.2 TGF- β

TGF- β is an essential element in the transition from inflammation to healing and fibrosis induced by its pleiotropic properties (Bujak and Frangogiannis 2007). Via activation of endothelial mononuclear cells, TGF- β inhibits the production of

pro-inflammatory cytokines and chemokines (Frangogiannis 2012). TGF- β stimulates fibroblast production of ECM proteins (collagen, fibronectin, tenascin, and proteoglycans) (Bassols and Massague 1988). Moreover, TGF- β reduces the expression of proteinases (plasminogen activator and collagenase) and enhances the production of proteinase inhibitors (plasminogen activator inhibitor and TIMP-1), leading to an inhibition in matrix breakdown (Laiho, Saksela et al. 1986, Frangogiannis 2012). As a result, TGF- β plays a dual role post-MI; 1) reducing production of pro-inflammatory cytokines and chemokines, and 2) increase of the deposition of ECM (Frangogiannis 2012). Research shows that post-MI TGF- β administration decreases myocardial injury, an effect that is associated with TNF- α inhibition (Lefer, Tsao et al. 1990). Other studies suggest that inhibiting the TGF- β signaling pathway using a TGF- β type II receptor antagonist modulates fibrotic tissue accumulation at the injured myocardium and attenuates adverse cardiac remodeling (Ikeuchi, Tsutsui et al. 2004, Okada, Takemura et al. 2005). However, research also shows that earlier suppression of TGF- β decreases survival rate and accentuates LV dilatation and heart failure, suggesting a potential role for TGF- β signaling in the inhibition of post-MI inflammatory pathways (Ikeuchi, Tsutsui et al. 2004, Frangogiannis 2012). These different observations most likely relate to the pleiotropic effects of TGF- β on the inflammatory and healing phase post-MI. TGF- β can activate both Smad-dependent and-independent pathways. In the inflammatory phase, Smad3 actively participates in post-MI inflammation resolution and fibrosis. While this signaling cascade does not seem to play a role in inflammation resolution in the healing phase, it facilitates interstitial fibrosis in the injured myocardium (Frangogiannis 2012).

1.1.1.7 Targeting macrophage phenotypes

The presence of pro-inflammatory monocytes/macrophages over a prolonged period in the myocardium induces infarct expansion (Courties, Heidt et al. 2014). Animal studies reveal that shifting macrophages to the reparative phenotype induces timely resolution of inflammation and enhances cardiac recovery (Weirather, Hofmann et al. 2014). Here, it is important to mention that

macrophage polarization is a multifactorial process dependent on interaction with other inflammatory cells, age, microenvironment, and time after injury. The balance between macrophage phenotypes is reversible (Ben-Mordechai, Palevski et al. 2015) and can be therapeutically harnessed to enhance cardiac healing after ischemic injury. Research shows that early intervention aimed at altering macrophage activation state or shifting the balance towards the reparative state substantially reduces the risk of developing heart failure (de Couto, Liu et al. 2015, Kain, Liu et al. 2017). However, studies in alternative macrophage activation in MI have had limited safety in humans. In the next sections, we will critically evaluate the different anti-inflammatory approaches previously tested following MI.

1.1.2 Anti-inflammatory treatments in MI

A balanced inflammatory response fundamentally regulate healing and remodeling after MI. However, therapeutic interventions targeting the systemic inflammatory pathways have yielded limited success in pre-clinical and clinical studies. Modulating the post-MI inflammatory response is an elusive target as studies have shown that the complete inhibition of inflammation is rather harmful (Frangogiannis 2014). Animal studies using non-steroidal anti-inflammatory drugs (NSAIDs) yielded contradictory results regarding protection against myocardial injury (Jugdutt, Hutchins et al. 1980, Romson, Hook et al. 1982, Flynn, Becker et al. 1984, Mullane, Read et al. 1984). Some studies revealed that NSAIDs were able to protect the heart from ischemic injury, albeit with limited success. These studies suggest that these protective effects are not related to the inhibition of the cyclo-oxygenase pathway in the myocardial tissue (Jugdutt, Hutchins et al. 1980, Romson, Hook et al. 1982). It was also observed that ibuprofen, a member of the non-steroidal family, inhibits PMN activation by the complement cascade and generation of ROS species (Flynn, Becker et al. 1984). Other parts of research show no effect of NSAIDs on neutrophil infiltration and no beneficial effect on cardiovascular inflammation, remodeling, or development of heart failure (Mullane, Read et al. 1984). In clinical trials, NSAID use in coronary artery disease patients correlated with higher mortality and recurrent MI (Schjerning Olsen, Fosbol et al.

2011). Another anti-inflammatory class of therapeutic agents, glucocorticosteroids, were found to delay wound healing and are associated with higher incidence of ventricular aneurysm development in MI patients and therefore their use should be avoided (Bulkley and Roberts 1974). With modern anti-inflammatory therapies, observations from animal experimental studies suggest that suppression of IL-1, IL-6, MCP-1, CC chemokines, CXC chemokines, or TNF- α improves the cardiac recovery and function post-MI. Research demonstrates that targeting integrin signaling (CD11/CD18) reduces neutrophil recruitment after MI and is effective in minimizing scar size (Arai, Lefer et al. 1996). Unfortunately, data from clinical trials after acute ischemic injury were disappointing (Faxon, Gibbons et al. 2002). Deletion of IL-1 and MCP-1 significantly decreased adverse cardiac remodeling without noticeable reduction in scar size, which suggests that the inflammatory response contributes to reduced cardiac function post-MI without necessarily altering cardiomyocyte death (Bujak and Frangogiannis 2009, Frangogiannis 2014). On the other hand, selective targeting of potential inflammatory mediators in MI such as IL-1 β using selective monoclonal antibodies demonstrate some success in clinical studies, albeit with high cost and modest benefit (Seropian, Toldo et al. 2014). Based on the above data, strategies aimed at modulating the inflammatory response rather than profoundly suppressing this response may represent promising therapeutic avenues to improve post-MI outcomes.

1.1.3 Macrolides as immunomodulatory drugs

Macrolides are antibiotic agents with a common structure that contain a macrolide ring (Zarogoulidis, Papanas et al. 2012). Macrolides include multiple FDA-approved members such as azithromycin, clarithromycin, erythromycin, roxithromycin, telithromycin, and dirithromycin, as well as non-FDA-approved agents such as carbomycin and josamycin (Zarogoulidis, Papanas et al. 2012). Of note, azithromycin (AZM) is an azalide derivative of macrolides, is not an inhibitor of CYP3A4, and has a lower potential to interact with food or other drugs, unlike other members. Macrolides have wider spectrum of coverage in comparison with penicillins and have lower risk of allergic reaction (Zarogoulidis, Papanas et al.

2012). The mechanism of action of macrolides as antibiotics is through inhibition of protein synthesis in microorganisms, caused by suppression of ribosomal translocation and inhibition of peptidyl-transferase action (Ungureanu 2010, Zarogoulidis, Papanas et al. 2012). They reversibly bind to the P site on the 50s subunit of the bacterial ribosome (Zarogoulidis, Papanas et al. 2012). Macrolides are bacteriostatic antibiotics, however; they become bactericidal at higher doses. Importantly, macrolides are highly concentrated in leukocytes, and thus, achieve high levels at sites of infection (Zarogoulidis, Papanas et al. 2012).

In addition to antimicrobial activity, macrolides also have immunomodulatory and anti-inflammatory properties, as recently demonstrated in a large number of studies (Rubin and Henke 2004, Feola, Garvy et al. 2010, Zarogoulidis, Papanas et al. 2012). These non-antimicrobial activities are dose-time-dependent, and the underlying molecular mechanisms remain poorly defined (Kano and Rubin 2010, Zarogoulidis, Papanas et al. 2012). Macrolides are able to reduce the time of inflammation and modulate immune system activation (Zarogoulidis, Papanas et al. 2012). Moreover, macrolides display immunomodulatory actions through polarization of immune cells to the anti-inflammatory activation state (Feola, Garvy et al. 2010), modifying the production of cytokines (Shinkai, Foster et al. 2006), reducing the generation of ROS, preventing neutrophil activation and migration, inducing neutrophil apoptosis, and inhibiting nuclear transcription factors (Kano and Rubin 2010, Zarogoulidis, Papanas et al. 2012). Hence, macrolides are immunomodulatory rather than immunosuppressive drugs as they exhibit a non-linear time-dependent inhibition of pro-inflammatory mediators (Kano and Rubin 2010).

1.1.3.1 Molecular mechanisms of immunomodulatory effects of macrolides

The mode of action of macrolides as immunomodulatory agents seems multifaceted (Kano and Rubin 2010). These mechanisms may include: a) normalization of the Ca^{+2} response and maintenance of normal intracellular Ca^{+2} concentration (Kano and Rubin 2010), b) inhibition of the mitogen-activated protein kinase (MAPK) pathway, specifically ERK1/2 (Kaneko, Yanagihara et al.

2003, Imamura, Yanagihara et al. 2004, Tsai, Rodriguez et al. 2004, Kanoh and Rubin 2010), and c) inhibition of NF- κ B and activator protein 1 (AP-1) signaling through inhibition of nuclear translocation and signaling (Aoki and Kao 1999, Desaki, Takizawa et al. 2000, Kanoh and Rubin 2010). The proposed modes of action of immunomodulatory effects of macrolides are summarized in Figure 1.2. The following will focus on the immunomodulatory and anti-inflammatory properties of AZM as it is the agent of choice in this project.

1.1.4 Azithromycin

AZM is highly concentrated in leukocytes, macrophages and neutrophils, where it reaches 5 to 100-fold higher concentrations than in serum (Foulds, Shepard et al. 1990, Zimmermann, Ziesenitz et al. 2018). The AZM modulation of macrophage activation state towards the anti-inflammatory phenotype and the cytokine profile to the anti-inflammatory state is well documented (Zarogoulidis, Papanas et al. 2012). AZM therapy also reduces glutathione S-transferases (GSTs), specifically GST theta 1 (GSTT1) and GST Mu 1 (GSTM1) levels in cystic fibrosis cell lines (Jiang, Finkbeiner et al. 1999, McGrath-Morrow and Stahl 2000, Leme, Raposo et al. 2010), implying that AZM may have novel antioxidant activity (Zarogoulidis, Papanas et al. 2012). Interestingly, IL-10 levels also decline with GST, suggesting a relationship between immunomodulatory effects and suppression of GST. AZM suppresses H292 cell line stimulation by N-3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL), a sensory signaling molecule produced by *P. aeruginosa*, due to inhibition of ERK1/2 phosphorylation (Imamura, Yanagihara et al. 2004, Kanoh and Rubin 2010).

Studies reveal that AZM reduces the expression of mRNA and protein levels of TNF- α , which may be attributed to inhibition of NF- κ B and AP-1 translocation to the nucleus and binding to DNA (Ivetic Tkalcevic, Bosnjak et al. 2006, Cigana, Assael et al. 2007, Zarogoulidis, Papanas et al. 2012). LPS-stimulated models also show reduction of TNF- α with AZM treatment (Ivetic Tkalcevic, Bosnjak et al. 2006, Zarogoulidis, Papanas et al. 2012). Research reports that AZM inhibits both TNF- α secretion and neutrophil recruitment to the lung in a murine model (Tsai,

Rodriguez et al. 2004, Feola, Garvy et al. 2010). Following neutrophil mobilization and activation to the site of injury, they undergo apoptosis, initiating inflammation resolution (Kanoh and Rubin 2010). AZM enhances neutrophil apoptosis (Koch, Esteban et al. 2000, Kanoh and Rubin 2010), and promotes macrophage phagocytosis of apoptotic neutrophils, resulting in inflammation resolution. This resolution is related to the alternatively activated macrophage state (Yamaryo, Oishi et al. 2003, Hodge, Hodge et al. 2006).

1.1.4.1 Immunomodulatory and anti-inflammatory effects of AZM in sterile inflammatory diseases

AZM modulates the inflammatory response through macrophage repolarization towards the reparative state (Murphy, Sundareshan et al. 2008, Cory, Birket et al. 2014), as demonstrated in models of inflammation and tissue injury such as spinal cord injury (Zhang, Bailey et al. 2015), lung inflammation (Feola, Garvy et al. 2010), and stroke (Amantea, Certo et al. 2016). In a model of lung inflammation, AZM reduces the production of pro-inflammatory cytokines (IL-6 and IL-12) while increasing IL-10. Additionally, AZM significantly decreases the expression of iNOS and pro-inflammatory macrophage receptor (CCR7) while increasing arginase activity and anti-inflammatory macrophage receptor (MR and CD23) expression (Murphy, Sundareshan et al. 2008). In an ischemic stroke model, which induces a sterile inflammatory response similar to MI, AZM shifts macrophages towards the reparative phenotype, leading to a limited blood brain barrier injury and improved neurological recovery (Amantea, Certo et al. 2016). Likewise, in an experimental retinal ischemia/reperfusion model, AZM is protective against neuronal injury. This protection is attributable to the anti-inflammatory properties of the drug, as evidenced by reduced MMP-9/2 expression and activity (Varano, Parisi et al. 2017). Research in a spinal cord injury model finds that pre-injury AZM treatment induces macrophage repolarization to the reparative phenotype as demonstrated by increased M2 macrophage (CD206, Arginase 1) and reduced M1 macrophage (CD86) marker expression (Zhang, Bailey et al. 2015). Accompanying the immunomodulatory effects of AZM is an increase in

tissue sparing and functional recovery (Zhang, Bailey et al. 2015). In this report, *in vitro* experiments confirm a well-defined shift in cytokine profile towards the anti-inflammatory state (IL-12 downregulation and IL-10 upregulation), indicating a more prominent M2 macrophage phenotype with AZM therapy. Subsequent work by the same group shows that post-injury AZM treatment significantly shifts macrophage state to the reparative phenotype (Gensel, Kopper et al. 2017) in macrophages isolated from spinal cord as well as in *in vitro* stimulated macrophages using a gene-array approach to provide a comprehensive and unbiased profiling of macrophage phenotype. Importantly, this work shows that AZM doses (10, 40, 160 mg/kg) suppress the expression of pro-inflammatory (M1) genes in macrophages, while two doses (10, 160 mg/kg) induce anti-inflammatory (M2) gene expression (Gensel, Kopper et al. 2017). These effects strongly suggest that AZM exerts its immunomodulatory effects in both high and low doses similarly, without causing off-target effects at higher doses.

1.1.5 Liposomes

Various therapeutic agents have attempted to alleviate cardiac remodeling post-MI through systemic administration, however; they achieved limited efficacy due to a lack of bioavailable cardiac tissue (Dasa, Suzuki et al. 2015). Furthermore, most drug delivery systems do not achieve sufficient accumulation in the damaged myocardium or in the cells of interest, which may increase potential off-target effects. Although local delivery may be an option in the setting of MI, it may require repeated catheterization, which is not an applicable and cost-effective procedure (Dasa, Suzuki et al. 2015). To avoid off-target effects and enhance efficacy, an encapsulated dosage form has been utilized successfully (Bornmann, Graeser et al. 2008, Dasa, Suzuki et al. 2015).

Liposomal dosage form, an FDA approved drug delivery platform, is a self-closed spherical structure made of one or more phospholipid bilayers surrounded by an aqueous core. Since they can hold both lipophilic and hydrophilic compounds, liposomes represent an attractive drug delivery system to achieve targeted drug delivery (Levchenko, Hartner et al. 2012). They improve drug

pharmacokinetics, minimize systemic side effects, decrease required dose, prolong circulation half-life, and reduce time to reach steady state (Oh, Nix et al. 1995). In addition, encapsulated therapeutic agents slowly release from the liposomes, providing continuous exposure to the targeted site and enhanced drug efficacy (Cheraghi, Negahdari et al. 2017). Non-cardiac applications show that liposomal delivery platforms can reduce doxorubicin drug toxicity by reducing the administered dose while keeping or enhancing antitumor effectiveness (Rose 2005). In this study, liposomal doxorubicin reaches steady state level faster and with fewer doses due to promoted accumulation in the targeted organs (Charrois and Allen 2003). After infarct, cellular and vascular permeability increase in the injured myocardium, facilitating the accumulation of nanoparticles such as liposomes in the damaged zones (Takahama, Minamino et al. 2009). Liposomes are a proven potent drug delivery system that can serve as a carrier to achieve higher drug concentration at sites of cardiac injury (Mueller, Marcus et al. 1981). Liposomes can also augment the local distribution of tracers in the infarcted myocardium (Caride, Twickler et al. 1984).

The post-infarction myocardium contains a large variety of diverse cells such as inflammatory phagocytes that may be successfully targeted, hypothetically, by liposomal delivery platforms (Oh, Nix et al. 1995). After parenteral administration, mononuclear phagocytes naturally take up liposomes. Liposomes can harness the physiological function of these cells to mediate targeted delivery, therefore promoting therapeutic agent efficacy (Kelly, Jefferies et al. 2011). Stroke research shows that parentally administered gene therapy localizes in infiltrated macrophages in the infarcted brain (Tanaka, Kitagawa et al. 2004). These observations replicate with macrophages in several studies (Scott, Rosano et al. 2009, Harel-Adar, Ben Mordechai et al. 2011, Cheraghi, Negahdari et al. 2017). Mononuclear phagocytes (neutrophils, monocytes, macrophages) play pivotal roles in the immunopathology of MI, and thus, represent an attractive target for liposomal drug delivery (Kelly, Jefferies et al. 2011), as demonstrated with liposomal celecoxib therapy (Margulis, Neofytou et al. 2015, Cheraghi, Negahdari et al. 2017).

In targeting cardiac macrophages with liposomes, research shows that parenteral administration of phosphatidylserine-presenting liposomes (PS-liposomes) shifts cardiac macrophage phenotype to the reparatory state (Harel-Adar, Ben Mordechai et al. 2011). These liposomes are taken up by macrophages both *in vivo* and *in vitro*, activating them to produce large amounts of anti-inflammatory cytokines (TGF- β and IL-10) and reduce production of pro-inflammatory cytokines (TNF- α). Moreover, mannose receptor, CD206, is upregulated, while pro-inflammatory macrophage marker, CD86, is downregulated, implicating a marked shift in the function from pro-inflammatory towards reparative state. This strategy is associated with significant improvement in scar size, angiogenesis, LV function, and cardiac remodeling in the PS-liposome treated group (Harel-Adar, Ben Mordechai et al. 2011).

1.1.5.1 Liposomal AZM

AZM precipitates in aqueous solution due to poor solubility, which has detrimental effects on drug efficacy (Peters, Friedel et al. 1992, Oh, Nix et al. 1995). To prevent AZM hydrolysis during storage and to achieve easier administration without co-solvents, a lyophilized drug-phospholipid dosage formulation may be used. Hydrophobic drugs such as AZM reside in the acyl hydrocarbon chain of liposomes, while hydrophilic drugs locate mostly in the aqueous interior. Therefore, liposomal formulations may be effective in increasing solubility, extending half-life, and improving the therapeutic index and stability of AZM (Cheraghi, Negahdari et al. 2017). Consequently, liposomes alter the biodistribution and the clearance rate of encapsulated drugs (Gabizon, Shmeeda et al. 2003, Cheraghi, Negahdari et al. 2017). Our research indicates that liposomes increase AZM solubility 75 times, suggesting superior potency due to higher drug availability. In agreement with this strategy, studies show that the antibacterial effects of liposomal AZM against intracellular *M. avium* growth in macrophages is 41-fold higher than free AZM (Oh, Nix et al. 1995). This may explain the success of liposomal preparation in promoting the pharmacodynamic effects of the drug. Although lack of efficient assays to precisely determine the

intracellular concentration of AZM limits our ability to test this hypothesis, this strategy successfully enhances the intracellular deposition of other antibiotics (ciprofloxacin) (Oh, Nix et al. 1995). Additionally, AZM substantially spreads throughout the body due to the large volume of distribution (27 liters/kg) (Oh, Nix et al. 1995), and as such, developing a strategy that constrains it to target cells may improve efficacy and prevent non-specific distribution and accumulation. In conclusion, liposomes are valuable tools in enhancing the pharmacokinetics, reducing off target side effects, and achieving targeted delivery of drugs post-MI. Furthermore, these liposomes decrease the exposure of other systems to the drug and thus reduce the potential microbial resistance for AZM.

1.2. STATEMENT OF THE PROBLEM

Myocardial infarction (MI) is a severe ischemic disease, causing sudden death and heart failure with prevalence rates promptly rising worldwide. Despite significant advances in medical care, mortality rate and comorbid complications remain high in MI patients. Heart failure is one of the most expensive chronic illnesses with medical costs constituting 1-2% of overall healthcare spending. Post-MI inflammatory changes in the heart play a critical role in the pathophysiology of MI and its related complications. Likewise, these inflammatory pathways regulated healing and remodeling after MI. Accordingly, targeting suppression of post-MI inflammation is a potential therapeutic approach in MI treatment. However, multiple clinical trials fail to demonstrate the effectiveness of traditional anti-inflammatory drugs such as corticosteroids, cyclosporine, and non-steroidal anti-inflammatory drugs in treating patients with MI. Additionally, studies that selectively target one signaling pathway (cytokine and/or receptor) fail to demonstrate robust clinical benefits.

A growing body of evidence suggests that modulation of post-MI inflammation via selective targeting of potential inflammatory mediators may hold the key in protecting the heart from adverse cardiac remodeling and chronic heart failure. However, the development of such therapies is a challenge as they must act as immunomodulatory rather than immunosuppressive agents since immunosuppressive agents have proven harmful in MI patients. Macrophages are key players in acute inflammation and chronic recovery, which present in MI in two activation states: pro-inflammatory and reparative. Pro-inflammatory macrophages contribute to adverse cardiac remodeling and heart failure while reparative macrophages enhance tissue healing. Recent research reveals that immunomodulation of the inflammatory response post-MI via macrophage repolarization towards the reparative state counterbalances pathophysiological mechanisms of adverse cardiac remodeling and prevents heart failure. However, translational approaches to harness this benefit are not clinically available.

In this work, we represent an approach to achieve this goal by using a small molecule drug. Research demonstrates that azithromycin (AZM) alters the inflammatory response and polarizes macrophages to the alternatively activated state in various inflammatory pathologies. AZM has an excellent safety profile and is approved for human use. We hypothesize that AZM therapy modulates the excessive inflammatory response post-MI, and thus, improves cardiac recovery.

1.3 Hypothesis

Azithromycin is a potential immunotherapy to improve inflammation and adverse cardiac remodeling after MI. To test this hypothesis, we performed the following studies:

1: Examine the immunomodulatory and cardioprotective effects of azithromycin post-MI (a proof-of-concept study).

Hypothesis: AZM therapy modulates the potent inflammatory response, and thus, improves cardiac recovery after infarction.

2: Determine the immunotherapeutic effects of low dose liposomal AZM delivered after infarction (clinically-relevant study).

Hypothesis: Low dose liposomal AZM (Lazm) treatment, started immediately after MI, yields an enhanced immunomodulatory potential and cardiac recovery with low risk of adverse effects

Table 1.1: Innate immune mechanisms in MI: contribution of studies using transgenic mice (Liaudet and Rosenblatt-Velin 2013)

Transgenic strain	Model of MI	Main findings
TNF alpha pathway		
TNF alpha KO	I/R (30'-120')	Reduced infarct size and contractile dysfunction. Reduced NF-kappaB activation, chemokine expression, PMN infiltration.
TNF alpha KO	P.O. (up to 7 D)	Improved cardiac function. Reduced myocardial ICAM-1 expression.
TNFR1-R2 KO	P.O. (24 h)	Increased infarct size, increased apoptosis of cardiac myocytes.
TNFR1 (p55) KO	P.O. 7-28 D	Reduced mortality, improved functional recovery.
TNFR2 (p75) KO	P.O. 7-28 D	Increased mortality, increased LV dysfunction.
IL-1 beta pathway		
IL-1R KO	I/R (1 h/6 h to 7 D)	Reduced chemokine, cytokine and MMP expression. Reduced PMN infiltration, LV dilation and LV fibrosis.
gp-130 mutant	P.O. (up to 2 W)	Persistent myocardial STAT3 activation, increased myocardial inflammation, LV dysfunction and LV rupture.
IL-10 pathway		
IL-10 KO	I/R (30'-2-24 h)	Increased PMN infiltration, ICAM-1/TNF expression and infarct size.
IL-10 KO	I/R (1 h/6 h to 7 D)	Increased expression of TNF and CCL2. No effect on LV dysfunction and remodeling.
TGF beta pathway		
Smad3 KO	I/R (1 h/6 h to D)	Reduced PMN infiltration and chemokine expression. Reduced dilative LV remodeling and diastolic dysfunction.
TSP-1 KO	I/R (1 h/3 h to 7 D)	Increased and prolonged expression of IL-1, IL-6 and TGF-beta. Enhanced LV adverse remodeling with increased fibrosis.
CXCR4 KO	P.O. (3 W)	No influence on LV systolic dysfunction and adverse remodeling
IP-10 KO	I/R (1 h/6 h to 7 D)	Expansion of fibrosis, increased remodeling and systolic dysfunction Increased PMN (after 24 and 72 h) and macrophages (after 3 to 7 D).
MCP-1 KO	I/R (1 h/6 h to 7 D)	Delayed macrophage recruitment and myofibroblast accumulation. Reduced cytokine expression. Reduced LV remodeling.

Table 1.1 (Continued)

CCR2 KO	P.O. (up to 28 D)	Reduced macrophage infiltration, MMP expression and collagen deposition. Reduced LV systolic dysfunction and LV remodeling.
CCR1 KO	P.O. (up to 21 D)	Reduced PMN infiltration, decreased apoptosis, increased proliferation of myofibroblasts in the infarcted tissue. Reduced LV remodeling.
ICAM-1 KO	I/R (30'/2 h-3 W)	Reduced reperfusion injury at 2 h.
ICAM-1/P-sel KO	I/R (30'-1 h/3-24 h)	Reduced PMN infiltration at 24 h, but no change in infarct size.
CD-18 KO	I/R (30'/2 h)	Reduced PMN infiltration and smaller infarct size (at 2 h reperfusion)
Cardiac restricted mutated IkappaB	P.O. (4 W)	Diminished NF-kB p65 activation and cytokine expression. Reduced LV remodeling, fibrosis and systolic function.
Cardiac-restricted mutated IkappaB	P.O. (24 h)	Increased infarct size and apoptosis. Reduced expression of anti-apoptotic proteins (Bcl-2 and c-IAP1).
p50 KO	I/R (30'/24 h)	Reduced PMN infiltration, decreased infarct size
p50 KO	P.O. (up to 8 W)	Reduced mortality, LV remodeling and systolic dysfunction
p50 KO	P.O. (4 W)	Increased expression of cytokines, chemokines and MMPs. Enhanced remodeling, fibrosis, hypertrophy and systolic dysfunction.
TLR2 KO	I/R (30'/60') (isolated hearts)	Improved post-ischemic functional recovery. Blunted myocardial expression of TNF alpha and IL-1 beta.
TLR2 KO	P.O. (1 to 4 W)	Reduced TGF beta expression and fibrosis. Reduced LV remodeling and systolic dysfunction.
TLR2 KO	I/R (30'/60')	Reduced myocardial PMN accumulation, ROS formation and IL-1 beta expression. Suppressed coronary endothelial dysfunction.
TLR4 deficient (C3H/HeJ mice)	P.O. (up to 4 W)	Reduced remodeling and preserved systolic function. Reduced fibrosis and expression of cytokines.
TLR4 KO	P.O. (1-4 W)	Reduced systolic dysfunction and remodeling. Decreased TGF beta expression. Reduced lymphocyte infiltration and apoptosis.

Table 1.1 (Continued)

TLR4 KO	I/R (1 h/24 h)	Reduced infarct size, myocardial PMN infiltration and oxidative stress.
MyD88 KO	I/R (1 h/up to 7 D)	Reduced infarct size, reduced LV dysfunction, reduced myocardial PMN infiltration, MCP-1 and ICAM-1 expression.
RAGE KO	I/R (30'/48 h)	Reduced infarct size, myocardial inflammation and dysfunction.
Inflammasome pathway		
Caspase-1 KO	P.O. (up to 9 D)	Reduced LV remodeling, MMP-3 and IL-18 expression and apoptosis.
ASC KO	I/R (30'/up to 2 W)	Reduced infarct size at 48 h. Reduced LV fibrosis, remodeling and systolic dysfunction. Reduced myocardial cytokine expression.

ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain; Bcl-2: B-cell CLL/lymphoma 2; c-IAP-1: inhibitor of apoptosis protein-1; CCL: chemokine (C-C motif) ligand; CCR: chemokine (C-C motif) receptor; CXCR: chemokine (C-X-C motif) receptor; D: Day; H: hour; ICAM-1: inter-cellular adhesion molecule-1; I κ B: inhibitor of KappaB; IL: interleukin; IL-1R: IL-1 beta receptor; IP-10: Interferon-inducing protein 10; I/R: Ischemia/Reperfusion; KO: Knockout; LV: left ventricle; MCP-1: monocyte chemoattractant protein-1; MMP: matrix metalloproteinase; MyD88: myeloid differentiation primary response gene 88; NF- κ B: nuclear factor kappaB; PMN: polymorphonuclear cell; P.O.: permanent occlusion; P-sel: P-selectin; RAGE: receptor for advanced glycation end-products; ROS: reactive oxygen species; SDF-1: stromal cell-derived factor-1; STAT: signal transducer and activator of transcription; TGF β : transforming growth factor beta; TLR: toll-like receptor; TNF α : tumor necrosis factor alpha; TNFR1: TNF receptor 1; TNFR2: TNF receptor 2; TSP-1: Thrombospondin-1; W: week.

Table 1.2: Gating strategies for immune cells (Ma, Mouton et al. 2018)

Gating strategy	Cells labeled	Species
Monocytes		
Ly6C ^{high} CCR2 ^{high} CX3CR1 ^{low} CD62 L ⁺	Classical monocytes	Mouse
Ly6C ^{low} CCR2 ^{low} CX3CR1 ^{high} CD62L ⁻	Nonclassical monocytes	Mouse
CCR2 ⁺ Ly6C ^{high}	Inflammatory blood monocytes	Mouse
CD14 ⁺ CD16 ⁻ , CD14 ⁺ CD16 ⁺	Blood monocytes	Human
B220 ⁻ F4/80 ⁺ CD115 ⁺ Ly6C ⁻ , B220 ⁻ F4/80 ⁺ CD115 ⁺ Ly6C ⁺	Blood monocytes	Mouse
MHCII ^{low} CCR2 ⁺	Cardiac monocytes	Mouse
CD11 b ⁺ F4/80 ⁻ Ly6G ⁻ Ly6C ^{high} , CD11 b ⁺ F4/80 ⁻ Ly6G ⁻ Ly6C ^{low}	Monocytes	Mouse
Lineage ⁻ CD11 b ⁺ F4/80 ^{low} Ly6C ⁺	Cardiac monocytes	Mouse
CD11 b ⁺ CD11C ⁻ MHCII ⁻ CD68 ⁻ Ly6C ^{low} , CD11 b ⁺ CD11C ⁻ MHCII ⁻ CD68 ⁻ Ly6C ^{high}	Blood and cardiac monocytes	Mouse
Macrophages		
CD45 ⁺ CD11 b ⁺ F4/80 ⁺ CD206 ⁻	M1 macrophages	Mouse
CD45 ⁺ CD11 b ⁺ F4/80 ⁺ CD206 ⁺	M2 macrophages	Mouse
CD45 ⁺ CD11 b ⁺ F4/80 ⁺ Ly6C ^{low}	Resident cardiac macrophages	Mouse
CD11 b ⁺ F4/80 ⁺ CD206 ⁺	Alternatively activated macrophages	Mouse
CD11 b ⁺ F4/80 ⁺ CD64 ⁺ Ly6C ⁺ MHCII ^{+/-}	M1 like macrophages	Mouse
CD11 b ⁺ F4/80 ⁺ CD64 ⁺ Ly6C ⁻ MHCII ^{+/-}	M2 like macrophages	Mouse
F4/80 ⁺ CD86 ⁺	M1 macrophages	Mouse
F4/80 ⁺ CD206 ⁺	M2 macrophages	Mouse
F4/80 ⁺ CD206 ⁻	M1 macrophages	Mouse
CD45 ⁺ CD68 ⁺	Cardiac, blood, and spleen macrophages	Rat
CD11 b ⁺ F4/80 ⁺ CD68 ⁺ Ly6C ^{low} , CD11 b ⁺ F4/80 ⁺ CD68 ⁺ Ly6C ^{high}	Monocyte-derived cardiac macrophages	Mouse
CD14 ⁺ CD64 ⁺ MERTK ⁺ F4/80 ⁺ CX3CR1 ⁺ MHCII ⁻ , CD14 ⁺ CD64 ⁺ MERTK ⁺ F4/80 ⁺ CX3CR1 ⁺ MHCII ⁺ , CD14 ⁺ CD64 ⁺ MERTK ⁺ F4/80 ⁺ CX3CR1 ⁻ MHCII ⁻ , CD14 ⁺ CD64 ⁺ MERTK ⁺ F4/80 ⁺ CX3CR1 ⁻ MHCII ⁺	Resident cardiac macrophages	Mouse
F4/80 ⁺ CD11 b ⁺ Ly6C ^{low} , F4/80 ⁺ CD11 b ⁺ Ly6C ^{medium} , F4/80 ⁺ CD11 b ⁺ Ly6C ^{high}	Alternatively activated macrophages	Mouse

Table 1.2 (Continued)

CD45 ⁺ CD11 b ⁺ F4/80 ⁺ Ly6C ⁻ MHCII ^{high} , CD45 ⁺ CD11 b ⁺ F4/80 ⁺ Ly6C ⁻ MHCII ^{low} , CD45 ⁺ CD11 b ⁺ F4/80 ⁺ Ly6C ⁺ MERTK ⁺ CD206 ⁺ , CD45 ⁺ CD11 b ⁺ F4/80 ⁺ Ly6C ⁺ MERTK ⁻ CD206 ⁻	Cardiac resident macrophages	Mouse
CD45 ⁺ F4/80 ⁺ MHC-II ^{low} CCR2 ⁻ , CD45 ⁺ F4/80 ⁺ MHC-II ^{high} CCR2 ⁻	Cardiac resident macrophages	Mouse
CD45 ⁺ F4/80 ⁺ MHCII ^{high} CCR2 ⁺	Monocyte-derived cardiac macrophages	Mouse
CD14 ⁺ CD16 ⁺ CD163 ⁺ CD204 ⁺ CD206 ⁺ CD209 ⁻	Anti-inflammatory M2c macrophages	Human

Table 1.3: Properties of M1 and M2 macrophages post-MI (Ma, Mouton et al. 2018)

	Pro-inflammatory	Anti-inflammatory
Stimuli	GM-CSF, IFN- γ , TNF- α , IL-1 β	Hydrogen sulfide, IL-4, IL-10, IL-13, IL-33, TGF- β 1, M-CSF
Transcription factors	AP-1, HIF-1 α , IRF3, IRF5, NF- κ B, STAT1	c-Maf, c-Myc, IRF4, JMJD3, KLF4, PPAR- γ , STAT3, STAT6
Markers	CCL2 (MCP1), CCL3 (MIP1a), CCL4 (MIP1b), CCL5 (RANTES), CCL7, CCL8, CCR2, CD80, CD86, CXCL1, CXCL2, CXCL6, CXCL8 (IL-8), CXCL9, CXCL10, CXCL11, CXCL16, IL-1 β , IL-6, IL-12, IL-23, iNOS, MHCII, RNS, ROS, S100a8, S100a9, TNF- α	Arg1, CCL1, CCL16, CCL17, CCL18, CCL22, CCL24, CXCL13, CXCL17, CXCL22, CXCL24, CXCR1, CXCR2, CD163, CD206 (MRC1), CD280 (MRC2), Cd301a (Clec10a, Mgl1), Cd301b (Mgl2), Dectin-1, Fizz1 (Retnla, Relmq), IL-10, PGE2, Spp1 (osteopontin), Stabilin1, TGF- β 1, VEGF, Ym1 (Chi3l3)
Cell physiology	Pro-inflammation; proteolysis; phagocytosis of debris; antigen presentation to lymphocytes	Anti-inflammation and resolution of inflammation; phagocytosis of apoptotic cells; pro-angiogenesis; ECM production and scar formation

Tabl1 1.4: Summary of markers of immune cells in humans and rodents (Ma, Mouton et al. 2018)

Marker	Location	Expressed by	Cell physiological function
CCR2/CD192	Cell surface	Monocytes, macrophages	Mediates Ly6C ^{high} monocytes recruitment and migration
CD11 b/ITGAM	Cell surface	Monocytes, macrophages, neutrophils, NK cells	Couples with CD18 to form integrin α M β 2 (also named Mac1 or complement receptor 3) to initiate immune response
CD14	Cell surface	Human monocytes	Mediates toll-like receptor 4 activation and production of IFN- β
CD16/FCGR3	Cell surface	Human monocytes	Binds to the Fc portion of IgG antibodies, antigen presentation, anti-inflammatory cytokine production
CD64/FcyR1	Cell surface	Monocytes, macrophages	Antibody-dependent phagocytosis, recognizes the Fc region of IgG
CD68/macrosialin	Endosomal/lysosomal compartment, cell surface	Monocytes, macrophages	Antigen processing and presentation, binds to oxidized low-density lipoprotein
CD163	Cell surface, secreted (soluble)	Macrophages, neutrophils	Hemoglobin/haptoglobin scavenger receptor, anti-inflammatory
CX3CR1	Cell surface	Monocytes, macrophages	Mediates Ly6C ^{lo} monocyte recruitment, inhibits proliferation of local macrophages
F4/80/EMR1	Cell surface	Macrophages	Promotes pro-inflammatory factor production, induces antigen-specific effector Treg cells

Table 1.4 (Continued)

Galectin 3/Mac2	Cell surface, secreted	Macrophages	Induces monocyte-macrophage differentiation, interferes with dendritic cell fate decision, regulates T cell apoptosis, inhibits B-lymphocyte differentiation into plasma cells
Ly6C/Gr-1	Cell surface	Monocytes	A specific marker for pro-inflammatory monocytes
Mac3	Cell surface	Macrophages	A glycoprotein
MERTK	Cell surface	Macrophages, phagocytes	Mediates phagocytosis, increases migration
MHCII	Cell surface	Macrophages, dendritic cells, B cells	Mediates antigen presentation

Table 1.5: Post-MI effect of perturbing inflammation components (Mouton, Rivera et al. 2018)

Perturbation	Effect	Mechanism
Reducing inflammation:	↑LV physiology	IL-1α null: ↓innate immune response in cardiac fibroblasts
IL-1α null TREM1 null NF-κB null TLR4 null TRPV2 null PS-presenting liposomes	↓LV remodeling	TREM1 null: ↓myeloid cell recruitment from the spleen and bone marrow; ↓CCR2 ⁺ Pro-inflammatory but not pro-reparative X3CR1 ⁺ monocyte infiltration TLR4 null: ↓neutrophil infiltration/activation TRPV2 null: ↓macrophage migration PS-liposomes: ↑macrophage secretion of IL-10 and TGF-β
Total neutrophil depletion	↓LV physiology	↓Necrotic myocyte removal
	↑Fibrosis	↑Macrophage inflammatory response
		↑Myofibroblast activation
Total macrophage depletion	↓LV physiology	Total: ↓necrotic/apoptotic cell removal
	↓Collagen deposition	↓Fibroblast and endothelial cell activation
M2-specific depletion	↓Neovascularization	M2: prolonged N1 neutrophil/M1 macrophage activation
Macrophage modulation:	↑LV physiology	Wntless: ↑reparative M2 macrophages, neovascularization
Wntless null Macrophage EP3R null Macrophage EP3R transgenic CD5L null	↓LV remodeling	EP3R null: monocyte TGF-β signaling/↓CX3CR1 and VEGF signaling, ↓Ly6C ^{lo} reparative monocyte activation EP3R transgenic: ↑angiogenesis CD5L null: ↓neutrophils, ↓collagen accumulation, ↓IL-1 receptor-associated kinase 4, NF-κB, myeloperoxidase, and inducible nitric oxide synthase

MI, myocardial infarction; LV, left ventricular; ↑, increase; ↓, decrease.

Table 1.6: Summary of clinical outcomes with anti-inflammatory agents post-MI (Seropian, Toldo et al. 2014)

Therapy	Outcome
Glucocorticoids	conflicting results, associated with impaired healing, scar thinning, ventricular aneurysm, and increased risk of ventricular rupture
NSAIDs	worse clinical outcome and ventricular rupture after AMI
rhuMAb CD18 (humanized monoclonal antibody against CD18)	failed to improve coronary reperfusion on angiography, ST-segment resolution, increased infections and bleeding complications
Pexelizumab (humanized antibody against C5)	failed to reduce infarct size or reduce major adverse cardiac events
Tocilizumab (humanized monoclonal antibody against the IL-6 receptor)	conflicting and inconclusive results regarding the role IL-6 in ventricular remodeling
Anakinra (competitive inhibitor of IL-1)	a favorable effect on C-reactive protein (CRP) levels and trend toward more favorable left ventricular remodeling and reduced incidence of HF at 3 months
Etanercept and infliximab (TNF- α blockers)	a dose-dependent increase in adverse cardiac events, significantly lowered the interest in these drugs for heart disease
PI3K inhibitor	no protective effect in ischemia-reperfusion model

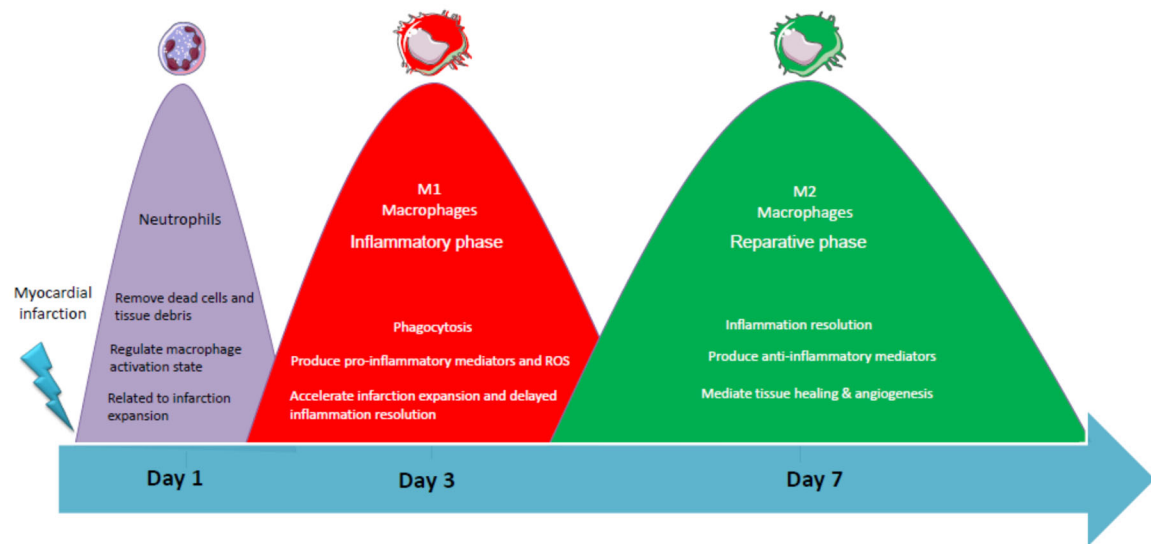


Figure 1.1: Primary immune cells at the site of infarction.

Neutrophil and macrophage responses after infarction. Shifting the response of M2 macrophage to the left by using AZM is beneficial in MI (Hypothesis).

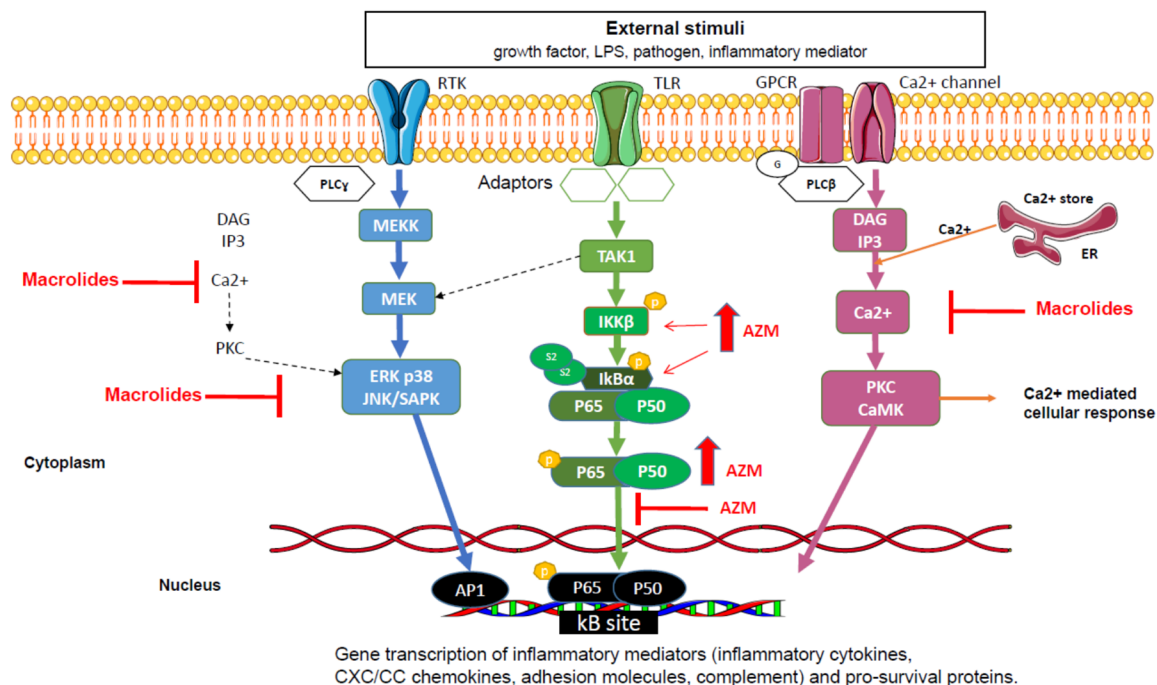


Figure 1.2: The likely underlying molecular mechanisms for the immunomodulatory effects of macrolides.

AP-1, activator protein 1; CaMK, calmodulin kinase; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GFR, cytokine receptor/growth factor receptor; GPCR, G-protein-coupled receptor; IKK, IκB kinase; IP₃R, inositol triphosphate receptor; IRAK, IL-1 receptor-associated kinase; MEK, MAPK/ERK kinase; PKC, protein kinase C; TAK1, transforming growth factor-activated protein kinase 1; TLR, Toll-like receptor. Big arrows, major pathways influenced by macrolides; dashed arrows, sub-pathways or cross-talk pathways; red lines, inhibition by macrolides; red arrows, levels are increased by AZM; AZM, azithromycin (Kano and Rubin 2010).

CHAPTER II. SPECIFIC AIM 1

Azithromycin therapy reduces cardiac inflammation and mitigates adverse cardiac remodeling after myocardial infarction: Potential therapeutic targets in ischemic heart disease

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2.1 Summary

Introduction: Acute myocardial infarction (MI) is a primary cause of worldwide morbidity and mortality. Macrophages are fundamental components of post-MI inflammation. Pro-inflammatory macrophages can lead to adverse cardiac remodeling and heart failure while anti-inflammatory/reparative macrophages enhance tissue healing. Shifting the balance between pro-inflammatory and reparative macrophages post-MI is a novel therapeutic strategy. Azithromycin (AZM), a commonly used macrolide antibiotic, polarizes macrophages towards the anti-inflammatory phenotype, as shown in animal and human studies. We hypothesized that AZM modulates post-MI inflammation and improves cardiac recovery.

Methods and results: Male WT mice (C57BL/6, 6–8 weeks old) were treated with either oral AZM (160 mg/kg/day) or vehicle (control) starting 3 days prior to MI and continued through day 7 post-MI. We observed a significant reduction in mortality with AZM therapy. AZM-treated mice showed a significant decrease in pro-inflammatory (CD45+/Ly6G-/F4-80+/CD86+) and increase in anti-inflammatory

(CD45+/Ly6G-/F4-80+/CD206+) macrophages, decreasing the pro-inflammatory/anti-inflammatory macrophage ratio in the heart and peripheral blood as assessed by flow cytometry and immunohistochemistry. Macrophage changes were associated with a significant decline in pro- and increase in anti-inflammatory cytokines. Mechanistic studies confirmed the ability of AZM to shift macrophage response towards an anti-inflammatory state under hypoxia/reperfusion stress. Additionally, AZM treatment was associated with a distinct decrease in neutrophil count due to apoptosis, a known signal for shifting macrophages towards the anti-inflammatory phenotype. Finally, AZM treatment improved cardiac recovery, scar size, and angiogenesis.

Conclusion: Azithromycin plays a cardioprotective role in the early phase post-MI through attenuating inflammation and enhancing cardiac recovery. Post-MI treatment and human translational studies are warranted to examine the therapeutic applications of AZM.

2.2 Introduction

Acute myocardial infarction (MI) is a leading cause of mortality and morbidity in the western world (van der Laan, Nahrendorf et al. 2012). MI provokes a profound coordinated inflammatory response, a process mediated by inflammatory bone marrow (BM) and peripheral blood (PB) cells, which has been linked to the development of end stage heart failure (HF), a highly frequent complication post-MI (van der Laan, Nahrendorf et al. 2012). The peri-infarct zone demonstrates dynamic cellular changes with the infiltration of various inflammatory cells including neutrophils, monocytes, and macrophages (Epelman, Liu et al. 2015). Monocytes infiltrate the peri-infarct zone and differentiate into macrophages, which play an important role in the initial inflammatory as well as the following reparatory phases (Dutta and Nahrendorf 2015). Two dominant patterns of macrophage activation are found: pro-inflammatory/classically activated macrophages (M1-like) and anti-inflammatory/alternatively activated/reparative macrophages (M2-like), with different cell markers and gene expression profiles (Epelman, Liu et al. 2015). In mice, the initial exaggerated

inflammatory response may actually confer long-term harm because reductions in the initial recruitment of inflammatory monocytes reduce infarct size and prevent adverse cardiac remodeling (de Couto, Liu et al. 2015, Protti, Mongue-Din et al. 2016). Pro-inflammatory macrophages trigger inflammation, damage of extra cellular matrix (ECM)(Gibbs, Shanley et al. 1999), production of reactive oxygen/nitrogen species and pro-inflammatory cytokines (IL-6, TNF- α , and IL-1 β) (Martinez, Sica et al. 2008). In contrast, anti-inflammatory macrophages promote ECM repair, angiogenesis, and production of anti-inflammatory cytokines (IL-4, IL-10, and IL-13) (Sunderkotter, Goebeler et al. 1991).

Azithromycin (AZM), a clinically approved macrolide antimicrobial agent, has an excellent safety profile in humans. AZM modulates the inflammatory response through macrophage polarization towards the reparative state (Murphy, Sundareshan et al. 2008, Cory, Birket et al. 2014), as demonstrated in models of inflammation and tissue injury such as spinal cord injury (Gensel, Kopper et al. 2017), lung infection (Feola, Garvy et al. 2010), and stroke (Amantea, Certo et al. 2016). In these clinically relevant scenarios, AZM reduces the production of pro-inflammatory cytokines (IL-6 and IL-12) and increases that of anti-inflammatory cytokines (IL-10)(Murphy, Sundareshan et al. 2008). Additionally, AZM significantly decreased the expression of iNOS and pro-inflammatory macrophage receptor (CCR7) while increasing arginase activity and anti-inflammatory macrophage receptors (MR and CD23) (Murphy, Sundareshan et al. 2008). In an ischemic stroke model, which induces a similar sterile inflammatory response to MI, AZM shifted macrophages from the pro-inflammatory to the reparative state leading to inhibition of blood brain barrier injury and improvement in neurological recovery (Amantea, Certo et al. 2016). Likewise, in a retinal ischemia/reperfusion experimental model, AZM was protective against neuronal injury. This protection was attributed to the anti-inflammatory properties of AZM, as evidenced by the reduction in MMP-9/2 expression and activity (Varano, Parisi et al. 2017). In addition to its immunomodulatory properties, AZM is well tolerated, achieves a

wide therapeutic index, and has well characterized pharmacokinetic and pharmacodynamic properties(Koch, Esteban et al. 2000).

Here, we provide the first evidence that AZM reduces the inflammatory response and mediates cardioprotection against adverse cardiac remodeling and HF post-MI. AZM produces its positive effects through increasing neutrophil apoptosis and switching macrophage activation towards an anti-inflammatory phenotype. Our findings present an important step towards designing clinically relevant strategies to reduce the risk of cardiac remodeling and HF after MI.

2.3 Materials and methods

Study Design. 8–10 week old male C57BL/6 mice (Jackson Laboratory, BarHarbor, ME) were treated with either AZM (Azithromycin tablets, USP, Sandoze, NDC0781194133, Princeton, NJ), (crushed tablets suspended in 2% methylcellulose), orally using gastric gavage at 160 mg/kg/day or vehicle (2% methylcellulose), starting 3 days prior to MI or sham surgery through day 7 post-MI (Fig 1A). Timing of AZM therapy was selected to ensure appropriate steady-state levels at the time of injury (Feola, Garvy et al. 2010). AZM administration continued for 7 days after surgery to cover the entire duration of post-MI inflammatory response (Heidt, Courties et al. 2014, Ramirez, Iyer et al. 2014). AZM treatment was not associated with changes in liver or kidney function throughout the period of administration (S1 Fig). All procedures were conducted under the approval of the University of Kentucky IACUC in accordance with the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. [NIH] 85–23, rev. 1996).

2.3.1 Murine model of myocardial infarction

Mice were anesthetized with 1–3% isoflurane using a small animal vaporizer system. Pain reflexes were examined to make sure that the mouse was adequately anaesthetized prior to surgery. Following a left thoracotomy between the fourth and fifth ribs and removal of pericardial sac, the heart was exposed and pushed out of the thorax under direct visualization. The left anterior descending

coronary artery (LAD) was identified under a surgical microscope and permanently ligated 3 mm below its origin using a 6–0 silk suture as previously described (Gao, Lei et al. 2010, Klyachkin, Nagareddy et al. 2015). The sham group underwent the same surgical procedure except the suture was passed under the LAD but not tied. Following LAD ligation, the heart was placed back into the intrathoracic space and the pneumothorax was manually evacuated. After muscle closure, the skin was sutured using 4–0 Prolene running sutures.

2.3.2 Humane endpoints

Animals were treated with pain medications for 24–48 hours after surgery. This duration may be prolonged if animals showed signs of pain, discomfort or reduced food or water intake. Animals were followed every 8 hours for 24 hours followed by daily for 1 week, for any signs of clinical deterioration or overt heart failure. Their water and food consumption is monitored carefully. Any animals that show signs of decompensation were resuscitated and if this is unsuccessful, they were humanely euthanized. We also monitored mice for additional signs of distress or weight loss >15–20% from baseline weight; hunched posture; ruffled coat; etc. If the mice exhibited any of these signs, they were euthanized without any subsequent experimental procedures. Most of the mortality in our MI group occurred in the first week after surgery. Necropsy showed blood filled thoracic cavity suggesting myocardial rupture as the cause of death.

The appearance of the surgical site is checked for signs of infection, undue sutures, and seroma formation. When possible, infection, sutures, or seroma formation is treated medically (antibiotics, if indicated) or surgically (replacing sutures or percutaneous drainage, respectively). In rare cases in which infection appears to have occurred, we consulted with DLAR veterinary for advise, treatment and management. In cases where complications are felt to be causing the animal significant distress, the animal is euthanized.

2.3.3 Method of euthanasia

Mice were euthanized using high dose isoflurane followed by cervical dislocation to confirm their euthanasia.

2.3.4 Flow cytometry

Peripheral blood tubes containing 1:5 ratios of ethylenediaminetetraacetic acid (EDTA)/citrate-theophylline-adenosinedipyridamole (CTAD) were used to collect blood samples at 1, 3 and 7 days after MI (4 MI mice and 3 sham mice /treatment group). Whole blood was centrifuged at 700g for 5 minutes, and plasma layer was collected in separated tubes and kept at -80°C. The remaining cell pack was incubated with 0.5 ml of 1X of red blood lysing buffer (BD pharm lyse) for 10 minutes with gentle agitation for RBC lysis followed by dilution using 0.5 ml of staining buffer (5% goat serum, 0.05% sodium azide in phosphate-buffered saline) to stop the reaction. The suspension was centrifuged at 400g for 5 minutes, and supernatant was discarded. This step was repeated if we observed residual red blood cells. The white blood cell pellet was resuspended in staining buffer, washed to remove any remaining lysis buffer, and centrifuged at 400g for 5 minutes. Cells from individual blood samples were split into two portions for flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR) based on cell counts so that each sample contained $\sim 10^6$ live (trypan blue-negative) cells. Cells for rtPCR were lysed using cell lysis buffer (Life technologies) for 10 minutes with strong agitation and kept at -80°C for gene analyses. Cells for staining were immediately incubated on ice for 30 minutes with conjugated primary antibodies against FITC-conjugated Ly6G/C (BD Pharmingen), APC-CY7-conjugated CD45 (Biolegend), PE-conjugated CD115 (Biolegend) for the monocyte panel. After incubation, cells were washed twice using flow buffer and analyzed using an LSR II (Becton Dickinson) in the University of Kentucky Flow Cytometry Core. Monocytes were classified as pro-inflammatory ($CD45^+/CD115^{hi}/Ly6-C^{hi}$) and anti-inflammatory ($CD45^+/CD115^{hi}/Ly6-C^{lo}$). Neutrophils were identified as $CD45^+/CD115^{lo}/Ly6G/C^{lo}$.

Heart tissue collected at the same time points was rapidly removed and placed in ice cold PBS (VWR International). The heart was manually minced using razor blades. Minced heart tissue was transferred into 15 ml tube with Collagenase B (Roche, Indianapolis, IN) and Dispase II (Roche, Indianapolis, IN) solution for 30 minutes in 37°C water bath, with manual agitation every 5–10 minutes. Then, the digestion reaction was terminated using cold staining buffer, and tubes were placed in ice. The digestion mixture was filtered through 70 µm cell strainers and centrifuged at 400g for 5 min at 4 °C, then the supernatant was aspirated and cells were resuspended in 0.5 ml of staining buffer. Cells from individual hearts were split into approximately three portions based on cell counting so that each sample contained ~10⁶ live (trypan blue-negative) cells for flow cytometry and RT-PCR as detailed above. Cells were incubated directly on ice for 30 minutes with conjugated primary antibodies against FITC-conjugated Ly6G (BD Pharmingen), PE-conjugated CD206 (Biolegend), PerCP-CY5.5-conjugated CD86 (Biolegend), PECY7-conjugated F4/80 (Biolegend), and APC-CY7-conjugated CD45 (Biolegend) for the macrophage panel, or the monocyte panel detailed above. After incubation, cells were washed twice using flow buffer and analyzed using an LSR II (Becton Dickinson). Macrophages were classified into pro-inflammatory (CD45⁺/Ly6G⁺/F4-80⁺/CD86⁺ cells) and ant-inflammatory (CD45⁺/Ly6G⁺/F4-80⁺/CD206⁺ cells). Laser calibration and compensation were carried out for every experiment utilizing unstained and single fluorescent controls.

Using the same protocols mentioned earlier, cells isolated from hearts and peripheral blood were incubated with APC-CY7-conjugated CD45 (Biolegend), FITC-conjugated Ly6G (BD Pharmingen), APC-conjugated Annexin 5 (Biolegend) and PE-conjugated propidium iodide (PI) (Biolegend) to identify apoptosis in neutrophils. Cells were considered apoptotic if they were PI^{lo}/Annexin V^{hi}. For flow analyses in all experiments we utilized FlowJo (version 7) software to generate dot plots and analyze the data.

2.3.5 Histology

Mice (N = 12-15/treatment group) were sacrificed 30 days after MI and hearts were collected in diastole by using saturated KCl and CdCl₂ (100 mM) given through the apex into the left ventricular (LV) cavity. Then we cannulated the ascending aorta and perfused the heart with PBS (VWR International) followed by 10% buffered formalin (VWR International) at 75 mmHg. Hearts were placed in 5 ml of 10% neutral buffered formalin (VWR) and fixed overnight at room temperature. Hearts were then sectioned into 2-mm cross-sectional slices followed by paraffin embedding. Tissue was transferred to 70% ethanol for overnight followed by sectioning into 4- μ m sections starting at the level of LAD ligation. Sections were then stained with Masson's trichrome to evaluate scar size. Digital images were taken, and areas were evaluated using NIH ImageJ (version 7). We measured the LV area, LV cavity area, and infarct area in stained sections, as previously described (Klyachkin, Nagareddy et al. 2015). The scar size was presented as a percentage of LV myocardial volume.

2.3.6 Immunohistochemistry

Mice (N = 4/treatment group) were sacrificed 3 days after MI and hearts were collected. Immunohistochemical assessments were carried out on deparaffinized and rehydrated sections as previously described (Klyachkin, Nagareddy et al. 2015). After deparaffinization and washing, slides were incubated with primary antibodies: rat anti-mouse CD86 (1:100, BD Biosciences), goat anti-mouse CD206 (1:1000, R and D Systems), rabbit anti-mouse YM1 (1:25, Stem Cell technologies), and goat anti-mouse IL-1 β (1:200 R & D System) overnight at 4°C. After washing, sections were incubated with secondary antibodies conjugated to Alexa Fluor 594,647 (1:500, Invitrogen, Carlsbad, CA), then incubated with Sudan Black B (Sigma Aldrich, St. Louis, MO) for 30 minutes. and subsequently incubated with DAPI nuclear counterstain. 10–15 adjacent areas in the pre-infarct and remote zones per section were analyzed (1–2 sections/animal) at 40x magnification using Nikon Confocal Microscope A1 in the University of Kentucky Confocal Microscopy facility. Only nucleated antibody positive cells were counted.

Calculations were performed using the Cell Counter plugin for ImageJ (version 1.51d). Data were presented as total positive cells per high power field in the region of interest.

A similar protocol was used to prepare heart sections from mice on day 30 for angiogenesis assessment using FITC- isolectin B4 (FL1201, Vector Labs, Burlingame, CA). 10–15 adjacent areas in the pre-infarct per section were examined (1–2 sections/animal) at 40x magnification using Nikon Confocal Microscope A1 in the University of Kentucky Confocal Microscopy facility. Data were presented as total capillary density per mm² in the peri-infarct region. All measurements were analyzed by blinded observers.

To examine apoptosis in heart tissue, we used a TdT dUDP Nick-End Labeling (TUNEL) Assay and Caspase-3 staining. Caspase 3 staining was done in deparaffinized and rehydrated sections using antibody against cleaved caspase 3 as previously described (Klyachkin, Nagareddy et al. 2015). TUNEL staining was performed in Biospecimen Procurement and Translational Pathology Shared Resource Facility (BPTP SRF) at the University of Kentucky. Only nucleated positive cells were counted in the peri-infarcted and infarcted areas. Calculations were done using the Cell Counter plugin for ImageJ (version 1.51d). Data were presented as total positive cells per high power field in the region of interest. All measurements were analyzed by blinded observers.

2.3.7 Real-time polymerase chain reaction

We utilized PureLink RNA Mini Kit (ThermoFisher Scientific) to collect total RNA from heart and blood cells according to the manufacturer's protocol. The extracted RNA was quantified using NanoDrop 8000 spectrophotometer (Thermofisher). Then, cDNA was synthesized using SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative RT-PCR was performed using a QuantaStudio 7 Flex real-time thermocycler (Applied Biosystems by life technology) to quantify the mRNA expression of markers identifying: inducible nitric oxide synthases (iNOS), tumor necrosis factor alpha (TNF- α), monocyte

chemotactic protein-1 (MCP-1), transforming growth factor beta (TGF- β), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), interleukin-4 (IL-4), chitinase-like3 Chil3 (YM1), Peroxisome proliferator-activated receptor gamma (PPAR γ). We used the comparative Ct method for relative estimation of mRNA expression which was normalized to 18s (a housekeeping gene). To overcome the possible errors due to augmentation of contaminated DNA: (a) we used primers adjusted to bridge an intron for specific cDNA augmentation; (b) we used proper negative control reactions (template free controls); (c) we reassessed the consistency of product augmentation through examination of the melting curve of augmented products (dissociation graphs); and (d) the melting temperature (T_m) was 57°C–60°C, and the probe T_m was at least 10°C more than the primer T_m . The primer sequences are listed in S1 Table.

2.3.8 Echocardiography

We used a Vevo 3100 system supplied with a 15-7-MHz linear broadband transducer and a 12-5-MHz phased array transducer to acquire Echocardiograms. We assessed cardiac function at baseline (before cardiac surgery) then at 48 hours after MI, and immediately before sacrifice at 30 days after MI. We utilized a heating pad to keep the body temperature at 37°C during the experiment; the temperature was measured using a rectal temperature probe. To determine the left ventricular function and volume in M-mode, two-dimensional and Doppler echocardiography modes we utilized modified parasternal long-axis and short-axis. We also used M-mode tracings at the mid-papillary level to estimate the systolic and diastolic parameters, and Teichholz formula at end-systole and end-diastole to measure the LV volumes. All mice were anaesthetized using 1%–3% isoflurane during Echocardiography to maintain a heart rate of 450–500 BPM for all echocardiographic acquisitions. Echocardiography imaging and analysis was performed by a blinded investigator.

2.3.9 Cell culture and hypoxic exposure

The murine macrophage cell line J774 (ATCC, Manassas, VA) was used for the *in vitro* studies on the anti-inflammatory effects of AZM. Cells were plated in 6 well plates at concentration 0.3×10^6 cells/well in media consisting of DMEM, 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, L-Glutamine, and Glucose. Following cell adhesion (4–6 hours) cells were treated with AZM (Sigma-Aldrich, St. Louis, MO) at concentration 30 μ M or DMSO (SIGMA-ALDRICH, St. Louis, MO) as control and incubated over night at 37 C with 5% CO₂. To induce hypoxia-reperfusion injury, cultured cells were established in a gas-tight modular chamber (STEMCELL Technologies Inc., Seattle, WA) containing 1% O₂, 5% CO₂, 94% N₂, or in normal oxygen tension with 20% O₂, 5% CO₂ as control for 24 hours at 37°C followed by reperfusion with 21% O₂, 5% CO₂ for 24 or 48 hours. Supernatants were collected at the end of these time points to assess the cytokines level and their ratio (TNF- α and IL-10).

2.3.10 ELISA assays

Protein concentrations of TNF- α and IL-10 are measured using standard ELISA kits (BD Biosciences, San Deigo, CA) according to the manufacturer protocol. Data are presented as individual cytokine values and their ratio.

2.3.11 Luminex assay

Plasma was collected according to the above mentioned protocols at days 3, and 7 post-MI. Inflammatory biomarkers (IL-12, IL-1 β , IL-1 α , IL-6, TNF- α , MCP-1, MIP-1a and MIP-1b) were assayed using the Milliplex mouse cytokine magnetic kit (MILLIPLEX MAP for Luminex xMap Technology, Millipore, USA) according to the manufacturer's protocol.

2.3.12 Liver and kidney function tests

Plasma was collected according to the above mentioned protocols at days 1, 3, and 7 post-MI. Samples were sent to ANTEC diagnostic to assay blood urea nitrogen, alanine aminotransferase (ALT), albumin, and albumin/globulin ratio.

2.3.13 Statistical analysis

Values are expressed as mean \pm standard error of mean (SEM). We used unpaired Student t test or analysis of variance (one-way or multiple comparisons) to estimate differences, as appropriate. We utilized two-sided Dunnett or Dunn tests for post hoc multiple comparison procedures, with control samples as the control category. Throughout the analyses, a P value less than 0.05 was considered statistically significant. All statistical analyses were performed using the Prism 7 software package (GraphPad, La Jolla, CA).

2.4 Results

2.4.1 AZM shifts macrophages away from the pro-inflammatory towards the reparative state post-MI

Macrophages are the predominant inflammatory cells type in cardiac tissue after cardiac injury, regulating its healing at multiple phases. It is well established that AZM can modulate inflammation through shifting macrophages towards the reparative phenotype (Murphy, Sundareshan et al. 2008, Feola, Garvy et al. 2010, Cory, Birket et al. 2014, Gensel, Kopper et al. 2017). To date, the effect of AZM on the inflammatory response after MI at the level of macrophages has not been characterized. We analyzed the phenotype of macrophages in cardiac tissue at several time points following induction of MI using flow cytometry (**Figure 2.1A**). We detected a significant reduction in the pro-inflammatory macrophages (CD45⁺/Ly6G⁺/F4-80⁺/CD86⁺) at 1 day following MI with AZM treatment (**Figure 2.1B**). This effect was associated with a significant increase in the reparative macrophages (CD45⁺/Ly6G⁺/F4-80⁺/CD206⁺) at day 1 and 3 after MI (**Figure 2.1C**). Overall, the ratio between pro- and anti-inflammatory macrophages was reversed towards an anti-inflammatory state with AZM treatment, particularly in the early phase after MI (**Figure 2.1D**).

Under physiological conditions, resident macrophages maintain tissue homeostasis (Epelman, Liu et al. 2015). However, after injury such as MI, monocytes infiltrate the heart and differentiate into macrophages. Monocytes

contribute to tissue healing and repair through coordinated activities of different monocyte subpopulations (Ly6C^{hi} vs. Ly6C^{lo}) (Nahrendorf, Swirski et al. 2007, Swirski, Nahrendorf et al. 2009). We assessed monocyte subpopulations (Ly6C^{hi} vs. Ly6C^{lo}) after MI in the PB and heart (**Figure 2.2A**). While we did not notice significant differences between blood monocyte subsets in AZM vs. vehicle treated mice, we detected a significant reduction in the Ly6C^{hi} (pro-inflammatory) monocytes (CD45⁺/Ly6C/G^{hi}/CD115^{hi}) in cardiac tissue after MI with AZM treatment (**Figure 2.2B**). There was no significant difference in the Ly6C^{lo} (anti-inflammatory) monocytes in cardiac tissue with AZM treatment. This suggests a reduction in pro-inflammatory monocyte infiltration to the heart or a reduction in their polarization in the myocardium. Since maintained Ly6C^{hi} monocytes impair healing after MI (Swirski, Nahrendorf et al. 2009), these results imply that AZM treatment reduces detrimental inflammatory response following myocardial ischemia.

2.4.2 AZM downregulates pro-inflammatory cytokines while upregulating anti-inflammatory cytokines following cardiac injury

Macrophages have distinctive cytokine profiles based on their inflammatory status. Pro-inflammatory macrophages are potent generators of toxic effector molecules (reactive oxygen species, nitric oxide) and pro-inflammatory cytokines (IL-1 β , TNF- α , IL-6)(Martinez, Sica et al. 2008). Conversely, reparative macrophages produce anti-inflammatory molecules (TGF- β and IL-10) and express scavenger, mannose, and galactose-type receptors (Cynamon, Klemens et al. 1992, Song, Ouyang et al. 2000). We conducted *in vitro* studies to examine the effect of hypoxia/reperfusion injury on the phenotype of J774 macrophages in the presence or absence of AZM. Following 24 hours hypoxia and reperfusion, we noted significant shift in macrophage cytokine production towards a pro-inflammatory phenotype with higher TNF- α levels compared to IL-10 levels. AZM treatment shifted the cytokine production towards an anti-inflammatory pattern with significant increase in IL-10/TNF- α ratio (**Figure 2.3**). We also examined the effect of AZM on cytokine expression following MI in the *in vivo* studies. Our data confirm

the change towards the anti-inflammatory state with AZM treatment. In heart tissue, AZM treatment was associated with significant reductions in the gene expression of iNOS and inflammatory cytokines (MCP-1, TNF- α , IL-6, and IL-1 β) while the anti-inflammatory cytokines/macrophage modulatory factors (TGF- β and IL-4, PPAR γ and YM1) were increased (**Figure 2.4A**).

A similar trend was noted in the gene expression of pro- and anti-inflammatory cytokines in PB cells at the same time points (**Figure 2.4B**). These gene expression results are corroborated by IHC and ELISA studies of the heart and plasma, respectively. Three days after MI, we quantified IL-1 β and YM1 levels in the peri-infarct zone. IL-1 β was remarkably downregulated with a concomitant elevation in YM1 in AZM treated mice compared to the control group (**S2.2 Figure**). In plasma, levels of inflammatory cytokines (IL-12, IL-1 β , IL-1 α , IL-6, TNF- α , MCP-1, MIP-1a and MIP-1b) were suppressed on days 3 and 7 post-MI as assessed by the Luminex assay (**S2.3 Figure**). Overall, these results imply that AZM treatment modulates the inflammatory response following myocardial ischemia by attenuating the pro-inflammatory changes and enhancing anti-inflammatory/repairative cytokine production.

2.4.3 AZM treatment is associated with alternative macrophages activation in the peri-infarct region

The balance between pro- and anti-inflammatory macrophages in the peri-infarct region plays an important role in infarct expansion and adverse cardiac remodeling (Harel-Adar, Ben Mordechai et al. 2011, Ben-Mordechai, Palevski et al. 2015, de Couto, Liu et al. 2015, Epelman, Liu et al. 2015). To evaluate the impact of AZM on the activation state of macrophages in the peri-infarct region, we quantified pro-inflammatory (CD86+) and anti-inflammatory (CD206+) macrophages 3 days post-MI using immunohistochemistry. Our results confirmed the shift towards an anti-inflammatory state with AZM treatment. AZM-treated mice had significantly lower numbers of CD86+ cells and higher numbers of CD206+ cells (**Figure 2.5**). Importantly, the pro-inflammatory/repairative macrophage ratio was decreased robustly in the AZM treated compared to the vehicle group. Of note,

macrophage inflammatory state is a spectrum from the pro- to the anti-inflammatory phenotypes. Nonetheless, we used the same strategy for assessing each population quantitatively to avoid any bias. We counted CD86 and CD206 separately and this could have led to double counting of cells in each category. These findings are in agreement with our flow cytometry data and implicate a potential therapeutic role for AZM in reducing post-MI cardiac inflammatory changes.

2.4.4 AZM reduces neutrophil counts after cardiac ischemia through enhancing apoptosis

In acute inflammation, neutrophils are key players in removing pathogens and debris, thus contributing to the return of normal tissue homeostasis (Carbone, Nencioni et al. 2013). Evolutionary mechanisms have developed to limit the initial inflammatory response after MI. These pathways include a robust switch of macrophages towards anti-inflammatory phenotype following ingestion of apoptotic neutrophils (Serhan and Savill 2005). Indeed, prior studies have implicated the ingestion of apoptotic cells as a mechanism for the anti-inflammatory changes observed with AZM (Hodge, Hodge et al. 2006). While our data as well as others have shown that AZM induces alternative polarization of macrophages towards the anti-inflammatory state, the mechanism of these changes is not entirely clear. We hypothesized that AZM promotes neutrophil apoptosis which likely initiates the anti-inflammatory changes. In support of this hypothesis, prior reports have confirmed the pro-apoptotic effects of macrolides on neutrophils (Aoshiba, Nagai et al. 1995, Rapp 1998). We therefore analyzed neutrophil numbers (CD45⁺/CD115^{lo}/Ly6G/C^{lo}) using the gating strategy outlined in Fig 2A in the heart and PB via flow cytometry. Treatment with AZM resulted in significant reductions of neutrophil counts in cardiac tissue and PB in the early phase after MI (**Figure 2.6A**). To further investigate the mechanism associated with neutrophil count reduction, we examined neutrophil apoptosis using Annexin V and PI staining in cardiac tissue using flow cytometry. AZM treatment was associated with a significant increase in the percentage of apoptotic neutrophils in

the early inflammatory phase after MI compared to vehicle-treated mice (**Figure 2.6B and 2.6C**). Our findings indicate that AZM treatment reduces neutrophil numbers after cardiac injury through pro-apoptotic effects, which could account for its post-MI immunomodulatory effect.

2.4.5 AZM diminishes cardiac cell death and scar size while promoting angiogenesis following cardiac ischemic injury

Alternatively activated macrophages are master regulators of the healing process after MI by regulating cardiomyocyte apoptosis, collagen deposition, and angiogenesis in the peri-infarct regions (Aurora, Porrello et al. 2014). To determine the effect of AZM effects on programmed cell death in the heart post-MI, we investigated cleaved caspase-3 positive staining of cells in the infarct zone, peri-infarct border, and remote (normal) zones 3 days post-MI. We found that apoptosis was significantly reduced within the infarct and border regions in the AZM-treated group compared to vehicle-treated group (**Figure 2.7**). In order to confirm our caspase 3 staining results, we performed TUNEL assay in the peri-infarct region. The results were consistent with the caspase 3 assay (**S2.4 Fig**), suggesting enhanced survival of cardiac cells, likely driven by AZM. These data corroborate the immunohistochemistry and real-time PCR results, demonstrating significant reduction in pro-apoptotic cytokines such as TNF- α . We then assessed the effect of AZM therapy on the infarct size using morphometric assessments on Masson's trichrome-stained cross-sections from the hearts (30 days post-MI). We observed significantly smaller scar size in mice treated with AZM compared to those treated with vehicle (**Figure 2.8A and 2.8B**). These data indicate that AZM protects the infarcted heart from the adverse cardiac remodeling that occurs chronically after ischemia. Alternatively activated macrophages produce multiple cytokines (including pro-angiogenic factors) that can enhance regeneration (Sunderkotter, Goebeler et al. 1991, Song, Ouyang et al. 2000). Angiogenesis is regarded as a major stimulator of tissue recovery in the heart after ischemic injury. To examine the vessels density in the peri-infarcted areas between groups, we assessed quantified isolectin (a marker of endothelial cells in blood vessels) 30 days

following MI. Mice treated with AZM had significantly higher capillary density in the peri-infarct region compared to vehicle-treated mice (**Figure 2.8C and 2.8D**). Taken together with the results detailed in Fig 7, AZM treatment reduces scar size after MI, which could be explained by the reduction in cellular apoptosis and enhanced angiogenesis in the ischemic heart.

2.4.6 AZM preserves cardiac function, reduces adverse remodeling and improves survival post-MI

Loss of balance between inflammatory and reparative phases post-MI can result in adverse cardiac remodeling and eventually systolic HF (de Couto, Liu et al. 2015). Our data suggest an immunomodulatory role for AZM on the heart post-MI. To examine the translation of these cellular effects on cardiac function, echocardiography was carried out on AZM and vehicle treated mice at baseline, and 2 and 30 days after MI. We observed preservation of LV ejection fraction as well as fractional shortening in AZM treated compared to vehicle mice (**Figure 2.9A-9C**). We also observed similar effects on parameters of LV remodeling such as LV end-systolic and end-diastolic diameters (**Figure 2.9D-9E**). In agreement with the noted reduction in scar size, we observed significantly less deterioration in infarct wall thickness in AZM treated group compared to controls (**Figure 2.9F**). Therefore, the beneficial effect of AZM extends beyond the enhancements in the inflammatory balance after MI to a favorable recovery of LV functional and remodeling parameters as summarized in **S2.2 Table**. Additionally, survival rates reflect these AZM-mediated functional improvements with a marked reduction in mortality in AZM-treated mice (**Figure 2.9G**).

2.5. Discussion

Cardiac inflammation is a crucial component of the recovery phase after MI. However, a tug-of-war between pro- and anti-inflammatory forces in the ischemic myocardium impacts cardiac recovery. Reversing the pro-inflammatory forces after MI can enhance cardiac recovery and survival. In this study, we provide first evidence that the macrolide, AZM, exerts immunomodulatory effects on macrophages after MI shifting them towards anti-inflammatory phenotype. This is demonstrated by significant reduction in pro-/anti-inflammatory macrophage ratio and pro-inflammatory genes expression in parallel with significant elevation in anti-inflammatory gene expression. These immunomodulatory effects resulted in reduced apoptosis and scar size, enhanced cardiac functional recovery, and improved survival. Taken together, AZM represents a novel and clinically available target for reducing cardiac inflammation and damage following MI.

The immunomodulatory effects of AZM after MI appear to be multifactorial including direct effects on macrophages as well as pro-apoptotic effects on neutrophils. It is well accepted that neutrophils play a critical role in tissue healing following MI through debris clearance from the injury site (Ortega-Gomez, Perretti et al. 2013). However, prolonged neutrophil accumulation in the heart post-MI is considered deleterious (Chia, Nagurny et al. 2009, Guasti, Dentali et al. 2011). On the other hand, aggressive neutrophil depletion following MI can disturb the healing process, leading to impaired functional recovery (Horckmans, Ring et al. 2017). Clinical data suggest that a delay in neutrophil apoptosis after MI contributes to the exaggerated inflammatory response (Garlichs, Eskafi et al. 2004). Here, we show that AZM enhances neutrophil apoptosis in cardiac tissue after MI, which could explain the reduction in neutrophil counts in our studies. This is in agreement with other reports in the literature that suggest a pro-apoptotic effect of macrolides on neutrophils (Aoshiba, Nagai et al. 1995, Inamura, Ohta et al. 2000). The pro-apoptotic effect of AZM is most noticeable on the first day after MI. Importantly, studies have shown that AZM is more concentrated in polymorphonuclear leukocytes, which will induce apoptosis in these cells through

arresting cell cycle in G2/M (Ribeiro, Hurd et al. 2009). Engulfing apoptotic neutrophils by macrophages stimulates the anti-inflammatory response through blocking pro-inflammatory cytokines and enhancing anti-inflammatory cytokines (IL-10 and TGF- β) and pro-resolving lipid mediators (Soehnlein and Lindbom 2010).

In addition to its effects on neutrophil apoptosis, AZM can have direct effects on macrophages. Our *in vitro* results indicate that AZM therapy attenuates the inflammatory changes seen in macrophages subjected to hypoxia/reperfusion injury. In addition, induction of programmed cell death in neutrophils results in decrease in the production of inflammatory cytokines and chemokines (Soehnlein, Zernecke et al. 2008), as demonstrated in our study. Indeed, our experiments show that AZM treatment decreased the expression of MCP-1 which promotes Ly6C^{hi} cells recruitment to the infarcted tissue. Ly6C^{hi} monocytes differentiate into pro-inflammatory macrophages in the peri-infarct border which eventually contribute to adverse cardiac remodeling (Panizzi, Swirski et al. 2010). Furthermore, through the reduction in neutrophil count and modulation of macrophage phenotype, the production and deleterious effects of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 were diminished (Saini, Xu et al. 2005). In parallel to the decreased pro-inflammatory cytokines, we observed significant increase in anti-inflammatory cytokines such as TGF- β and IL-4 which have been linked to favorable cardiac remodeling and functional recovery after MI (Lambert, Lopez et al. 2008).

The immunomodulatory effects of AZM have been demonstrated in various inflammatory and tissue injury scenarios (Zhang, Bailey et al. 2015, Gensel, Kopper et al. 2017). Among them, acute stroke and spinal cord injury have close resemblance to the inflammatory reactions (biphasic macrophage response and chemokine profile) happening post-cardiac injury (Gensel and Zhang 2015, Amantea, Certo et al. 2016). AZM treatment after spinal cord injury led to significant decrease in pro-inflammatory macrophages and increase in anti-inflammatory macrophages (Zhang, Bailey et al. 2015, Gensel, Kopper et al.

2017). Interestingly, and in accordance with our data, the shift in macrophage phenotype was associated with marked functional improvements in mice. The exact molecular mechanisms through which AZM exerts its immunomodulatory effects are not fully understood. However, transcription factors such as NF- κ B and AP-1 are likely targets (Kano and Rubin 2010). Our unpublished data suggest that AZM reduces NF- κ B activation through decreasing the translocation of phospho-p65 to the nucleus. Furthermore, there is a simultaneous increase in IKK β and I κ B α with AZM therapy. This does not exclude immunomodulatory actions mediated through other intracellular signaling pathways (Kano and Rubin 2010).

Macrophages are essential players in the myocardial environment post-MI and their role extends beyond the inflammatory phase to tissue regeneration and healing (Godwin, Pinto et al. 2013, Petrie, Strand et al. 2014). The heart possesses a remarkable regenerative capacity in the early stages of postnatal life, an ability lost by day 7 after birth (Porrello, Mahmoud et al. 2011, Porrello, Mahmoud et al. 2013). It has been postulated that this regenerative capacity is related to the naivety of the immune system in which macrophage modulation plays a central role (Song, Ouyang et al. 2000). Indeed, anti-inflammatory macrophages have been shown to interact with stem cells following cardiac injury leading to enhance cardiac recovery (de Couto, Liu et al. 2015). Overall, AZM therapy is associated with higher numbers of anti-inflammatory macrophages (CD206⁺ cells) after MI with the majority of changes occurring in the peri-infarct region as shown in our immunohistochemistry data. This phenotypic shift is associated with smaller scar, better cardiac function, and enhanced angiogenesis after MI. Additionally, we observed a significant reduction in apoptotic heart cells in both infarct and peri-infarct areas at day 3 post-MI, which could be attributed to the survival factors released from reparative macrophages (Lambert, Lopez et al. 2008, Murray, Allen et al. 2014). In summary, the early shift in macrophages towards the reparative phenotype by AZM treatment can limit acute ischemic damage and promote chronic cardiac recovery.

The balance between macrophage phenotypes is reversible (Ben-Mordechai, Palevski et al. 2015) and can be therapeutically harnessed to enhance cardiac healing after ischemic injury. Animal studies show that early interventions aimed at altering macrophage activation state or shifting the balance towards the anti-inflammatory phenotype can substantially reduce the risk of HF development (de Couto, Liu et al. 2015, Kain, Liu et al. 2017). Kain et al initiated a phenotypic switch in macrophages after myocardial injury by using either free or encapsulated 15-epi-Lipoxin A4. They demonstrated upregulation of the reparative macrophages as well as the anti-inflammatory/macrophage modulatory genes (*Mrc-1*, *Ym-1*, *Arg-1*). Moreover, cardiac function was improved in 15-epi-Lipoxin A4 treated mice compared to controls (Kain, Liu et al. 2017). These findings were confirmed in multiple studies using different therapeutic approaches, including formyl peptide receptor 2 (Heo, Kwon et al. 2017), cardiosphere-derived cells (de Couto, Liu et al. 2015), and endogenous Annexin-A1 (Qin, Finlayson et al. 2017). However, studies on alternative macrophage activation in MI utilized experimental therapeutics with limited safety data in humans. Conversely, our strategy of using AZM is more clinically relevant and can be safely translated to human studies.

Healing and remodeling after MI are fundamentally regulated by the inflammatory pathways. Modulating the inflammatory response post-MI is an elusive target as studies have shown that complete inhibition of inflammation is rather harmful (Frangogiannis 2014). Indeed, therapeutic interventions targeting the systemic inflammatory pathways have yielded limited success in pre-clinical and clinical studies (Frangogiannis 2014). Delayed healing and development of ventricular aneurysm were reported with glucocorticosteroids, and their uses should be avoided post-MI (Bulkley and Roberts 1974). On the other hand, NSAIDs use in coronary artery disease patients has been correlated with higher mortality and recurrent myocardial infarction (Schjerning Olsen, Fosbol et al. 2011). Selective targeting of inflammatory mediators such as IL-1 β using selective monoclonal antibodies demonstrated success in clinical studies, albeit with high cost and modest benefit (Seropian, Toldo et al. 2014). Therefore, strategies aimed

at modulating the inflammatory response rather than its suppression provides promising therapeutic modalities. In this study, we show that AZM as a small immunoregulatory molecule provides more specific modulation to the post-MI inflammation through increasing the abundance of anti-inflammatory macrophages without completely blunting the pro-inflammatory mediators.

In this study, we initiated AZM 3 days prior to MI to ensure appropriate steady-state levels at the time of injury (Feola, Garvy et al. 2010). Based on published literature (Kano and Rubin 2010), we anticipate rapid accumulation of AZM in macrophages and fast immunomodulatory actions. Hence, initiating AZM following MI could be beneficial as well and this is currently being studied in our lab. Another limitation in our study is the use of relatively higher dose of AZM (160mg/kg/day) than the clinically prescribed dose (10-45mg/kg) (Zhang, Bailey et al. 2015). As a proof of concept, we aimed to achieve a dose consistent with our *in vitro* studies. However, multiple human studies have demonstrated AZM safety at higher doses and its wide therapeutic window. Given the allometric scaling on AZM, lower doses than those used in our study may be effective in humans (Rapp 1998, Petrie, Strand et al. 2014). Finally, we sought to examine the extremes of macrophage polarization by quantifying the pro- and anti-inflammatory macrophages based on gene expression and surface markers. While this is a helpful approach for examining the effects of AZM, we acknowledge that it oversimplifies the macrophage landscape and categorizes them into only two main groups (pro- and anti-inflammatory) which may lead to overestimating either of the categories (Nahrendorf and Swirski 2016). Future studies are warranted to provide a comprehensive examination of the effect of AZM on the various macrophage subtypes after MI.

Conclusions

Cardiac inflammatory changes following MI are mediated, at least in part, by pro-inflammatory macrophages, which have detrimental effects on cardiac function and survival. This is the first study to demonstrate the efficacy of AZM as an immunomodulatory pharmacological agent for macrophages after MI. Our

results indicate that the anti-inflammatory effects of AZM are related to its direct effects on macrophages and its pro-apoptotic effects on neutrophils. Systematic assessment of cardiac and systemic changes demonstrates that the beneficial effects of AZM result in significant reduction in scar size, adverse cardiac remodeling, cardiac function, and survival post cardiac ischemia. AZM has a wide therapeutic window, can be administered orally, has low side effect profile and has been approved for human use. These criteria make it an ideal agent for novel and effective therapeutic interventions to improve survival and reduce HF following MI in humans.

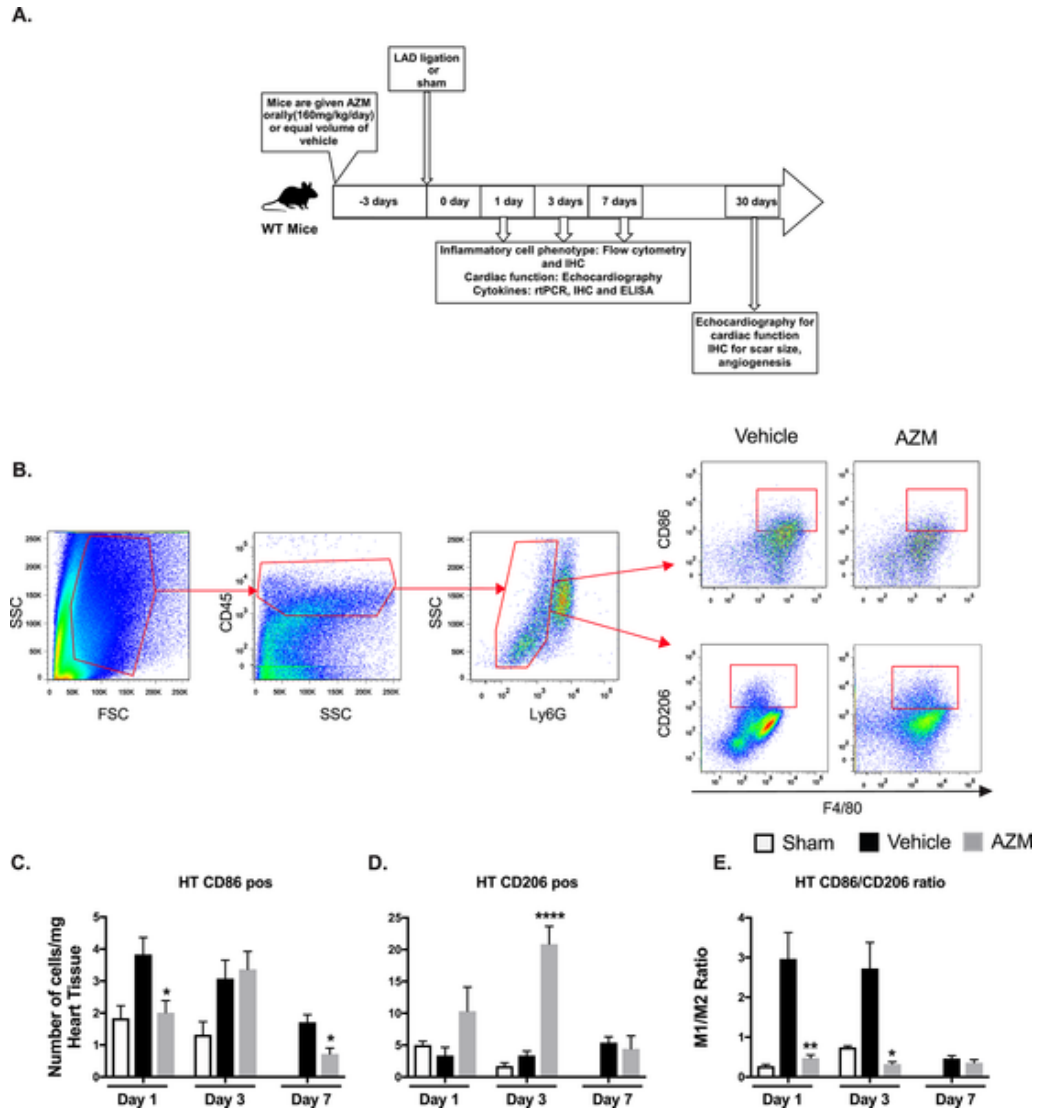


Figure 2.1. AZM therapy shifts macrophages towards the anti-inflammatory phenotype in the heart after myocardial infarction.

Experimental design for the *in vivo* study (Panel A). Representative FACS plots demonstrating the gating strategy for pro-inflammatory (CD45⁺/Ly6G⁺/F4-80⁺/CD86⁺) and anti-inflammatory (CD45⁺/Ly6G⁺/F4-80⁺/CD206⁺) macrophages (Panel B). Quantitative analyses of pro-inflammatory subpopulations (CD86⁺) and anti-inflammatory subpopulations (CD206⁺) are presented in Panels C and D, respectively, at different time points following MI in AZM and vehicle treated groups. There is a significant reduction in the inflammatory macrophages in the first day after MI in AZM-treated mice, which is associated with a significant increase in anti-inflammatory ones. These changes translate into a significant reduction in the pro-/anti-inflammatory (CD86⁺/CD206⁺) ratio at days 1 and 3 after MI (Panel E) (n = 4 MI and 3 sham mice/group/time point, *P<0.05, **P<0.01 and ****P<0.001 compared to vehicle controls). Data presented as mean ± SEM. AZM, azithromycin; IHC, immunohistochemistry; LAD, left anterior descending coronary artery; rtPCR, real-time Polymerase Chain Reaction.

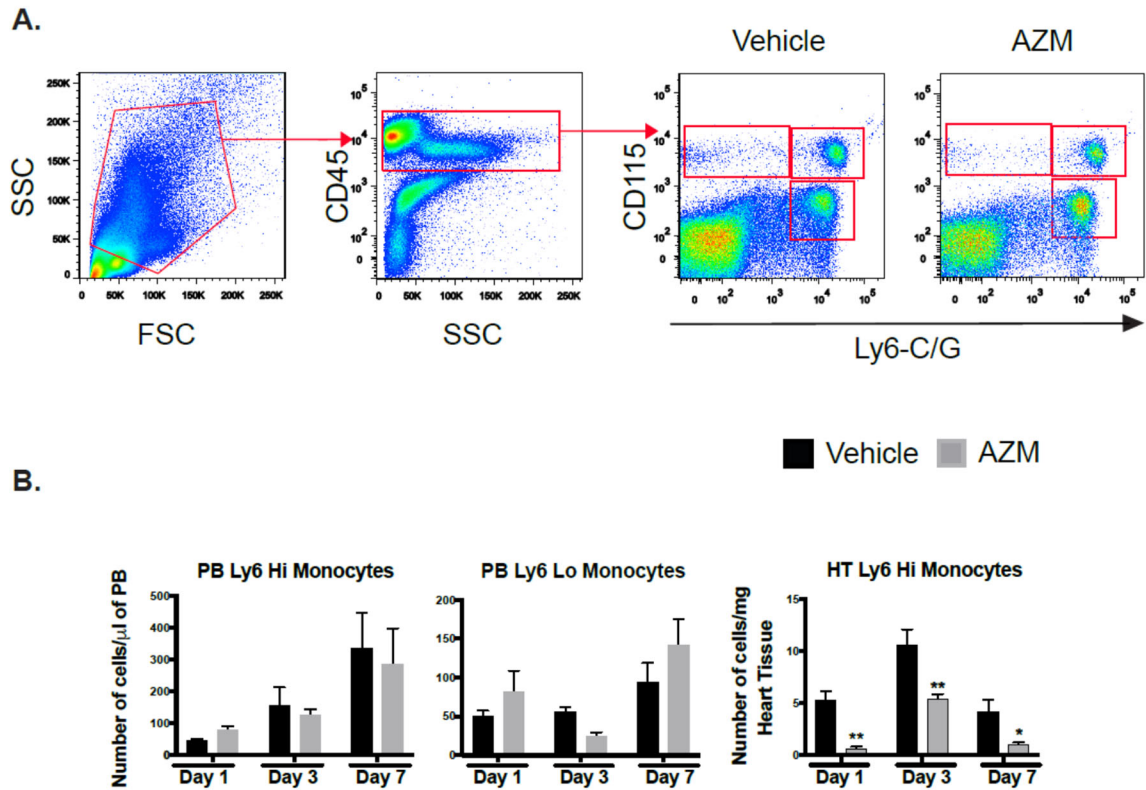


Figure 2.2. AZM therapy reduces cardiac inflammatory monocytes after MI.

Representative FACS plots demonstrating the gating strategy for Ly6C^{hi}(CD45⁺/CD115^{hi}/Ly6-C^{hi}) and Ly6C^{lo}(CD45⁺/CD115^{hi}/Ly6-C^{lo}) monocytes (Panel A). Quantitative analyses of monocyte subpopulations in PB and heart tissue demonstrating no significant changes in PB monocyte subpopulations but significant reduction in cardiac Ly6C^{hi} population throughout the different time points after MI in AZM treated mice (Panel B) (n = 4 MI and 3 sham mice/group/time point, *P<0.05 and **P<0.01 compared to vehicle controls). Data presented as mean ± SEM. AZM, azithromycin; PB, peripheral blood.

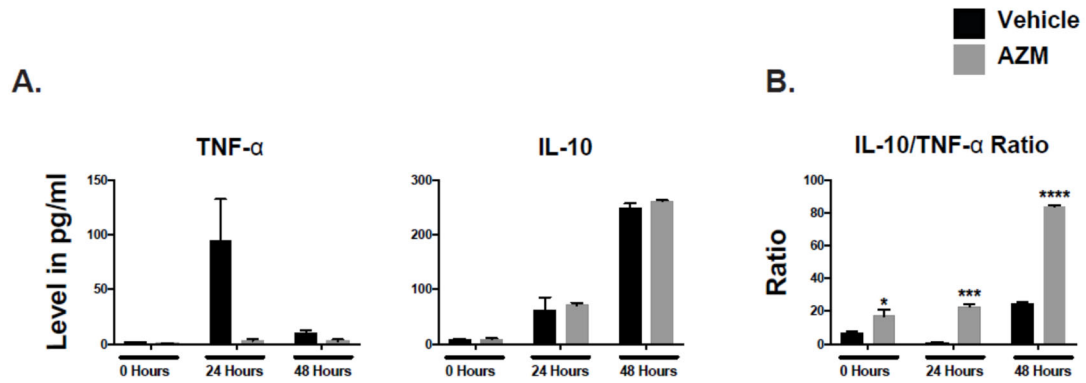


Figure 2.3. AZM therapy reduces the production of inflammatory cytokines in macrophages subjected to ischemia/reperfusion injury.

Quantitative analyses of pro-inflammatory cytokine, TNF- α , and the anti-inflammatory cytokine, IL-10, production from J774 macrophages subjected to 24 hypoxia followed by 24 and 48 hours of reperfusion. The analysis demonstrates reduction of TNF- α compared to vehicle both at 24 and 48 hours. No significant changes were noted in IL-10 production. Overall, the IL-10/TNF- α ratio was significantly higher in macrophages treated with AZM (Panel B) (two independent experiments and 4 replicates/time point, * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$ compared to vehicle controls). Data presented as mean \pm SEM. AZM, azithromycin; IL-10, interleukin 10; TNF- α , tumor necrosis factor- α .

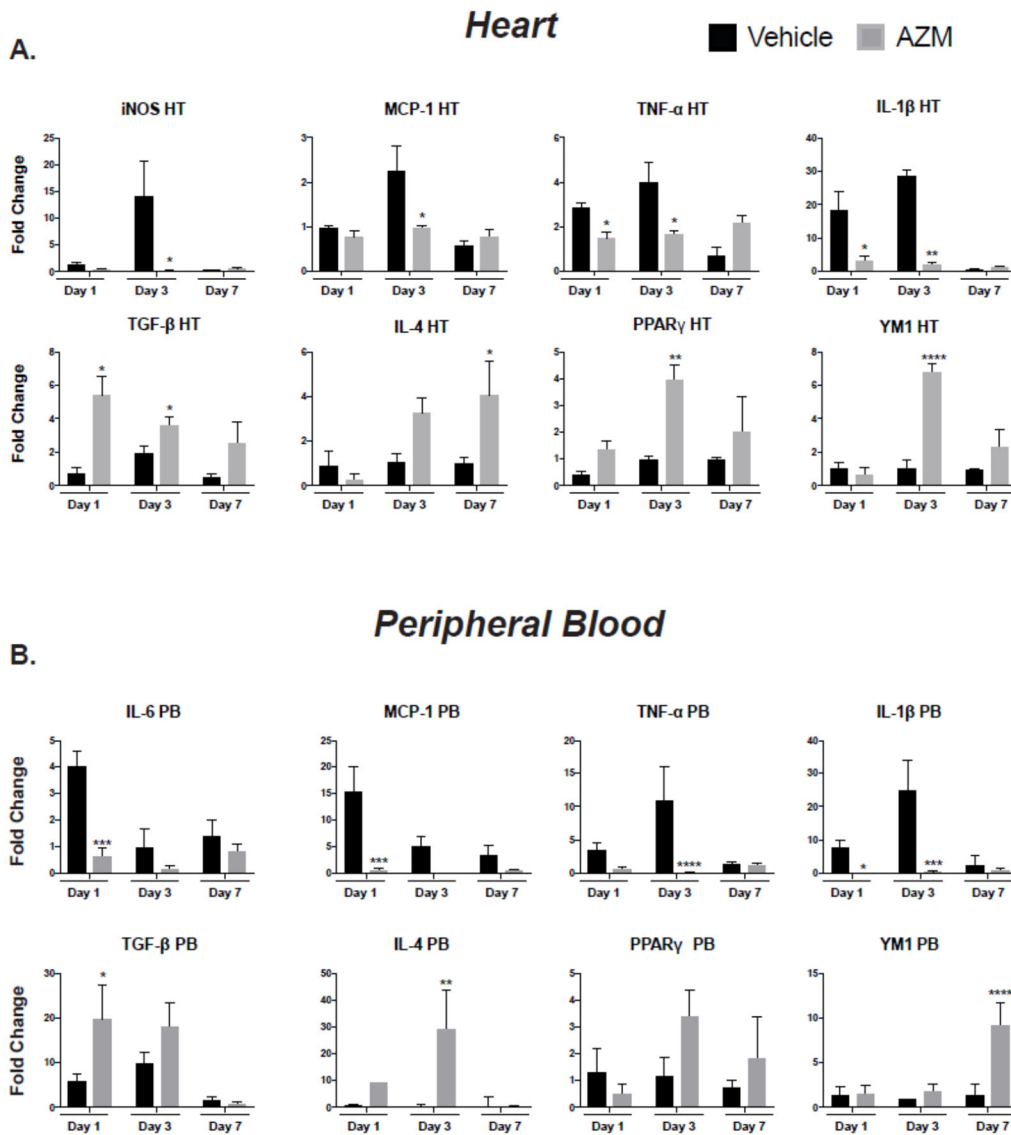


Figure 2.4. AZM treatment exerts immunomodulatory effects on cytokines expression following MI.

mRNA expression of pro-inflammatory cytokines in HT (Panel A) and PB (Panel B), demonstrate significant reduction in gene expression of these cytokines in the early inflammatory phase following injury with AZM therapy compared to vehicle controls (red line demarcates the level of gene expression in sham operated mice). The mRNA expression of anti-inflammatory cytokines is augmented with AZM therapy compared to vehicle control ($n = 4$ mice/group/time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to vehicle controls). Data presented as mean \pm SEM. AZM, azithromycin; HT, heart; IL-1 β , interleukin 1 beta; IL-6, interleukin 6; IL-4, interleukin 4; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; PB, peripheral blood; PPAR γ , peroxisome proliferator-activated receptor gamma; TGF-1 β , tissue growth factor 1 beta; TNF- α , tumor necrosis factor-alpha; YM1 (Chil3), chitinase-like 3.

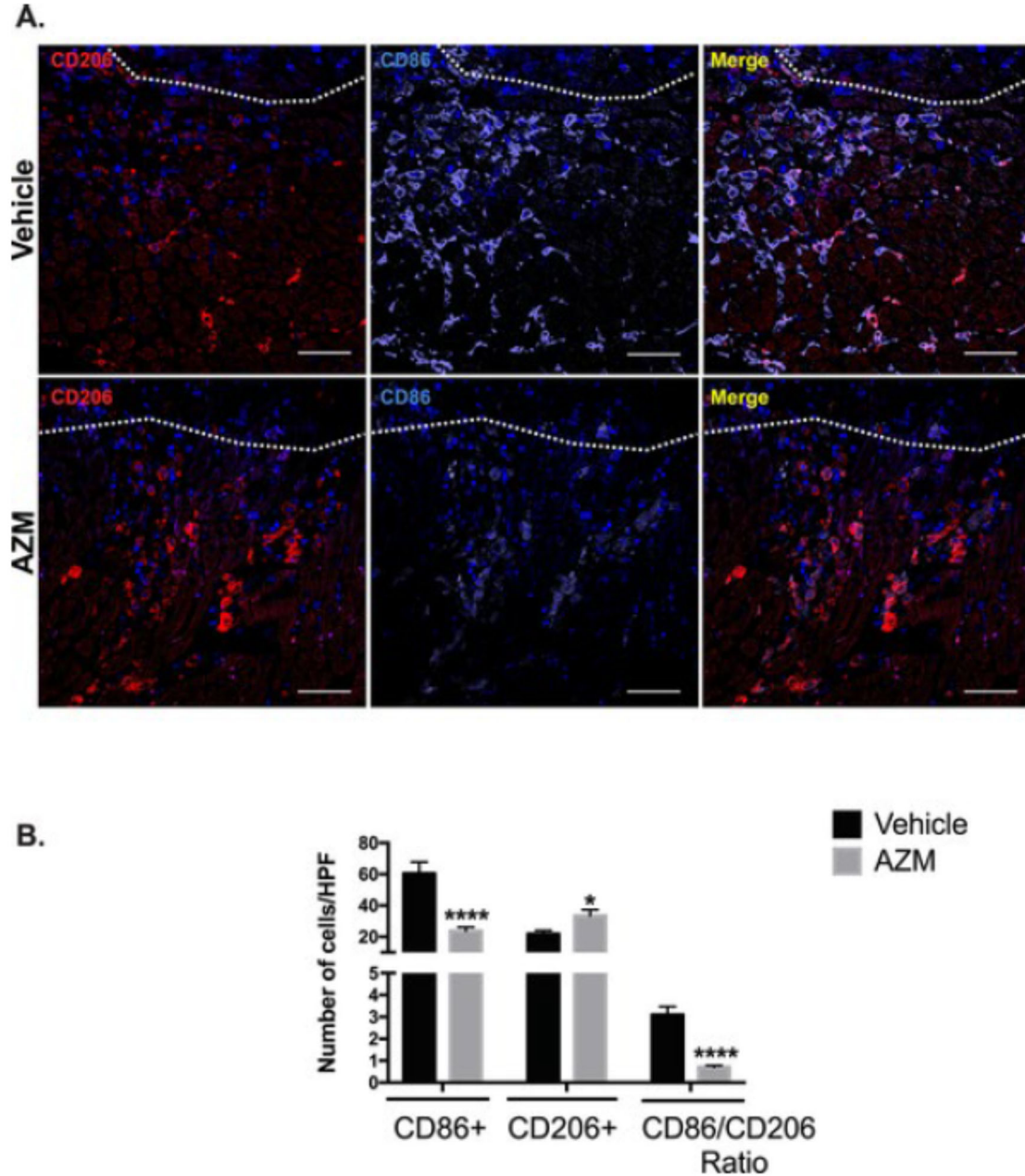


Figure 2.5. AZM treatment enhances alternative macrophage activation in the peri-infarct border of the injured heart.

Immunohistochemical assessment of the content of pro-inflammatory (CD86+) and reparative macrophages (CD206+) markers 3 days post-MI. Panel A shows representative images from vehicle- and AZM-treated mice demonstrating higher density of CD86+ compared to CD206+ cells in the peri-infarct border in vehicle-treated mice. White line demarcates the infarct border. Panel B shows quantitative assessment of CD86+, CD206+ and the markers ratio 3 days post-MI. The difference in pro-inflammatory and anti-inflammatory macrophages lead to a significant shift towards an anti-inflammatory state and the reduction in their ratio in AZM-treated mice ($n = 4$ animals/group, $*P < 0.05$ and $****P < 0.0001$ compared to vehicle controls). Scale bars represent 50 μm . Data presented as mean \pm SEM. AZM, azithromycin; HPF, high power field.

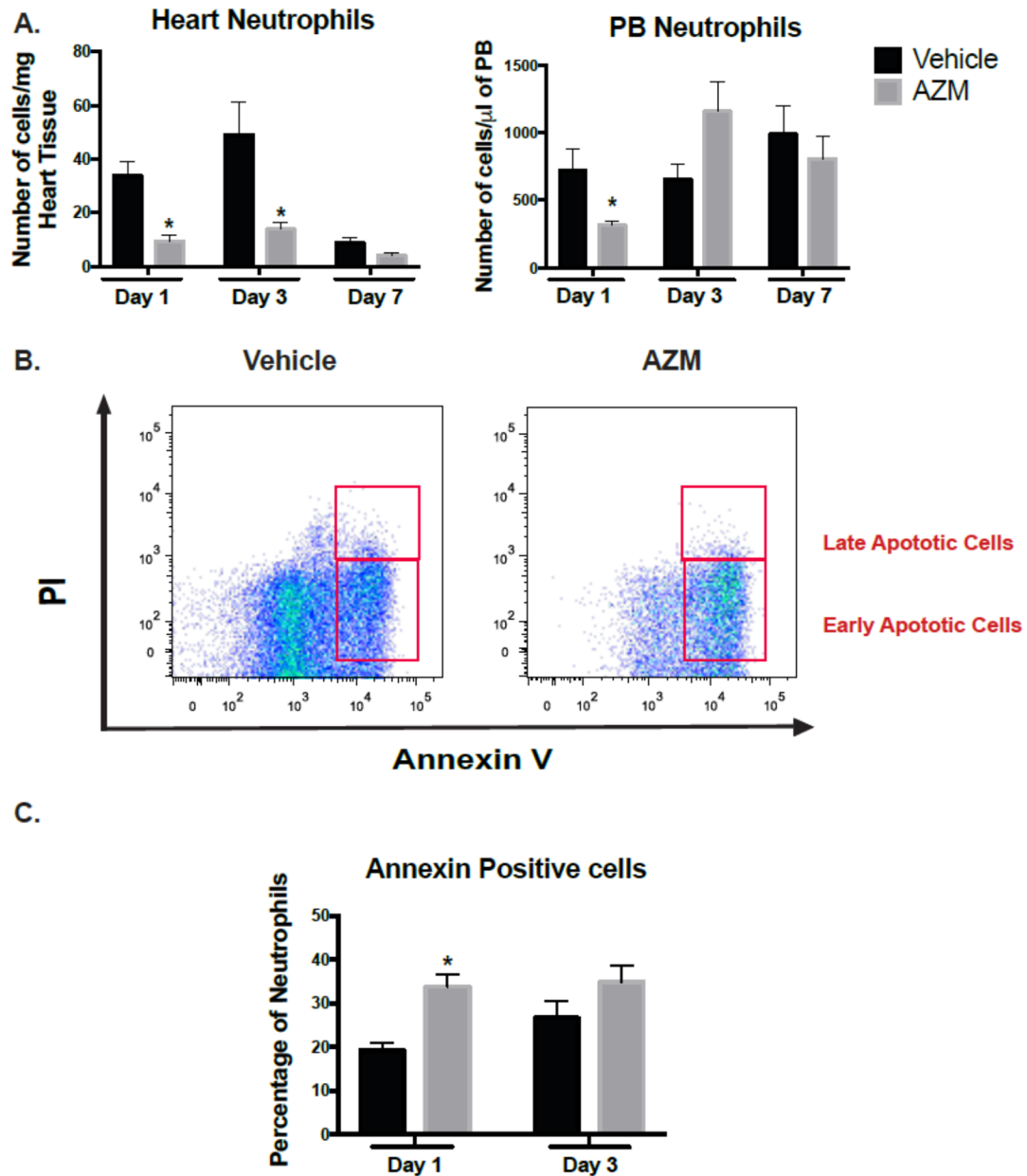


Figure 2.6. Cardiac neutrophils are reduced with AZM treatment due to apoptosis.

Panel A summarizes the quantitative assessment of neutrophil ($CD45^{+}/CD115^{lo}/Ly6G-C^{lo}$) numbers and demonstrates significant reduction in the AZM-treated group relative to controls in the heart and peripheral blood during the early inflammatory stage post-MI. Panel B illustrates the flow cytometry analyses of neutrophil populations stained against annexin V and PI showing higher percentage of early apoptotic neutrophils in AZM-treated mice. Panel C summarizes the quantitative assessment of early apoptotic neutrophils and demonstrates significantly higher percentage in the heart in the AZM-treated group during the early stage following MI ($n = 4$ mice/group/time point, $*P < 0.05$ compared to vehicle controls). Data presented as mean \pm SEM. AZM, azithromycin; HT, heart; PB, peripheral blood; PI, propidium iodide.

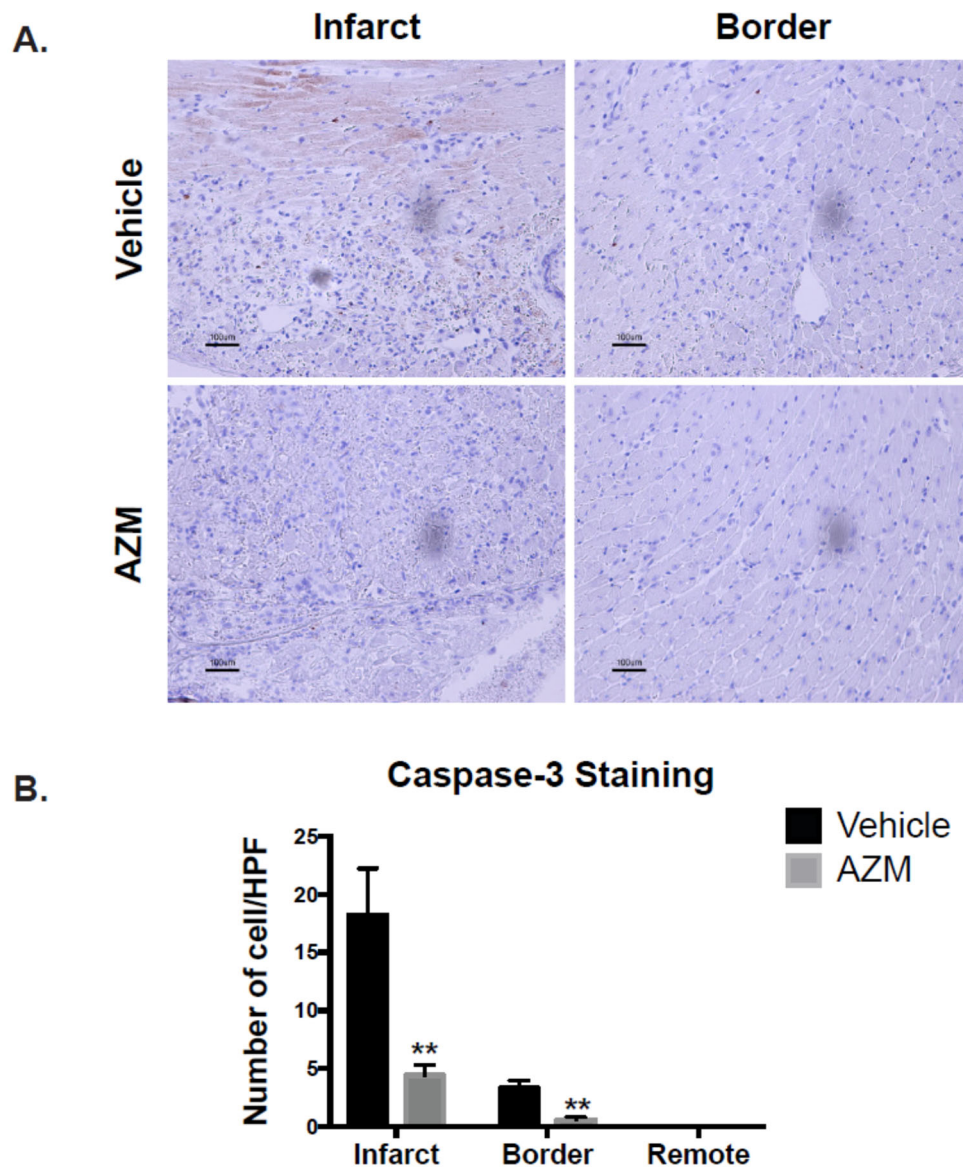


Figure 2.7. AZM reduces apoptosis post-infarction.

Panel A shows representative light microscope images of cleaved caspase-3 staining for infarcted and border regions in AZM- and vehicle-treated mice 3 days after MI. Quantitative analyses (Panel B) of apoptosis reveal a remarkable reduction in caspase-3 activation in the infarct and border regions of AZM-treated group compared to the control group (n = 4 animals/group, **P<0.01 compared to vehicle controls). Scale bars represent 100 μ m. Data presented as mean \pm SEM. AZM, azithromycin; HPF, high power field.

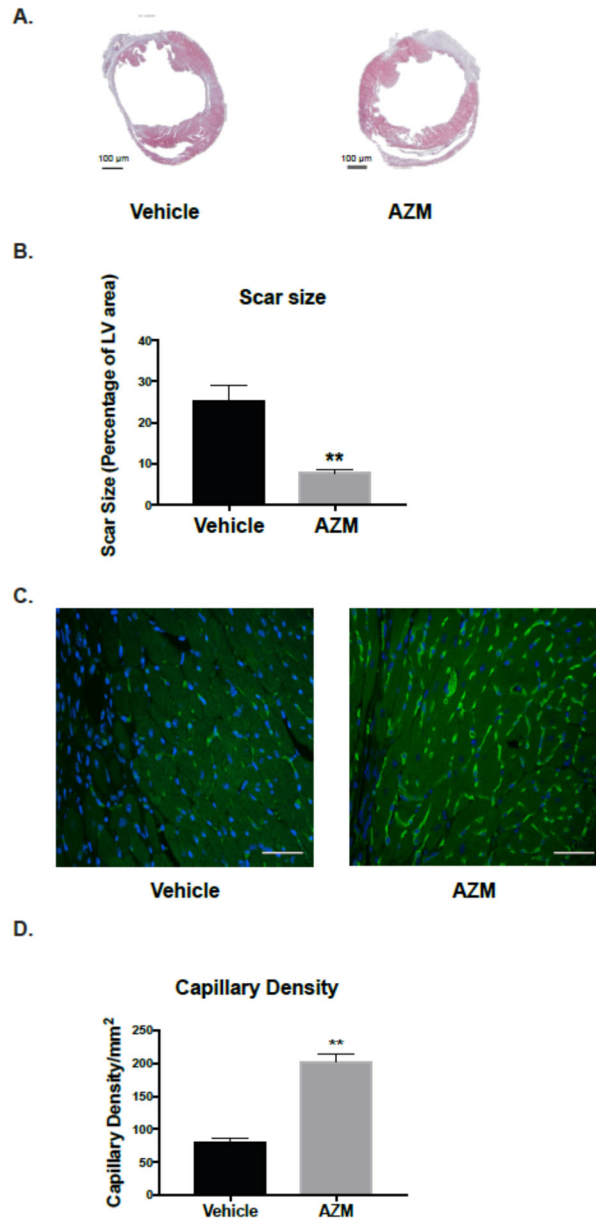


Figure 2.8. AZM treatment reduces scar size and enhances angiogenesis after myocardial injury.

Representative Masson's trichrome staining at 30 days after myocardial injury in vehicle- and AZM-treated groups (Panel A). Quantitative analysis of scars as percentage of LV area shows significant reduction in AZM group relative to the control group (Panel B) (vehicle-treated, $n = 15$ vs. AZM-treated, $n = 12$, $**P < 0.01$ compared to vehicle controls). Representative isolectin staining (Green) for capillary density in the peri-infarct region in AZM- and vehicle-treated animals demonstrates higher capillary density in AZM group compared to control (Panel C). Quantitative analysis of capillary density confirms the higher angiogenesis rate and capillary density in AZM-treated group (Panel D) ($n = 4$ animals/group, $**P < 0.01$ compared to vehicle controls). Scale bars represent $50 \mu\text{m}$. Data presented as mean \pm SEM. AZM, azithromycin; MI, acute myocardial infarction; LV, left ventricular.

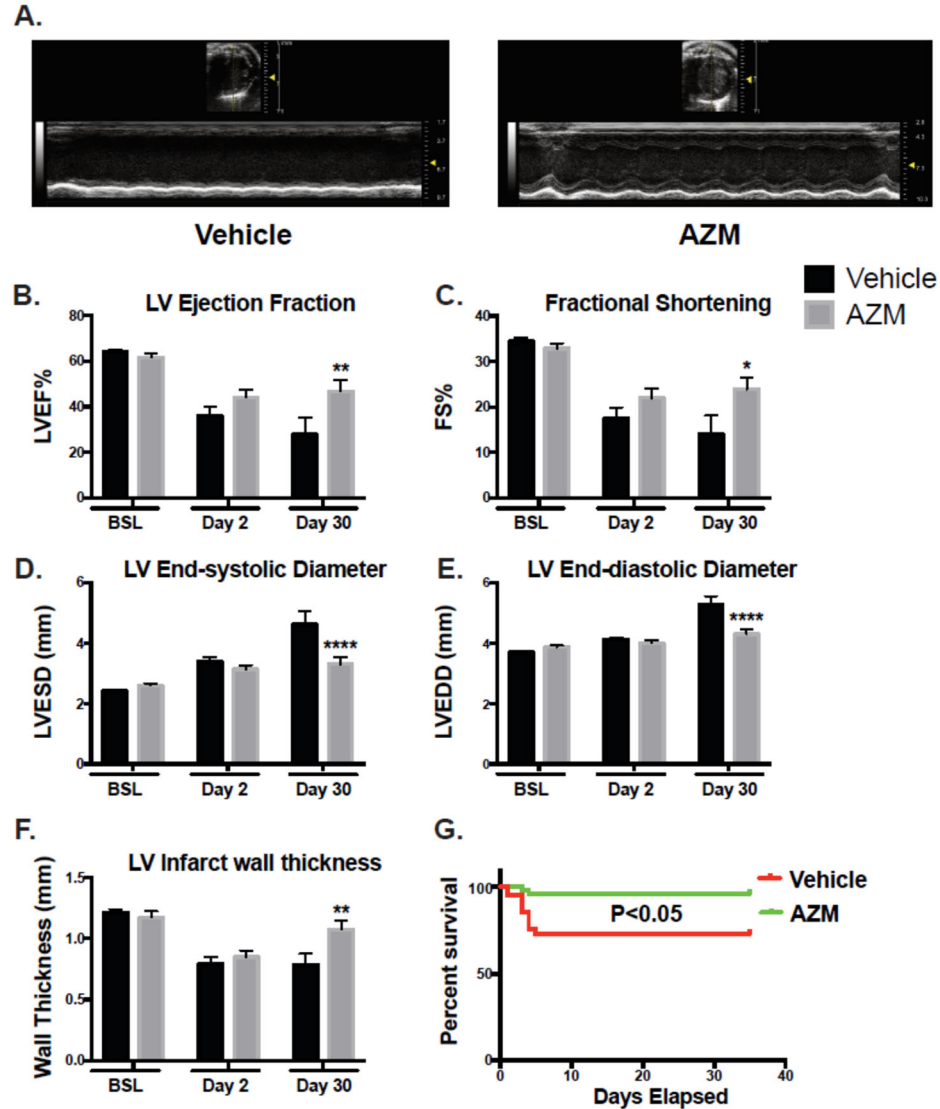
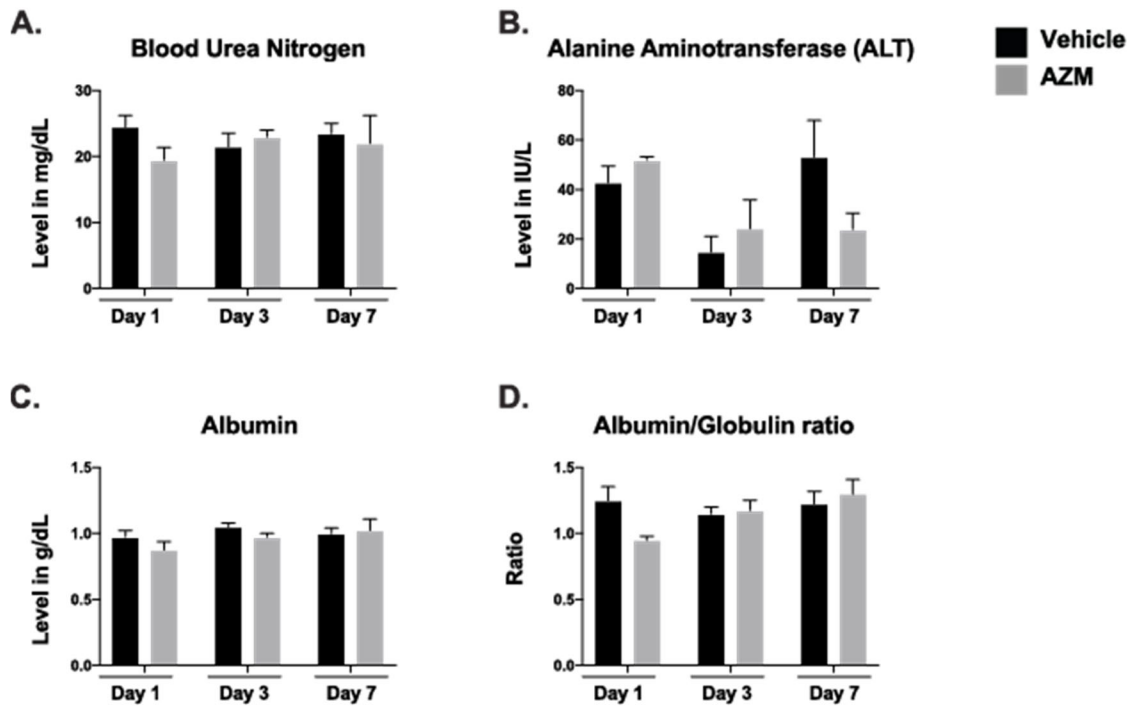
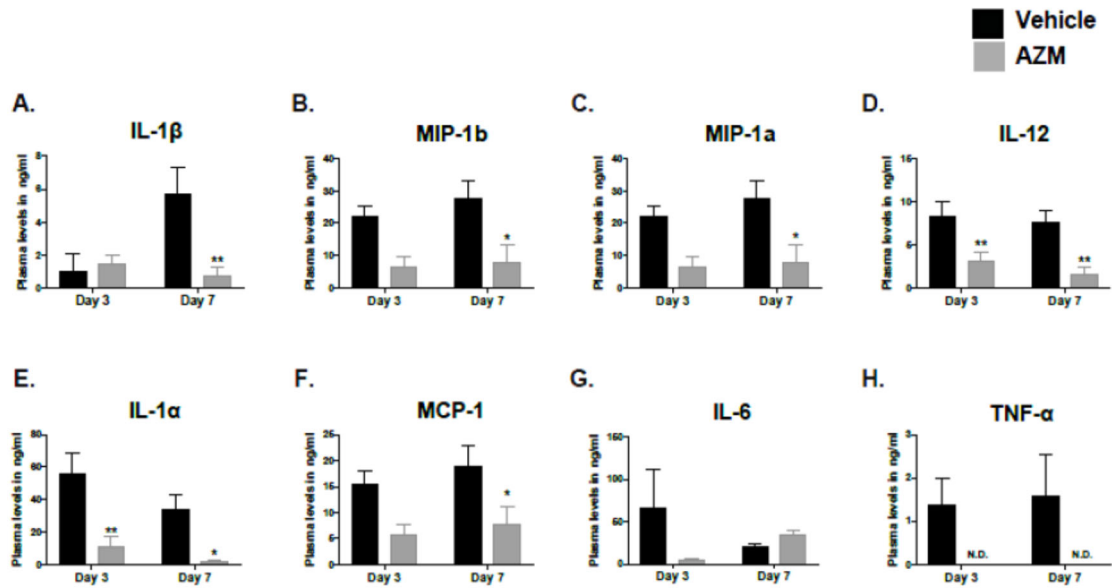


Figure 2.9. AZM treatment improves chronic cardiac remodeling and survival post-MI.

30 day following MI, transthoracic echocardiography using M-Mode (Panel A) and 2D echocardiography was performed on AZM- and vehicle-treated animals to evaluate left ventricular function and remodeling parameters. Quantitative analyses demonstrate significant recovery in LV function as assessed by ejection fraction (LVEF) (Panel B) and fractional shortening (FS) (Panel C). Data also shows significant improvements in LV adverse remodeling parameters such as end-systolic diameter (LVESD) (Panel D) and end-diastolic diameter (LVEDD) (Panel E). Additionally, we observed significantly thicker infarct walls suggesting of enhanced recovery and regeneration (Panel F). Survival curves of AZM and vehicle-treated mice 30 days post-MI demonstrate a significant improvement in survival with AZM treatment (Panel G). (Echo data: vehicle-treated, n = 15 vs. AZM-treated, n = 12; survival data: vehicle-treated, n = 48 and AZM-treated, n = 53; *P<0.05, **P<0.01 and ****P<0.001 compared to vehicle control). Data presented as mean \pm SEM. AZM, azithromycin; MI, acute myocardial infarction.

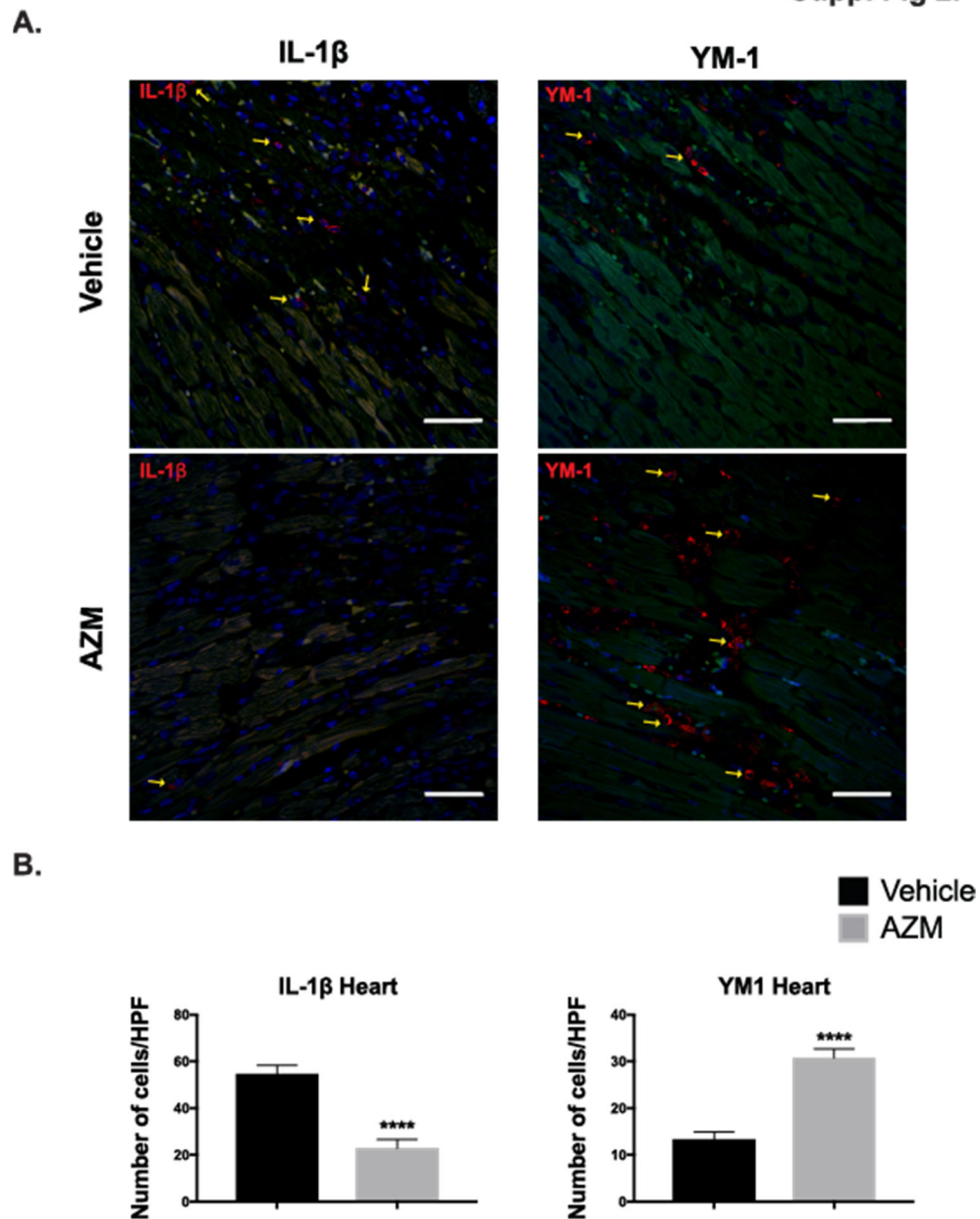


Supplemental Figure 2.1. AZM does not alter kidney or liver function with prolonged use after myocardial infarction. The levels of **plasma Albumin, Albumin/Globulin, Blood Urea Nitrogen or ALT** all these parameters were not significantly different between vehicle and AZM-treated groups. (n=4/group/time point). ALT, alanine aminotransferase; AZM, azithromycin.



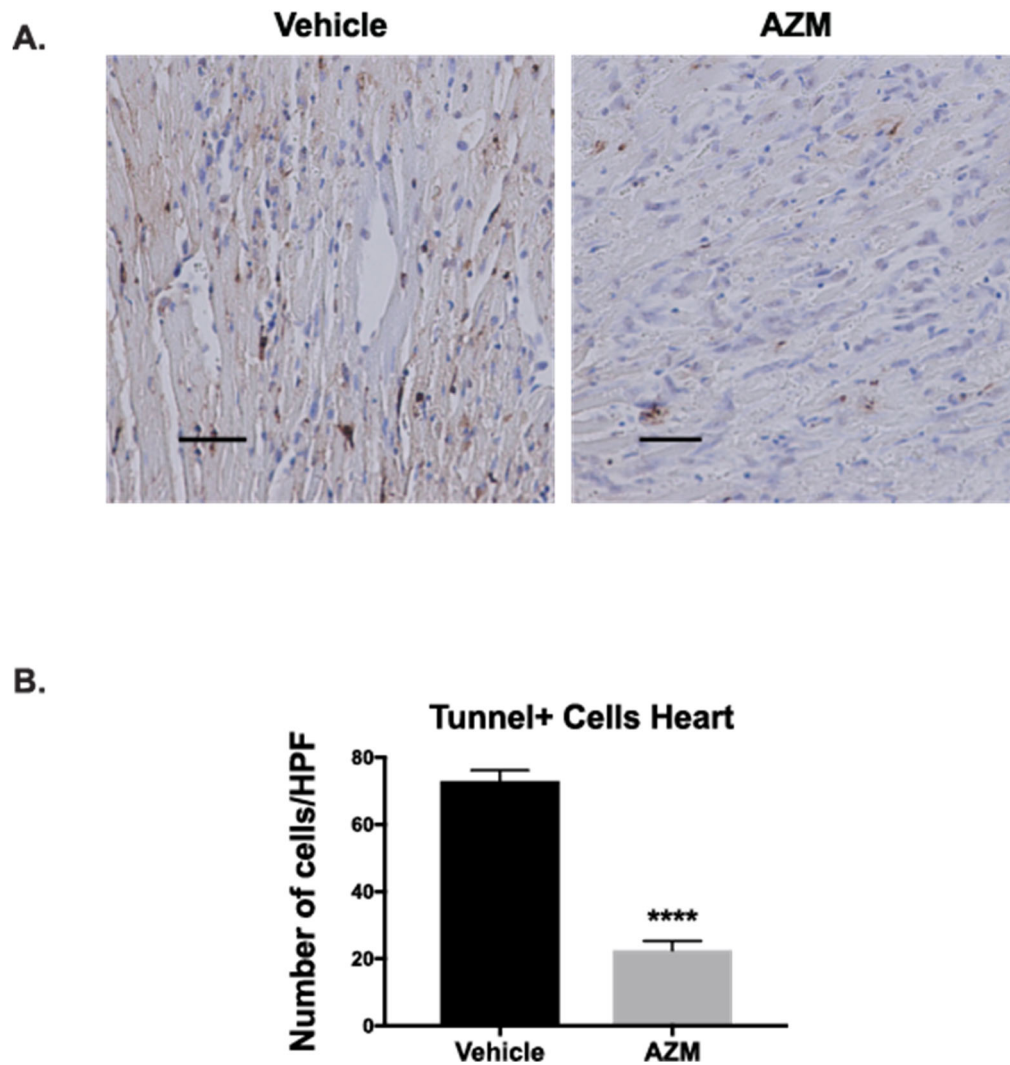
Supplemental Figure 2.2. AZM treatment reduces IL-1 β and increases YM1 expression in the peri-infarct border of the injured heart. Immunohistochemical assessment of cells expressing IL-1 β and YM1 3 days post-MI. **Panel A** shows representative images from vehicle and AZM-treated mice demonstrating higher density of IL-1 β compared to YM1 in the peri-infarct border in vehicle-treated mice. Yellow arrows point to positive cells. **Panel B** shows quantitative assessment of IL-1 β and YM1 3-days post-MI, which indicate significant reduction in IL-1 β and elevation in YM1 positive cells. (n=4 animals/group/time point, ****P<0.0001 compared to vehicle controls). Scale bars represent 50 μ m. Data presented as mean \pm SEM. AZM, azithromycin; HPF, high power field; IL-1 β , interleukin 1 beta; YM1 (Chil3), chitinase-like 3.

Suppl Fig 2.



Supplemental Figure 2.3. AZM therapy reduces the secreted inflammatory cytokines. Quantitative analyses of cytokines (IL-12, IL-1 β , IL-1 α , IL-6, TNF- α , MCP-1, MIP-1a and MIP-1b) in vehicle and AZM-treated mice. The analysis demonstrates reduction of pro-inflammatory cytokines on days 3 and 7 post MI compared to vehicle. (n=4 mice/group/time point, *P<0.05 and **P<0.01 compared to vehicle controls). Data presented as mean \pm SEM. AZM, azithromycin; IL-1, interleukin 1; IL-12, interleukin 12; MCP-1, monocyte chemoattractant protein-1; MIP, Macrophage Inflammatory Proteins; N.D., non-detectable; TNF- α , tumor necrosis factor-alpha.

Suppl Fig 4.



Supplemental Figure 2.4. AZM reduces apoptosis in the infarcted heart. Panel A shows representative light microscope images of a TdT dUDP Nick-End Labeling Assay (TUNEL) for the border regions in AZM and vehicle-treated mice 3 days after MI. Quantitative analyses (**Panel B**) of apoptosis reveal a reduction in TUNEL positive cells in the border regions of AZM-treated group compared to the control group (n=4 animals/group, ****P<0.0001 compared to vehicle control). Scale bars represent 100 μ m. Data presented as mean \pm SEM. AZM, azithromycin.

S2.1 Table. Forward and reverse primer sequence used in the experiments.

Primer	Forward	Reverse
18S	CCAGTGGTCTTGGTGTGCTG	GGAGAACTCACGGAGGACGA
i-NOS	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
IL-6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGA AG
MCP-1	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCTTCTTG
TNF-α	TGGAAGTGGCAGAAGAGG	AGACAGAAGAGCGTGGTG
IL-1β	CAACCAACAAGTGATATTCTCCA TG	GATCCACACTCTCCAGCTGCA
TGF-β	CGGAGAGCCCTGGATACCACCT A	GCCGCACACAGCAGTTCTTCT CT
IL-4	ACAGGAGAAGGGACGCCAT	GAAGCCCTACAGACGAGCTC A
PPARγ	GCCAGTTTCGATCCGTAGAA	AATCCTTGGCCCTCTGAGAT
Chil3 (YM1)	AGGCTTTGCGGTCCTGAT	CCAGCTGGTGAAGTAGCAGA

S2.2 Table. Echocardiographic morphometric parameters at 30 days post-MI.

Primer	Vehicle		AZM	P value
Left ventricular ejection fraction	27.9±7.4		46.8±4.9	0.04
Fractional shortening	14.1±4		23.9±2.6	0.049
Left ventricular end-systolic diameter	4.6±0.4		3.3±0.2	0.02
Left ventricular end-diastolic diameter	5.3±0.3		4.3±0.2	0.01
Left ventricular end-systolic volume	108.8±21.7		48.4±10.5	0.02
Left ventricular end-diastolic volume	136.2±17.8		84.7±8.7	0.01
Infarct wall thickness	0.78±0.09		1.1±0.07	0.02

CHAPTER III. SPECIFIC AIM2

Liposomes Enhance the Immunotherapeutic Efficacy of Azithromycin in Myocardial Infarction and Reduces the Risk of Adverse Effects

3.1 Summary

Introduction: There is a growing body of evidence showing that altering the inflammatory response by alternative macrophage polarization towards the reparative phenotype is protective against acute ischemic injury and improves cardiac functional recovery. Research shows that AZM, a macrolide antibiotic, alters inflammation and polarizes macrophages to the alternatively activated state in inflammatory conditions. We demonstrated that free AZM treatment (160 mg/kg/day, orally), initiated 3 days pre-MI, enhances post-MI cardiac recovery as a result of shifting macrophages to the reparatory state. Due to the limited clinical translational perspective (pre-MI treatment and relatively high dose of AZM) of our initial proof-of-concept study, we planned this study using a liposomal formulation of AZM (Lazm) to achieve rapid onset of action via targeted accumulation in inflammatory phagocytes. Based on our preliminary studies with Lazm, we hypothesized that Low dose of liposomal AZM (Lazm) treatment, started after MI, yields enhanced immunomodulatory potential and cardiac recovery with low risk of adverse effects.

Methods and results: Low dose free and liposomal AZM (40 or 10 mg/kg, IV) were administered immediately post-MI. We observed a significant shift favoring anti-inflammatory/reparatory macrophages with both AZM formulations (free and liposomal). A significant decrease in cardiac inflammatory neutrophils with AZM treatment was paralleled with significant reduction in the infiltration of inflammatory monocytes. Modulation of the inflammatory cell response was associated with a significant reduction in pro-inflammatory and an equivalent increase in anti-

inflammatory gene expression. Taken together, AZM treatment induced a systemic shift in post-MI inflammatory response. AZM effects resulted in reduced cardiac cell death and scar size, and enhanced angiogenesis. These protective effects led to improvement of the functional/structural complications usually seen post-MI. Importantly, the liposomal formulation of AZM was protective from its cardiac off-target effects.

Conclusion: We demonstrate that AZM is a potent immunomodulatory agent for the detrimental post-MI inflammatory response. Moreover, our studies are the first to demonstrate that targeted AZM delivery using liposomal formulations achieves more effective immunomodulatory effects with less cardiac side effects. To our knowledge, this is the first evidence of the immune modulation properties of liposomal AZM. Our findings strongly support clinical trials using AZM as a novel and clinically relevant therapeutic target to improve cardiac recovery and reduce heart failure post-MI in humans.

3.2 Introduction

Myocardial infarction (MI) remains a primary cause of morbidity and mortality globally, albeit hospitalization rates reduced in the last 15 years (Spatz, Beckman et al. 2016). Heart failure resulting from post-infarction remodeling is a major driver of cardiac death (Lindsey, Saucerman et al. 2016, Spatz, Beckman et al. 2016). Cardiomyocyte death and extracellular matrix (ECM) breakdown at the area of cardiac injury induce a potent and poorly controlled inflammatory response (Timmers, Pasterkamp et al. 2012), interfering with healing and scar formation processes (Entman and Smith 1994, Frangogiannis, Youker et al. 1998, Mehta and Li 1999). Innate immune cells such as neutrophils, monocytes, and macrophages are key players in this response (Hulsmans, Sam et al. 2016). After infarction, neutrophils are the first inflammatory cells to infiltrate the infarcted myocardium (Reimer, Murry et al. 1989). While neutrophils play an important role in removing dead cells and debris, their byproducts may exacerbate cardiac damage (Reimer, Murry et al. 1989, Ortega-Gomez, Perretti et al. 2013). A recent study reveals that there are two activation states of neutrophils in the post-ischemic

heart: pro-inflammatory (N1) and anti-inflammatory (N2) neutrophils (Ma, Yabluchanskiy et al. 2016). N1 neutrophils dominate the heart immediately after infarction and contribute to the development of adverse cardiac remodeling and heart failure, while N2 neutrophils increase gradually after MI and have cardio-protective effects (Ma, Yabluchanskiy et al. 2016). Additionally, neutrophils can negatively impact post-MI healing through preferential recruitment of pro-inflammatory (Ly6C^{hi}) (Soehnlein, Zernecke et al. 2008), rather than anti-inflammatory monocytes (Ly6C^{lo}) (Nahrendorf, Swirski et al. 2007).

Macrophages are essential in organizing the early post-MI inflammatory and subsequent reparative phases. General classification of macrophages is into pro-inflammatory/classically activated/M1 and anti-inflammatory/alternatively activated/reparative/M2 macrophages based on gene profile and function (Nahrendorf and Swirski 2016, Ma, Mouton et al. 2018). Pro-inflammatory macrophages are dominant days 1-3 post-MI (Ma, Mouton et al. 2018) and mediate the inflammatory process (Sica, Matsushima et al. 1990, Ma, Mouton et al. 2018). Following this early inflammatory phase, on days 5-7, reparative macrophages are the most prevalent and are characterized by high expression levels of IL-10, IL-1Ra, and decoy type II receptors (Dinarello 2005, Yan, Anzai et al. 2013, Ma, Mouton et al. 2018). Due to the production of these mediators, M2 macrophages play a central role in inflammation resolution and demonstrate robust anti-inflammatory effects, potentially involved in tissue remodeling and angiogenesis (Gombozhapova, Rogovskaya et al. 2017). There is a growing body of evidence showing that repolarization of macrophages to the reparative phenotype is protective against early development of ischemic lesion and its subsequent cardiac remodeling (Schnoor, Cullen et al. 2008, Harel-Adar, Ben Mordechai et al. 2011, de Couto, Liu et al. 2015, Kain, Liu et al. 2017, Qin, Finlayson et al. 2017). Previous research from our lab demonstrates that azithromycin (AZM), a commonly used antibiotic with immunomodulatory effects, alters post-MI inflammation through repolarization of macrophages to a reparative phenotype, which ultimately leads to increased protection against adverse cardiac remodeling and heart failure (Al-Darraj, Haydar et al. 2018). However, these

findings have limited clinical translation given the pre-MI treatment and relatively high dose, which our non-targeted delivery of AZM dictated (Al-Darraj, Haydar et al. 2018). It remains unclear whether low dose AZM immediately after cardiac injury is effective in shifting macrophage phenotype or enhancing cardiac recovery post-MI. The goal of this translational study is to demonstrate pre-clinical findings showing the efficacy of AZM as immunotherapeutic agent in a clinically translatable model of MI therapy.

Liposomes are formed of one or more phospholipid bilayers surrounded by an aqueous core (Levchenko, Hartner et al. 2012), which can hold both lipophilic and hydrophilic compounds. Given their biocompatibility and ease of use, they rapidly became an attractive drug delivery system (Levchenko, Hartner et al. 2012). Research shows that liposomes reduce the ED50 of the cytotoxic drug, doxorubicin, in preventing drug toxicity while promoting anti-tumor efficacy (Rose 2005). Doxorubicin liposomes reach steady state faster and with fewer doses due to intensified localization at targeted organs (Charrois and Allen 2003). Additionally, liposomes are highly effective carriers, achieving higher drug concentration at sites of cardiac injury (Mueller, Marcus et al. 1981). Increased cellular and vascular permeability in the injured myocardium enhances the specific capacity of liposomes to deliver therapeutic agents to the site of injury (Takahama, Minamino et al. 2009). Research shows that liposomes facilitate the local distribution of tracers in infarcted myocardium (Caride, Twickler et al. 1984). Additionally, post-infarction medium contains a large variety of diverse cell types, which can be targeted to improve outcomes of the accumulated therapeutics (Dasa, Suzuki et al. 2015). Liposomal dosage forms are effective as a drug delivery strategy for phagocyte-targeted therapy, producing lower immunogenic reaction, higher biocompatibility and specificity, and greater drug stability. After parenteral administration, liposomes are naturally taken up by mononuclear phagocytes (Kelly, Jefferies et al. 2011). Liposomes can harness the physiological function of these cells to mediate a targeted delivery and promote the therapeutic efficacy of the agent (Kelly, Jefferies et al. 2011). Therefore, we designed this study to overcome the limitations of our previous work and to improve outcomes

of AZM cardioprotective therapy. Additionally, we expect the encapsulated dosage form to reduce potential off-target effects of AZM. In this report we test the hypothesis that low dose liposomal AZM (Lazm) treatment, started immediately post-MI, yields an enhanced immunotherapeutic potential and cardiac recovery with low risk of adverse effects.

3.3 Materials and methods

3.3.1 Study Design

C57BL/6 male mice (Jackson Laboratory, BarHarbor, ME), age 6-8 weeks, were treated with one of two doses of liposomal or free AZM (10mg/kg/day or 40mg/kg/day) or vehicle (PBS or empty liposomes) using retro-orbital injection, starting immediately after MI or sham surgery for 7 days (**Fig 3.1A**). The doses used represent commonly prescribed comparable human dosages (Evans, Smith et al. 2005, Evans, Rogers et al. 2011), as well as provide effective distribution and tissue accumulation in animals (Shepard and Falkner 1990). Treatment continued for 7 days to span the duration of the post-MI inflammatory period (Heidt, Courties et al. 2014). All procedures were conducted under the approval of the University of Kentucky IACUC in accordance with the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. [NIH] 85–23, rev. 1996).

3.3.2 Murine model of myocardial infarction

Anesthesia was induced through a small animal vaporizer system with 1–3% isoflurane. Pain reflexes were appropriately tested to confirm adequate anesthesia before and during the surgical procedure. Lateral thoracotomy between the left fourth and fifth ribs was performed, the pericardial space was accessed, and the heart protruded from the intercostal space. The left anterior descending coronary artery (LAD) was identified and permanently ligated with 6–0 silk suture 3 mm distal to its origin, as previously described (Al-Darraj, Haydar et al. 2018). Sham surgery consisted of the same procedure without LAD ligation. The heart was immediately returned to the intrathoracic cavity and air manually evacuated to

prevent pneumothorax. Finally, the muscle and skin were sutured using a 4–0 Prolene running suture.

3.3.3 Preparation of liposomes

Liposomal formulations were prepared according to previously established protocol (Kierstead, Okochi et al. 2015), using the thin film hydration method with equal molar ratios of distearyl phosphatidylcholine (DSPC), distearyl phosphatidylglycerol (DSPG) and cholesterol (Avanti polar lipids). AZM was included at 10 and 30 mol% based on phospholipid content. Formulations containing fluorophores were prepared in the same manner using 0.5 mol% of the lipophilic dye with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD). Lipids and AZM were mixed in chloroform and methanol at the desired ratio and the organic solvents removed by rotary evaporation to yield a thin film. After placing under a vacuum overnight, the lipid/drug films were hydrated in phosphate buffered saline (pH 7.4) and sonicated at 65°C for 30 min. The resulting unilamellar vesicles were extruded through 200 nm polycarbonate membranes to obtain uniform particle size. Liposome size, polydispersity and stability over time were determined by dynamic light scatter testing.

3.3.4 Flow cytometry

3.3.4.1 Blood

Cell phenotype, in addition to gene profile, analyses of monocytes and neutrophils were investigated in peripheral blood as previously described (Al-Darraj, Haydar et al. 2018). Briefly, blood was collected in tubes with a 1:5 ratio of ethylenediaminetetraacetic acid (EDTA)/citrate-theophylline-adenosinedipyridamole (CTAD) on days 1, 3 and 7 post-MI. Whole blood was centrifuged for 5 minutes at 500 x g, and the plasma layer was separated and reserved at -80°C. To lyse red blood cells, the residual cell pellet was incubated with 0.5 ml of diluted red blood lysing buffer (BD pharm lyse) for 10 minutes with mild agitation. To end the lysis process, 0.5 ml of staining buffer (5% goat serum, 0.05% sodium azide in phosphate-buffered saline) was added to the suspension

of red blood cell lysed blood. Then, the suspension was centrifuged at 400 x g for 5 minutes, and the supernatant was removed. This step was repeated if the red blood cells were still detectable in the cell pellet. White blood cell pellet was washed twice in staining buffer to remove any residual lysis buffer. After supernatant removal, the pellet was resuspended in a pre-determined volume of staining buffer and counted. Cells of each blood sample were divided into two parts depending on cell count to have $\sim 10^6$ live (trypan blue-negative) cells in each part. One portion was collected for surface marker staining, and the other part incubated with cell lysis buffer (Life technologies) to lyse cells for 10 minutes with multiple aggressive agitations, and then kept at -80°C for further gene expression analysis. Cells for staining were incubated immediately with conjugated primary antibodies against PERCPY5.5-conjugated Ly6G/C (BD Pharmingen), PECY7-conjugated F4/80 (Biolegend), brilliant violet 421-conjugated CD11b (Biolegend), APC-CY7-conjugated CD45 (Biolegend), and PE-conjugated CD115 (Biolegend) for 30 minutes on ice. Following incubation, cells were washed twice with staining buffer and analyzed by an LSR II (Becton Dickinson) in the University of Kentucky Flow Cytometry Core. Using unstained cells and single fluorescent controls, laser calibration and compensations were performed for all experiments. We identified monocytes with the $\text{CD45}^{\text{hi}}/\text{CD115}^{\text{hi}}/\text{Ly6-C}^{\text{hi}}$ profile as classical (pro-inflammatory), and cells with the $\text{CD45}^{\text{hi}}/\text{CD115}^{\text{hi}}/\text{Ly6-C}^{\text{lo}}$ profile were identified as non-classical (anti-inflammatory). Neutrophils were identified as $\text{CD45}^{\text{hi}}/\text{CD115}^{\text{lo}}/\text{Ly6-C}/\text{G}^{\text{lo}}$.

3.3.4.2 Heart

Phenotypic cell analysis of macrophages and neutrophils and gene expression analysis was conducted in the heart as previously described (Al-Darraj, Haydar et al. 2018). Briefly, mice were sacrificed, and hearts were rapidly isolated and placed in ice cold PBS (VWR International). Using a razor blade, the heart was manually minced. After mincing, tissue was incubated with a Collagenase B (Roche, Indianapolis, IN) and Dispase II (Roche, Indianapolis, IN) mixture at 37°C for 30 minutes, with gentle agitation every 5-10 minutes. Tissue digestion ceased using cold staining buffer and the suspension was placed on ice

and filtered using a 70 µm cell strainer, followed by centrifugation at 400 x g for 5 mins at 4°C. Supernatant was discarded and the pellet was resuspended in 0.3 ml of staining buffer. Individual heart cells were divided into approximately three equal portions depending on cell count with $\sim 10^6$ live (trypan blue-negative) cells in each. Two portions were used for cell staining while the remainder was incubated with cell lysis buffer (Life technologies) using multiple aggressive agitations, for 10 minutes and reserved at -80°C for further gene expression analysis. Cells for flow cytometry were incubated immediately with conjugated primary antibodies against FITC-conjugated Ly6G (BD Pharmingen), PE-conjugated CD206 (Biolegend), PECY7-conjugated F4/80 (Biolegend), brilliant violet 421-conjugated CD11b (Biolegend), brilliant violet 510-conjugated CD11c (Biolegend), APC-CY7-conjugated CD45 (Biolegend), or PERCPY5.5-conjugated Ly6G/C (BD Pharmingen), PECY7-conjugated F4/80 (Biolegend), brilliant violet 421-conjugated CD11b (Biolegend), APC-CY7-conjugated CD45 (Biolegend), and PE-conjugated CD115 (Biolegend) for 30 minutes on ice. Cells were then washed twice with staining buffer and analyzed by an LSR II (Becton Dickinson) in the University of Kentucky Flow Cytometry Core. Using unstained cells and single fluorescent controls, laser calibration and compensations were performed for all experiments. CD45^{hi}/Ly6G^{lo}/F4-80^{hi} cells were identified as macrophages and further classified as pro-inflammatory or reparative based on the expression of CD206 and CD11c. Neutrophils were defined as CD45^{hi}/CD115^{lo}/Ly6G^{lo}, CD206 was used to further identify N1 neutrophils.

3.3.5 Histology

Histological analysis was performed on deparaffinized and rehydrated sections as previously described (Al-Darraj, Haydar et al. 2018). Briefly, 30 day post-MI mice (N = 6-10/treatment group) were sacrificed under isoflurane anesthesia and hearts were isolated. Hearts were perfused first with PBS (VWR International), then by 10% buffered formalin (VWR International). Perfused hearts were placed in 10% neutral buffered formalin (VWR) overnight at room temperature, then sectioned into 3-mm cross-sections starting at the level of

coronary ligation then embedded in paraffin and sectioned into 4- μ m sections. Sections were stained with Masson's trichrome to evaluate scar size as previously described (Al-Darraj, Haydar et al. 2018). Digital images of stained sections were acquired, and areas of interest were assessed using NIH ImageJ (version 7) software. The LV area, LV cavity area, and infarct area were measured as previously described (Al-Darraj, Haydar et al. 2018). Scar size was presented as a percentage of the total LV volume.

3.3.6 Immunohistochemistry

Heart sections (N = 4/treatment group) were prepared as described in the previous section. Immunostaining of heart sections was performed on deparaffinized and rehydrated sections as previously described (Al-Darraj, Haydar et al. 2018). Briefly, sections were incubated with primary antibodies: rabbit anti-mouse IBA1 (Wako), goat anti-mouse CD206 (R and D Systems), and/or goat anti-mouse IL-1 β (R & D Systems) overnight at 4°C, then washed and incubated with secondary antibodies conjugated to Alexa Fluor 488 or 594 (Invitrogen), followed by incubation with DAPI. In the peri-infarct area, 10-15 adjacent zones per section (1–2 sections/animal) were imaged at 40x magnification utilizing a Nikon Confocal A1 Microscope in the University of Kentucky Confocal Microscopy facility. Nucleated cells that were antibody-positive were quantified using Cell Counter plugin for Nikon NIS-Elements (version AR 3.2). Findings are presented as total number of positive cells per high power field in the area of interest.

By using a similar protocol to the above, heart sections (N=5-7/treatment group) were prepared from mice sacrificed at day 30 post-MI. Sections were stained with FITC-conjugated isolectin B4 (FL1201, Vector Labs, Burlingame, CA). In the peri-infarct region, 10-15 adjacent zones per section (1–2 sections/animal) were imaged at 40x magnification utilizing Nikon Confocal Microscope A1. Quantification was performed using Cell Counter plugin for Nikon NIS-Elements (version AR 3.2). Findings are presented as total capillary density per mm² in the peri-infarct zone. All measurements were performed in the peri-infarcted areas only and analyzed by blinded observer.

Cell apoptosis was examined on deparaffinized and rehydrated sections from mice (N = 4/treatment group) sacrificed at day 3, as previously described (Al-Darraj, Haydar et al. 2018). TUNEL and caspase-3 staining was performed in the Biospecimen Procurement and Translational Pathology Shared Resource Facility (BPTP SRF) at the University of Kentucky. Nucleated positively stained cells were estimated. Quantification was performed using Cell Counter plugin for ImageJ (version 1.51d). Findings are presented as total positive cells per high power field in the peri-infract region. All measurements were obtained in the peri-infarcted areas only and analyzed by blinded observer.

3.3.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR).

PureLink RNA Mini Kit (ThermoFisher Scientific) was used to isolate total mRNA from heart and blood cells according to manufacturer protocol. Isolated RNA was quantified using a NanoDrop 8000 spectrophotometer (ThermoFisher). Next, cDNA was generated using the SuperScript VILO cDNA synthesis kit (Invitrogen). Using a QuantaStudio 7 Flex real-time thermocycler (Applied Biosystems by life technology), Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed to measure the mRNA expression of markers identifying: inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF- α), monocyte chemotactic protein-1 (MCP-1), transforming growth factor beta (TGF- β), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), interleukin-4 (IL-4), chitinase-like3 Chil3 (YM1), interleukin-10 (IL-10), arginase-1 (ARG-1), and Peroxisome proliferator-activated receptor gamma (PPAR γ). We used the comparative Ct method for relative estimation of mRNA expression, which was normalized to 18s (a housekeeping gene). Certain strategies to avoid bias and error inducible by contaminated DNA were taken: (a) primers were adjusted to bridge an intron for specific cDNA augmentation, (b) appropriate negative control reactions (template free controls) were used, (c) careful examination of the melting curve of augmented products (dissociation graphs) for consistency was performed, and (d) the probe T_m was at least 10°C more than the primer T_m, while the melting temperature (T_m) was 57°C–60°C.

3.3.8 Echocardiography

Mice were anaesthetized using 1–3% isoflurane during echocardiography to maintain a heart rate of 450-500 BPM during imaging. A Vevo 3100 system coupled with a 15-7-MHz linear broadband transducer and a 12-5-MHz phased array transducer was used to perform echocardiogram analyses. Heart function was examined at baseline (before cardiac surgery) and then remeasured at one and 4 weeks post-MI. During acquisition, a heating pad was used to preserve the body temperature at 37°C and the temperature was continuously assessed by rectal temperature probe. Using modified parasternal long-axis and short-axis probe placement, two-dimensional and Doppler echocardiography was used to assess the LV function and volume in M-mode. Tracings at the mid-papillary level were used to investigate the systolic and diastolic parameters and Teichholz formula was applied to quantify LV function. Echocardiographic imaging and analyses were carried out by a blinded investigator.

3.3.9 Luminex assay

At 1 and 3 days post-MI, plasma was collected using the PB collection protocol detailed earlier. Inflammatory biomarkers (IL-12, IL-1 β , IL-1 α , TNF- α , MIP-1, MIP-1 α , MIP1 β , MIP2, IP-10, G-CSF, RANTES, and KC) were quantified using the Milliplex mouse cytokine magnetic kit (MILLIPLEX MAP for Luminex xMap Technology, Millipore, USA) according to the manufacturer's protocol.

3.2.10 Maestro *in vivo* fluorescence imaging

Mice were placed inside the Maestro imaging system under isoflurane anesthesia. They were positioned so that the chest and abdomen of the animal were in the field of view. We used an orange filter (excitation 605nm, emission 675nm long pass) to capture the APC signal that comes from liposomes. Acquisition settings were in range from 640 to 820nm in 10-nm steps. To enhance the quality of the images we adjusted various variables like stage height, focus, and exposure length, within time points. Images were taken with side-by-side Lazm and vehicle-

injected control animals and were analyzed using Maestro software (Cambridge Research & Instrumentation, INC. (CRI), USA).

3.3.11 Cell culture

We used a murine macrophage cell line, J774 monocyte/macrophage cell line (ATCC, Manassas, VA), for *in vitro* experiments examining the immunomodulatory effects of free and liposomal AZM. Using DMEM media (supplemented with 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, L-Glutamine, and Glucose), cells were plated in 6 well plates at a concentration of 0.3×10^6 cells/well. After adhesion, cells were treated with 30 μ M free or liposomal AZM (Sigma-Aldrich, St. Louis, MO) and 20 ng/ml IFN γ (eBioscience 14-8311-63) diluted in DMEM. Following overnight incubation at 37°C with 5% CO $_2$, cells were stimulated using 100 ng/ml of LPS (Invivogen) diluted in DMEM. Supernatant was collected 48 hours after stimulation to quantify pro- and anti-inflammatory cytokine (TNF- α and IL-10) concentration.

3.3.12 ELISA assays

Levels of TNF- α and IL-10 were assessed in the supernatant from *in vitro* cell culture experiments using standard ELISA kits (BD Biosciences, San Deigo, CA) according to the manufacturer protocol. Results are presented for each cytokine (picogram/ml) with different treatments.

3.3.13 Non-invasive electrocardiogram (ECG) system

Mice were anesthetized with 2% isoflurane, then placed on the ECG platform. Electrodes were placed through the skin on the torsos followed by ECG recording for 2-3 minutes. Once the recording was completed, the data were analyzed using Chart software (PhysioTel).

3.3.14 Statistical Analysis

Data are presented as mean \pm standard error of the mean (SEM). Unpaired Student t test or analysis of variance (one-way or multiple comparisons) were used for group comparisons, as appropriate. Two-sided Dunnett or Dunn tests for post hoc multiple comparison procedures were used, with control samples as the control category. P value less than 0.05 was considered statistically significant during the analyses. Statistical analyses were performed using the Prism 8 software package (GraphPad, La Jolla, CA).

3.4 Results

3.4.1 Lazm formulation accumulates in the injured myocardium, particularly in immune cells.

Several therapeutic agents have been tested to alleviate cardiac remodeling post-MI through systemic administration; however, they suffer limited efficacy due to poor bioavailability in the heart or dose-limiting adverse effects (Dasa, Suzuki et al. 2015). The increased vascular permeability post-MI facilitates the accumulation of nanocarriers such as liposomes in the injured myocardium (Takahama, Minamino et al. 2009). In our studies, we elected to use non-PEGylated liposomes which are readily engulfed by phagocytes at sites of tissue injury (Oh, Nix et al. 1995). First, we were interested in determining whether Lazm accumulates in the injured heart after parenteral administration. We utilized a Maestro EX *in vivo* imaging system in combination with APC-labeled liposomes for visualization (**Figure 3.1B**). We found that Lazm accumulates in the injured heart as early as the first day after infarct and peaks at day 3 after injury (**Figure 3.1B**). The *ex vivo* imaging shows that these liposomes more likely accumulate in the infarct and peri-infarct regions of the heart, which is consistent with geographic accumulation of infiltrating phagocytes (**Figure 3.1B**). Importantly, no signal was determined in the heart of sham-operated mice either *in vivo* or *ex vivo*.

Post-infarcted myocardium contains diverse cell populations that may be targeted therapeutically to enhance cardiac recovery (Dasa, Suzuki et al. 2015). Liposomal formulations have been extensively tested as targeted delivery tools for

phagocytic cells, with lower immunogenicity, higher bioavailability and specificity, and greater drug stability (Kelly, Jefferies et al. 2011). We examined liposomal accumulation in the heart at a cellular level using flow cytometry. FACS analysis of labeled Lazm shows that liposomes concentrate in phagocytes such as neutrophils and macrophages beginning on day 1 post-MI. Importantly, less than 1% of non-immune cells in the heart show liposomal uptake, implying preferential liposomal accumulation in our cells of interest (**Figure 3.2**). We confirmed these observations using Celigo analyses, which show an enhanced liposomal colocalization with phagocytic cells isolated from the heart after MI (**Figure 3.3**). These findings strongly indicate that our designed non-PEGylated liposomes accumulate at the peri-infarct region, particularly in phagocytic cells, early after MI. Liposomal accumulation exhibits temporal trends consistent with infiltration of inflammatory cells.

3.4.2 Liposomal formulation reduces the risk of AZM side effects.

Studies show that AZM produces a negative inotropic effect and delays ventricular repolarization. These hemodynamic effects of AZM are likely mediated by a Ca^{+2} channel-independent pathway and delayed rectifier K^{+} current (Ohara, Nakamura et al. 2015). Additionally, the cardiac action potential is much shorter in mice compared to humans (Perlman 2016). Therefore, we were interested in examining the potential arrhythmogenic and hemodynamic effects of AZM in the heart when administered post-MI. Baseline parameters do not differ among the monitored groups. The retro-orbital administration of free AZM at 40mg/kg remarkably reduces heart rate, RR, ejection fraction, and cardiac output interval compared with baseline (**Figure 3.4A and B**). Moreover, we note that the QT interval is also prolonged after free AZM administration (**Figure 3.4C**). In contrast, administration of Lazm at the same dose does not induce any noticeable conduction or electrical changes *in vivo* (**Figure 3.4A, B, and C**). These findings imply that the liposomal dosage form of AZM is indeed protective against hemodynamic drug modifications, which is most likely attributed to the specific delivery of AZM to immune cells, avoiding cardiomyocytes. It is likely that

encapsulation of AZM rescues other adverse drug effects as well, making it an attractive method of delivery for clinical practice, especially in high risk patients.

3.4.3 Lazm therapy recruits reparative macrophages to the infarcted myocardium.

Macrophages are the primary cardiac immune cells during steady state and after ischemic injury, where they organize the ensuing inflammatory and healing events (Hulsmans, Sam et al. 2016) ((Ben-Mordechai, Palevski et al. 2015). We previously identified a cardioprotective role of oral free AZM in the setting of MI via its immunomodulatory effects on macrophages in post-MI inflammation. However, the pre-MI administration and high dose of AZM limit the clinical translation of this study. Therefore, here we used an encapsulated dosage form in an attempt to promote early cardiac bioavailability of AZM therapy. This is the first study to examine the anti-inflammatory and immunomodulatory properties of liposomal AZM, which may potentially expand to other inflammatory pathologies. First, we find significantly lower heart weight in Lazm treated groups, suggestive of less tissue edema and potentially less inflammatory change (**Figure 3.5**). In our investigation of macrophage phenotype in the injured heart at multiple timepoints after injury using flow cytometry (**Figure 3.6**), we observe a decrease in pro-inflammatory macrophages (CD45⁺/Ly6G⁺/F4-80⁺/CD11c⁺) at day 1 with a more profound effect on day 3 post-MI in Lazm treated groups compared to free AZM and control groups (**Figure 3.6A**). Conversely, reparative macrophages (CD45⁺/Ly6G⁺/F4-80⁺/CD11c⁻/CD206⁺) significantly increase at day 3 in Lazm treated groups compared to free AZM and control groups (**Figure 3.6B**). Collectively, these changes translate into a significant reduction in the ratio of pro-/anti-inflammatory macrophages in Lazm groups, indicating a shift in macrophage phenotype towards the reparative state (**Figure 3.6C**).

Repolarizing macrophages towards the anti-inflammatory activation state in peri-infarct zones can protect more cardiomyocytes from death, reducing infarct expansion and the subsequent adverse cardiac remodeling (de Couto, Liu et al. 2015) (Ben-Mordechai, Palevski et al. 2015). To confirm the immunomodulatory

effects of AZM on macrophages in the peri-infarct zone, we quantified cells expressing a general macrophage marker (Iba1), a reparative macrophage marker (CD206⁺), and IL-1 β as a marker of pro-inflammatory macrophages on day 3 post-MI using immunohistochemistry. We found a significant increase in reparative (CD206⁺) macrophages in free and liposomal AZM treated mice compared to control groups (**Figure 3.7A**). At the same time, we observe significantly higher numbers of pro-inflammatory (Iba1⁺IL-1 β ⁺) macrophages in the control groups (**Figure 3.8A**). Generally, the total number of macrophages (Iba1⁺ cells) were not different between groups, implying that Lazm therapy does not affect total macrophage count, but rather influences polarization in favor of the reparative phenotype, and this effect is increased with liposomal AZM (**Figure 3.7B**). This shift in alternatively activated macrophages in AZM treated groups is observed in both our immunohistochemical and flow cytometric data. These effects may be therapeutically harnessed to resolve the detrimental inflammation post-MI.

Monocytes dominate the infarcted heart following MI, providing a rich source of macrophages (Zlatanova, Pinto et al. 2016). Recruitment of the two subsets of monocytes (Ly6C^{hi} and Ly6C^{lo}) to the heart occurs in two waves: a first wave of Ly6C^{hi} and a subsequent wave of Ly6C^{lo}. These subpopulations mediate essential activities in early inflammation and the later reparative process (Swirski, Nahrendorf et al. 2009) (Nahrendorf, Swirski et al. 2007). In the heart (**Figure 3.9A**), we find that the Ly6C^{hi} (CD45⁺/Ly6C/G^{hi}/CD115^{hi}) monocytes (pro-inflammatory) significantly decrease in the first day after injury in liposomal and free AZM treated groups, an effect that disappears at later timepoints. In the blood (**Figure 3.9B**), the Ly6C^{hi} monocytes were considerably low on day 3 with comparable numbers on other days in the Lazm groups. These observations suggest that the liposomal and free AZM treatments can modulate other members of the immune response to MI both locally in the heart and systemically.

3.4.4 Lazm treatment reduces neutrophils, particularly N1.

Neutrophils orchestrate the clearance of dead cells and debris (Ortega-Gomez, Perretti et al. 2013). Additionally, apoptotic neutrophils initiate anti-

inflammatory changes in macrophages and hence contribute to the resolution of inflammation (Horckmans, Ring et al. 2017). Neutrophil (CD45⁺/CD115^{lo}/Ly6G/C^{lo}) analysis in the heart and blood using flow cytometry reveals that Lazm treatment significantly reduces neutrophil counts during their peak at day 1 (**Figure 3.10A**). However, we did not see significant changes in mice treated with free AZM.

To further investigate the immunomodulatory actions of AZM, we examined the distribution of N1 neutrophils (CD11b⁺/Ly6G⁺/CD206⁻) in the heart over time. We found a substantial decrease in N1 neutrophils at day 1 in Lazm treated groups (**Figure 3.10C**), which may explain the reduction in total neutrophil number. These findings may represent a novel therapeutic avenue in the treatment of MI patients who experience prolonged neutrophilia after infarct.

3.4.5 Lazm treatment shifts inflammatory gene expression towards the anti-inflammatory state.

The gene expression profile of macrophages relates to the activation status of these cells. Pro-inflammatory macrophages produce large amounts of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-6) and toxic effector molecules (reactive oxygen species, nitric oxide) (Martinez, Sica et al. 2008). On the other hand, reparative macrophages are potent generators of anti-inflammatory cytokines (TGF- β and IL-10) in addition to the expression of scavenger, mannose, and galactose-type receptors (Song, Ouyang et al. 2000). To identify whether liposomal formulation enhances the anti-inflammatory effects of AZM, we conducted *in vitro* experiments to identify the production of pro-inflammatory/anti-inflammatory cytokines in the J774 macrophage cell line. After 48 hours of stimulation, we observed accentuated immunomodulatory effects with liposomal AZM, confirmed through significant shift in the production of TNF- α and IL-10 towards the anti-inflammatory pattern (**Figure 3.11**).

We also assessed the mRNA expression of inflammatory and reparative genes in the heart and blood cells using RT-PCR. We observed a significant shift in the gene expression profile towards the anti-inflammatory state associated with

liposomal/free AZM treatment. In cardiac tissue, mRNA levels of a pro-inflammatory macrophages (iNOS) and pro-inflammatory cytokines (MCP-1, TNF- α , IL-6, and IL-1 β) are substantially downregulated in mice treated with free and liposomal AZM, particularly on days 1 and 3 post-infarct (**Figure 3.12A**). Conversely, the expression of anti-inflammatory cytokines and reparative macrophages (TGF- β and IL-10; IL-4, ARG, Fizz1, PPAR γ and YM1) are significantly upregulated in the same groups of mice with consistent effects up through day 7 (**Figure 3.12B**). Interestingly, we observe a similar trend in the gene expression of cytokine and macrophage markers in PB cells in the same groups (**Figure 3.12C and D**), suggesting systemic anti-inflammatory and immunomodulatory effects of Lazm therapy in addition to local effects at the site of injury. Additionally, we observe that inflammatory cytokine and chemokine (IL-12, IL-1 β , IL-1 α ITNF- α , MIP-1, MIP-1 α , MIP1 β , MIP2, IP-10, G-CSF, RANTES, and KC) concentrations are reduced in plasma of AZM treated groups (**Figure 3.13**). Based on our prior published studies, we see no changes at day 7 so we did not run that analysis. Collectively, these consistent results from multiple *in vitro* and *in vivo* studies suggest that AZM may have therapeutic clinical potential in MI patients.

3.4.6 Lazm therapy reduces cell death and scar size, and increases angiogenesis in the infarcted heart.

To identify the pro-survival effect of AZM therapies on cardiac cells post-MI, we assessed apoptosis in peri-infarct borders 3 days post-MI using caspase-3 (early apoptosis) and TUNEL (late apoptosis) assays. We observed that free and liposomal AZM therapy markedly reduce apoptosis in comparison to controls (**Figure 3.14A and 3.15A respectively**), likely related to pro-survival factors produced by reparative macrophages (Aurora, Porrello et al. 2014). We next assessed the infarct size 30 days post-MI using Masson's trichrome-stained sections from the heart. We found that Lazm treatment significantly reduced scar size in comparison to controls (**Figure 3.16**). These data indicate that AZM

preserves the heart from acute injury after ischemia as well as reduces susceptibility to chronic scar enlargement.

Reparative macrophages, through secretion of protective cytokines and pro-angiogenic factors such as VEGF, may promote reparative power (Aurora, Porrello et al. 2014). To determine blood vessel density at the infarct borders, we quantified isolectin-positive cells (a marker for endothelial cells) in the peri-infarct region 30 days post-MI. We observed a significant increase in density of newly formed blood vessels in the Lazm groups compared to controls (**Figure 3.17**). This is another important factor in AZM/Lazm therapy that may contribute to reduced scar size. The- shift in macrophage phenotype may explain the reduction in apoptotic cells and enhanced angiogenesis and provide the mechanism for reduced scar size observed in these groups.

3.4.7 Lazm therapy enhances cardiac functional recovery after MI.

Adverse cardiac remodeling and the ensuing heart failure post-MI are likely related to an imbalance between pro-inflammatory and pro-reparative phases after injury (de Couto, Liu et al. 2015). By modulating the inflammatory response, we investigated the role of AZM in cardiac recovery after MI. We performed echocardiography at 1- and 4-weeks post-MI and analyzed cardiac function and remodeling. We observed a significant attenuation of LV functional decline in Lazm treated groups compared to controls (**Figure 3.18A**). We noted a similar trend in other cardiac remodeling parameters such as LV end-systolic and LV end-diastolic diameters in the same groups (**Figure 3.18B and C**). Finally, survival rates indicate that the cardioprotective effects of free/liposomal AZM translated into a remarkable reduction in mortality (**Figure 3.19**).

Collectively, the immunomodulatory effects of Lazm therapy were potent at inducing favorable resolution of the overactive and/or extended pro-inflammatory cascades and their associated cardiac damage and impaired recovery. As a result, we found significant improvement in the structural/functional complications post-MI.

3.5 Discussion

Macrophages are the primary immune cells in the heart post-MI, organizing the early inflammatory stage and the later reparative events. The two activation states of macrophages (pro-inflammatory vs. reparatory) make them an attractive therapeutic target to induce earlier resolution of post-MI inflammation. We recently found that azithromycin (AZM) improves cardiac remodeling and recovery post-MI through a shift in macrophages to the reparatory state using a proof-of-concept experimental model. In this study, we utilized a clinically relevant experimental design using targeted liposomal AZM delivery to potentiate its bioavailability and immunomodulatory effects post-MI. Lazm enhanced resolution of post-MI inflammation as evidenced by a shift of pro-inflammatory to reparatory monocytes/macrophages. Neutrophils were substantially decreased as well, particularly the pro-inflammatory N1 neutrophils. These observed cellular changes occurred primarily at the site of cardiac injury and were paralleled by a shift towards anti-inflammatory cytokine production and cellular preservation, leading to reduced scar size, enhanced angiogenesis, and improved cardiac functional recovery. In addition to these findings, we observed that the liposomal formulation is protective from adverse drug effects such as conduction abnormality. These findings imply that post-MI treatment with low dose Lazm is a promising therapeutic target in patients with MI.

Several therapeutic agents have been tested to alleviate cardiac remodeling post-MI through systemic administration, however; they had limited efficacy due to poor accumulation in the target site/cells, incompatible pharmacokinetic properties with MI, or dose-limiting adverse effects (Dasa, Suzuki et al. 2015). As a result, an optimal drug delivery system capable of favorably accumulating therapeutic agents in the injured myocardium while upholding a reservoir that is not intensely processed by other non-targets (Dasa, Suzuki et al. 2015) is urgently needed. In the present work, *in vivo* imaging and flow cytometric analyses confirm that liposomes accumulate preferentially in the peri-infarct and infarct regions post-MI beginning in the first 24 hours after infarct, indicating specific localization to the damaged myocardium. Flow cytometric analysis

investigating the accumulation of liposomes in cardiac cells indicates that liposomes concentrate in immune cells, with less than 1% of non-immune cells testing positive for liposomes. This distribution strongly suggests that liposomes increase the on-target effects (enhanced efficacy), as well as reduce the off-target effects (less potential of adverse effects). Indeed, our *in vivo* electrophysiological monitoring of the heart confirmed the observed reduction of conduction abnormalities with Lazm formulation compared to free AZM. By using non-PEGylated liposomes, inflammatory cells phagocytize Lazm in the leaky vasculature of the infarcted myocardium. However, more studies are needed to further refine drug delivery and release in the setting of MI.

We previously demonstrated the immunomodulatory effectiveness of AZM as a potential therapy for cardioprotection after ischemic injury (Al-Darraj, Haydar et al. 2018). In this earlier proof-of-concept study, pre-MI treatment provided time for the drug to reach steady levels before MI induction (Feola, Garvy et al. 2010). We also used a relatively high dose of AZM (160 mg/kg/day), consistent with previous animal studies (Feola, Garvy et al. 2010, Zhang, Bailey et al. 2015). We are currently working with our collaborators to prepare a biotinylated form of AZM for both *in vivo* and *in vitro* experiments to enable its quantitative tracking in cells and tissue using different encapsulation strategies. However, AZM administration may be related to cardiac conduction and hemodynamic changes. Therefore, we stopped pursuit of a long-term follow up study with free AZM due to profound adverse effects (Fig 3.4).

To avoid these translational barriers and warrant post-MI low dose AZM, we considered an encapsulated dosage form of AZM, which may enhance the immunomodulatory efficacy and facilitate faster mode of action of drugs as previously shown with other compounds (Cynamon, Klemens et al. 1992, Oh, Nix et al. 1995). Our data confirm enhanced pharmacological effects with low dose of Lazm compared to free AZM. Furthermore, our studies reveal that liposomal formulation is protective against adverse effects of free AZM such as the observed reduction in heart performance, presumably by reducing the uptake of AZM in non-immune cells in the heart.

It is widely accepted that neutrophils are the first immune cells to infiltrate the infarcted heart and their extended existence is associated with exacerbation of cardiac injury after ischemia. We showed in our first study that neutrophil counts substantially decrease in mice receiving AZM, due to enhanced apoptosis (Al-Darraj, Haydar et al. 2018). Recently, research shows that there are two activation states of neutrophils in the ischemic heart: early pro-inflammatory (N1) and late anti-inflammatory (N2) (Ma, Yabluchanskiy et al. 2016). Interestingly, PB neutrophils are CD206⁻, becoming CD206⁺ in the heart, which indicates the plasticity of neutrophils and their ability to change in response to the surrounding microenvironment. Here, we observed that N1 neutrophils significantly decrease with Lazm therapy, perhaps attributable to the apoptotic effects of AZM or reduced neutrophil recruitment due to suppressed production of chemokines such as Macrophage Inflammatory Protein (MIP) and Keratinocyte Chemoattractant (KC). It is possible that removal of apoptotic cells from the injured myocardium is an essential cellular mechanism to resolve inflammation by shifting macrophage phenotype (Huynh, Fadok et al. 2002, Nathan and Ding 2010). Additionally, we observed a significant reduction in cardiac Ly6C^{hi} monocytes with liposomal and free AZM, suggesting a possible mechanism for the reduced number of pro-inflammatory macrophages. This phenomenon is most likely related to the diminished expression of MCP-1 in the heart and blood.

The post-MI healing phase is primarily organized by reparative macrophages (Ma, Mouton et al. 2018). This could explain the attenuated cardiac recovery with macrophage depletion and the improved outcomes with adoptive transfer of alternatively activated macrophages (Leor, Rozen et al. 2006, van Amerongen, Harmsen et al. 2007). Furthermore, multiple studies demonstrated the therapeutic utility of shifting macrophages to the reparative phenotype post-MI (Weirather, Hofmann et al. 2014, ter Horst, Hakimzadeh et al. 2015). We found that liposomal and free AZM treatment were associated with higher anti-inflammatory (Iba1+CD206⁺ cells) and lower pro-inflammatory (Iba1+IL-1 β +) macrophages after MI in peri-infarct regions. Additionally, we observed a significant reduction in apoptosis at day 3 post-MI in the same regions, possibly

attributable to pro-survival factors released from reparative macrophages (Lenardo and Baltimore 1989, Lakshminarayanan, Beno et al. 1997). These data confirm our observations and analysis in flow cytometry and inflammatory mediators. Changes in post-MI inflammation translate into smaller scar, better cardiac function, and more newly formed blood vessels after MI. Collectively, we observed a global trend with both AZM and Lazm in shifting immune cells to the anti-inflammatory state.

Immune cell modulation was associated with significant downregulation in the inflammatory genes, and upregulation in the anti-inflammatory genes. Inflammatory cytokines IL-1 β , TNF- α , IL-6, and MCP-1, as well as M1 macrophages marker, iNOS, were markedly reduced in the early time points post-MI. These changes could explain the reduced infiltration of inflammatory cells into the heart. As an example, MCP-1 drives mobilization of Ly6C^{hi} monocytes to the heart after infarction where they differentiate into M1 macrophages. Furthermore, reduction of MCP-1 promotes alternative activation of macrophages (Carbone, Nencioni et al. 2013). Moreover, IL-1 β promotes generation of chemotactic mediators inducing myocardial injury via increased recruitment of immune cells to the heart (Bujak and Frangogiannis 2009). Recent, data shows that targeting IL-1 β with the monoclonal antibody, canakinumab, significantly decreases cardiovascular event reoccurrence (Ridker, Everett et al. 2017). In agreement, we observed a robust decrease in the expression of IL-1 β , which contributes to the improvement of outcomes in this study, at least partially.

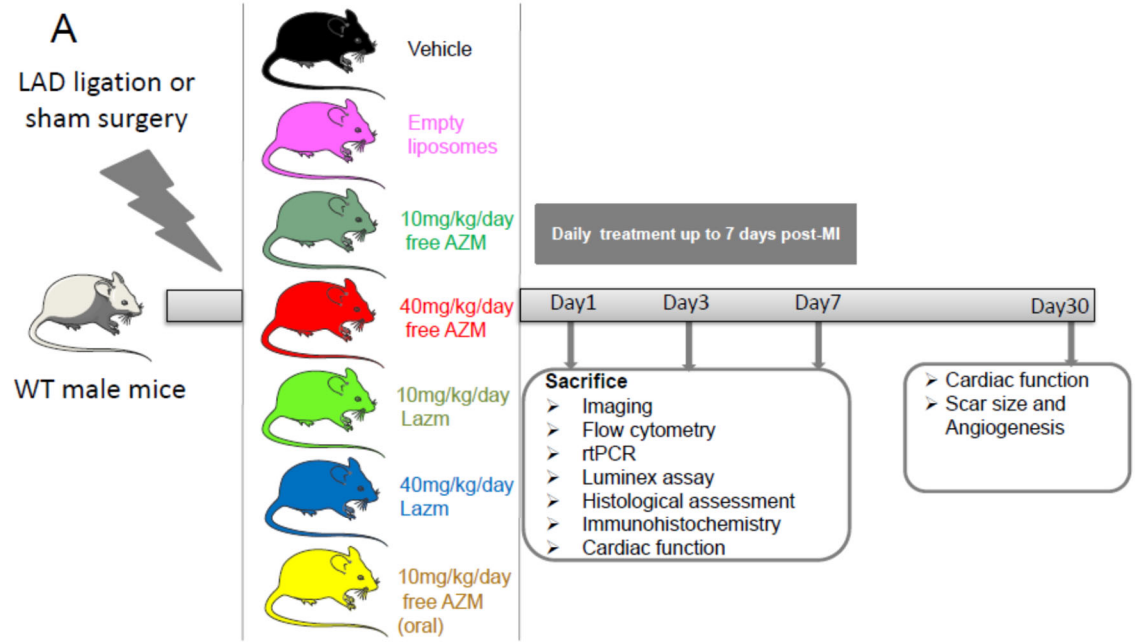
Simultaneously, anti-inflammatory genes such as TGF- β , IL-4, and IL-10, as well as M2 macrophage markers FIZZ1, YM1, PPAR- γ , and ARG are upregulated by free and liposomal AZM treatments. These mediators have been shown to exert multiple beneficial functions in the myocardium following MI. IL-10 modifies monocyte-macrophage function, morphology, and phenotype. Secretion of IL-1 α , IL-1 β , TNF- α , IL-6, and IL-8 is inhibited by IL-10 in activated monocytes (Frangogiannis 2012). Furthermore, IL-10 could participate in matrix remodeling via enhancement of tissue inhibitor of metalloproteinase 1 (TIMP-1) synthesis, thus stabilizing the ECM (Lacraz, Nicod et al. 1995, Frangogiannis 2012). TGF- β is an

essential regulator of both the transition from inflammation to healing and organized scar formation (Bujak and Frangogiannis 2007). Through activation of endothelial mononuclear cells, TGF- β inhibits production of various pro-inflammatory cytokines and chemokines (Frangogiannis 2012). Additionally, TGF- β stimulates fibroblasts to produce ECM proteins (collagen, fibronectin, tenascin, and proteoglycans) (Bassols and Massague 1988). Moreover, TGF- β reduces the expression of proteinases (plasminogen activator and collagenase) and enhances the production of proteinase inhibitors (plasminogen activator inhibitor and TIMP-1), suppressing matrix breakdown and helping scar maturation (Laiho, Saksela et al. 1986, Frangogiannis 2012). We noticed increased expression of IL-10 and TGF- β with free and liposomal AZM therapy, which may explain the reduction in heart failure and mortality observed in our study. Interestingly, changes in pro-inflammatory and anti-inflammatory genes are consistent between heart and peripheral blood, suggesting a systemic effect moderating the severity of post-MI inflammation. This feature is also important from a clinical prospective since it provides a more feasible way to monitor the pharmacological effects of AZM.

Conclusion

The present study is the first to demonstrate the immunomodulatory properties of liposomal AZM, which has wide therapeutic applications beyond the cardiovascular field. It is expected that liposomal AZM will be beneficial as a carrier and anti-inflammatory drug in other sterile inflammatory diseases that share a similar inflammatory profile with MI, such as ischemic stroke and spinal cord injury (Zhang, Bailey et al. 2015, Amantea, Certo et al. 2016). Immunomodulatory effects of AZM are time- and dose-dependent and are enhanced by improving the pharmacokinetics and pharmacodynamics of the drug through liposomal formulation. We note that low dose Lazm, started after MI, effectively resolves post-MI inflammation and improves adverse cardiac remodeling with lower risk of adverse effects. Interestingly, we observe also that the liposomal encapsulation is truly protective from the potential unwanted effects of AZM, which is of utmost importance to clinical application. The facts that AZM is a widely prescribed

antibiotic and liposomes are effective drug delivery systems for multiple therapeutic agents promotes rapid clinical translation of our findings. Finally, researchers can harness the method of preparing liposomes used in this study to deliver other therapeutic agents to the injured myocardium. Our findings strongly support clinical trials using AZM as a novel and clinically relevant therapy to improve cardiac recovery and reduce heart failure post-MI in humans.



LAD: permanent ligation of coronary artery
Lazm: labeled liposomal AZM

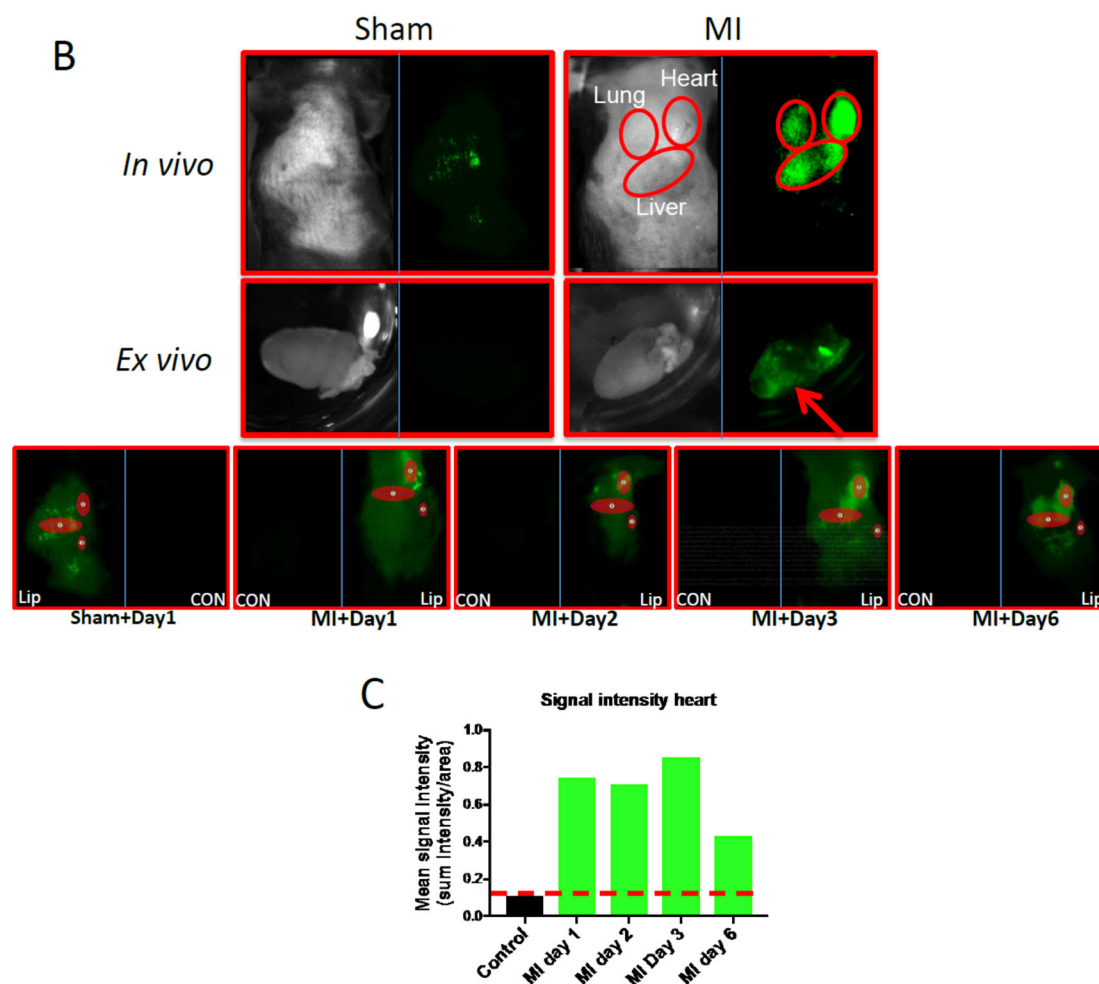


Figure 3.1. Experimental design and targeting myocardium with labeled Lazm after infarction.

(A) Experimental design for the *in vivo* study. **(B)** Imaging system images of mice (*in vivo*) injected with labeled Lazm 1, 2, 3, and 6 days after infarction. Hearts were collected 24 hours after injection and imaged (*ex vivo*). Images show that Lazm liposomes are accumulated in the heart starting the first day after infarction, an effect persisting during the time of follow-up (6 days). No signal was determined from the heart of the mouse with sham surgery either *in vivo* or *ex vivo*. The *ex vivo* imaging shows that liposomes are preferably accumulated in the infarct and peri-infarct regions of the heart (**red arrow**). **(C)** Represents quantitative assessments of signal intensity emitted from the infarcted heart day 1, 2, 3, and 6 after infarction in comparison to the **control** (CON) (a mouse injected with vehicle), which shows a marked increase in the intensity of the signal during all time points. Lip, labeled liposomal azithromycin.

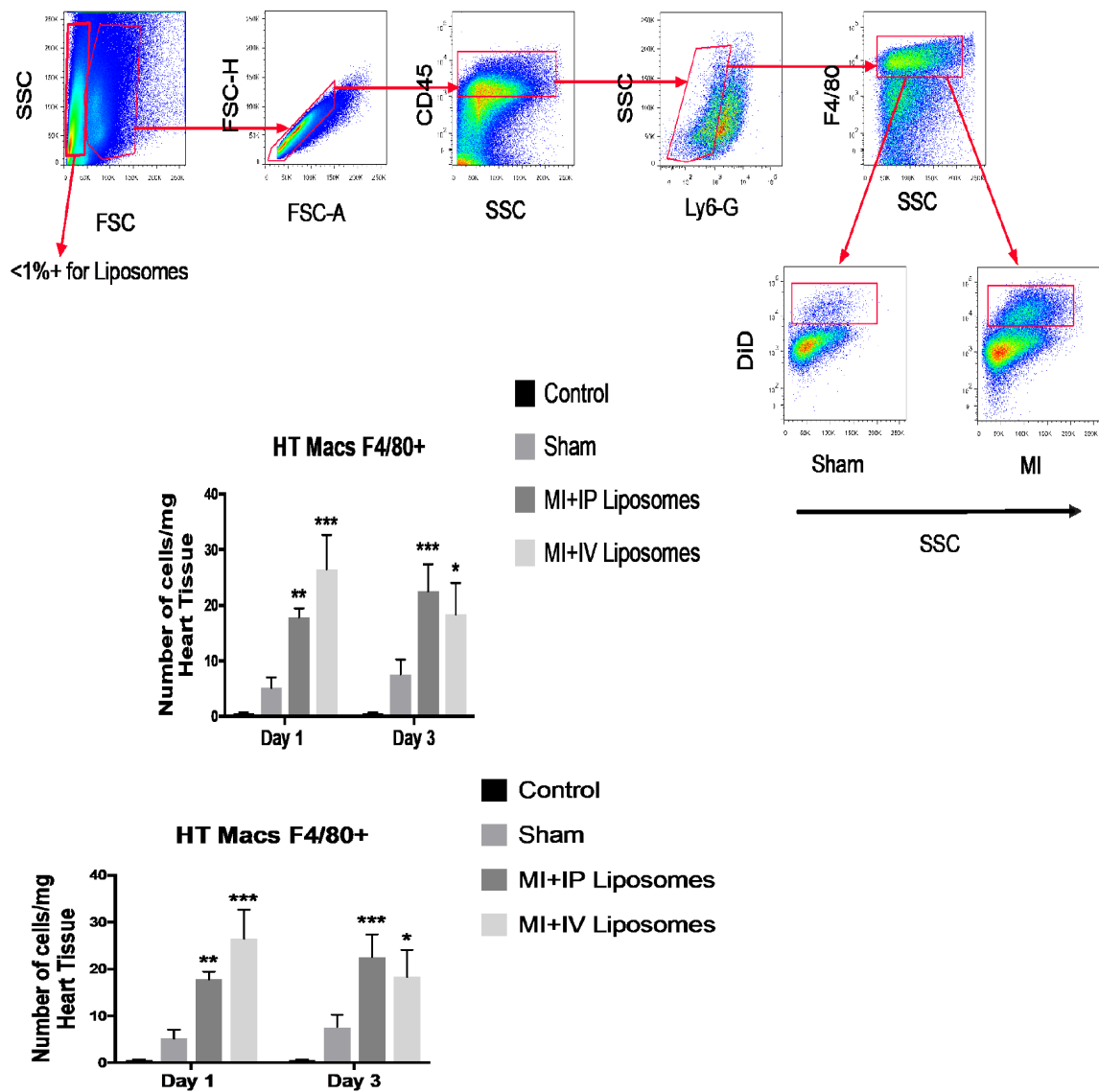


Figure 3.2. Targeting immune cells with labeled Lazm after infarction.

FACS analysis with labeled Lazm at days 1 and 3 post-MI shows liposomes are accumulated in cardiac immune cells after IP or IV administration, with more significant effect after IV administration. Data suggests that Lazm liposomes begin to concentrate in the immune cells on day 1 post-MI using IV but not IP route. No significant accumulation was noted in non-immune cells ($N = 3$ animals/group/timepoint, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, and $****P < 0.0001$ compared to sham). Data presented as mean \pm SEM. IV, intravenous; IP, intraperitoneal; Lazm, liposomal azithromycin; Macs, macrophages; Control, mice did not receive liposomes.

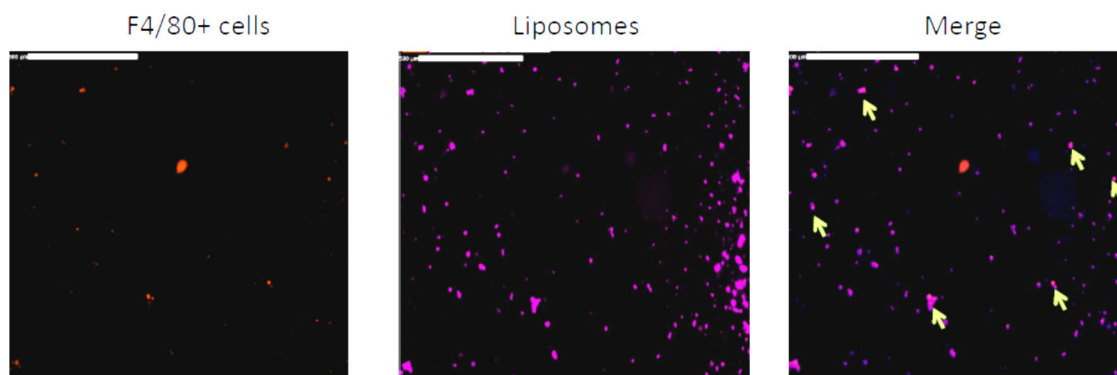


Figure 3.3. Liposomes co-localize with cardiac macrophages.

Representative images from Celigo of heart cells of Lazm- treated mice show spatial localization of macrophages (orange) and liposomes (purple). Images show a remarkable localization of macrophages with liposomes. Lazm, liposomal azithromycin.

In this experiment, a similar protocol to flow cytometry was used to prepare cell suspension; however, Celigo was used instead of flow cytometry to identify co-localization of different cell populations with liposomes (N=3 mice).

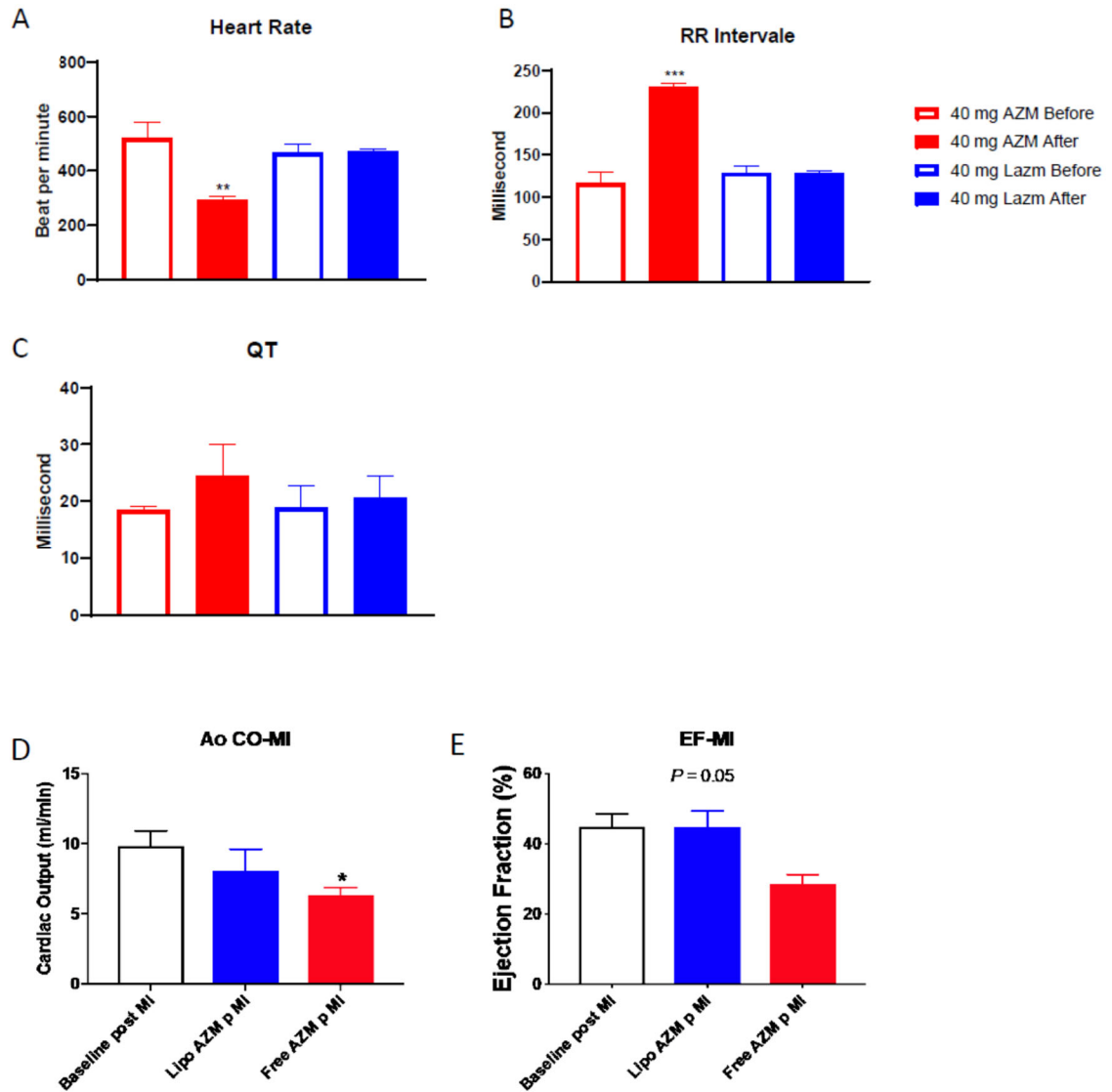


Figure 3.4. Hemodynamic Effects of free and liposomal AZM. Changes in heart rate (**Panel A**), RR interval (**Panel B**), QT interval (**Panel C**), ejection fraction (**Panel D**), and cardiac output (**Panel E**) after retro-orbital administration of various doses of Lazm or free AZM. Data shows that the liposomal formulation of AZM is protective against hemodynamic changes induced by free AZM. (N=3, **P<0.01 and *** P<0.001 compared to baseline of the corresponding group). Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin.

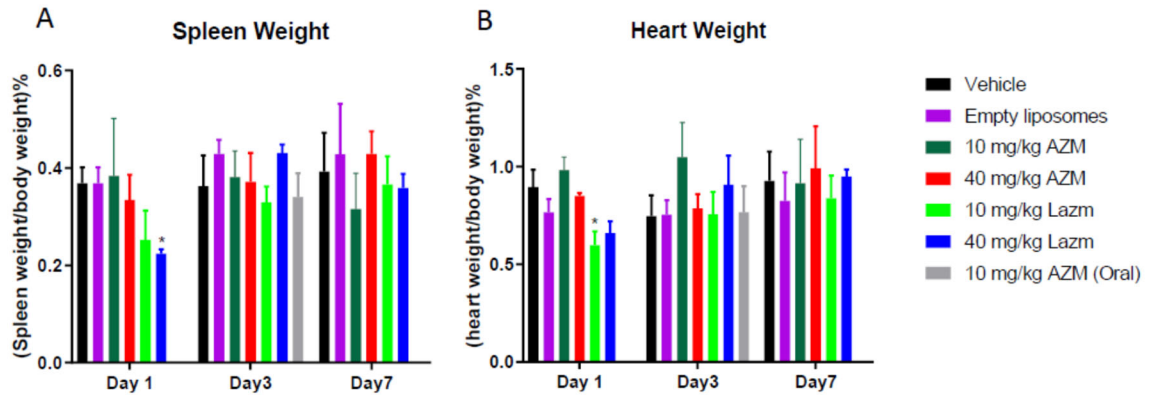


Figure 3.5. Lazm therapy rescues heart and spleen from inflammatory induced hypertrophy. Quantitative assessment of spleen (**Panel A**) and heart (**Panel B**) as a percent of body weight suggests that Lazm therapy is protective against heart and spleen enlargement after infarction, indicative of less potent inflammation. (n = 4 animals/group/timepoint, *P<0.05 and **P<0.01 compared to the vehicle control). Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; HT, heart.

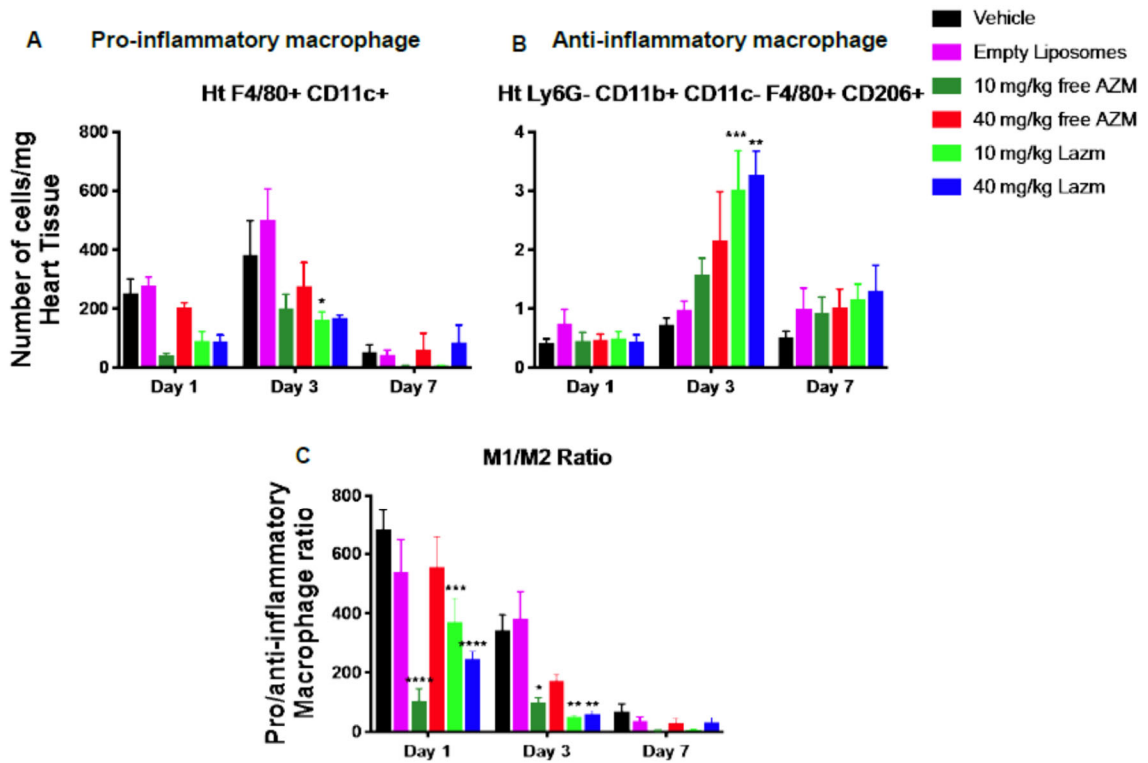


Figure 3.6. Lazm treatment shifts macrophages to the reparatory phenotype in the injured myocardium.

Immunomodulation of macrophages by Lazm in heart tissue post-MI. FACS analyses of the relative expression of macrophage markers to assess their activation state in vehicle, free AZM, and Lazm treated groups. Quantitative data suggests that inflammatory (F4/80+/CD11c+) macrophages markedly decrease in Lazm treated groups relative to controls starting at day1, with a more profound effect on day 3 post-MI (**Panel A**). In contrast, the reparatory macrophages (CD206+) increase significantly at day 3 post-MI in the same groups of mice (**Panel B**). The immunomodulatory effects translate into a substantial reduction in the pro-/anti-inflammatory (CD11c+/ CD206+) ratio at all tested timepoints post-MI (**Panel C**). Less powerful immunomodulatory changes were noted with free AZM treatments. (N = 4 animals/group/timepoint, **P<0.01, ***P<0.001, and ****P<0.0001 compared to the vehicle control). Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; Ht, heart.

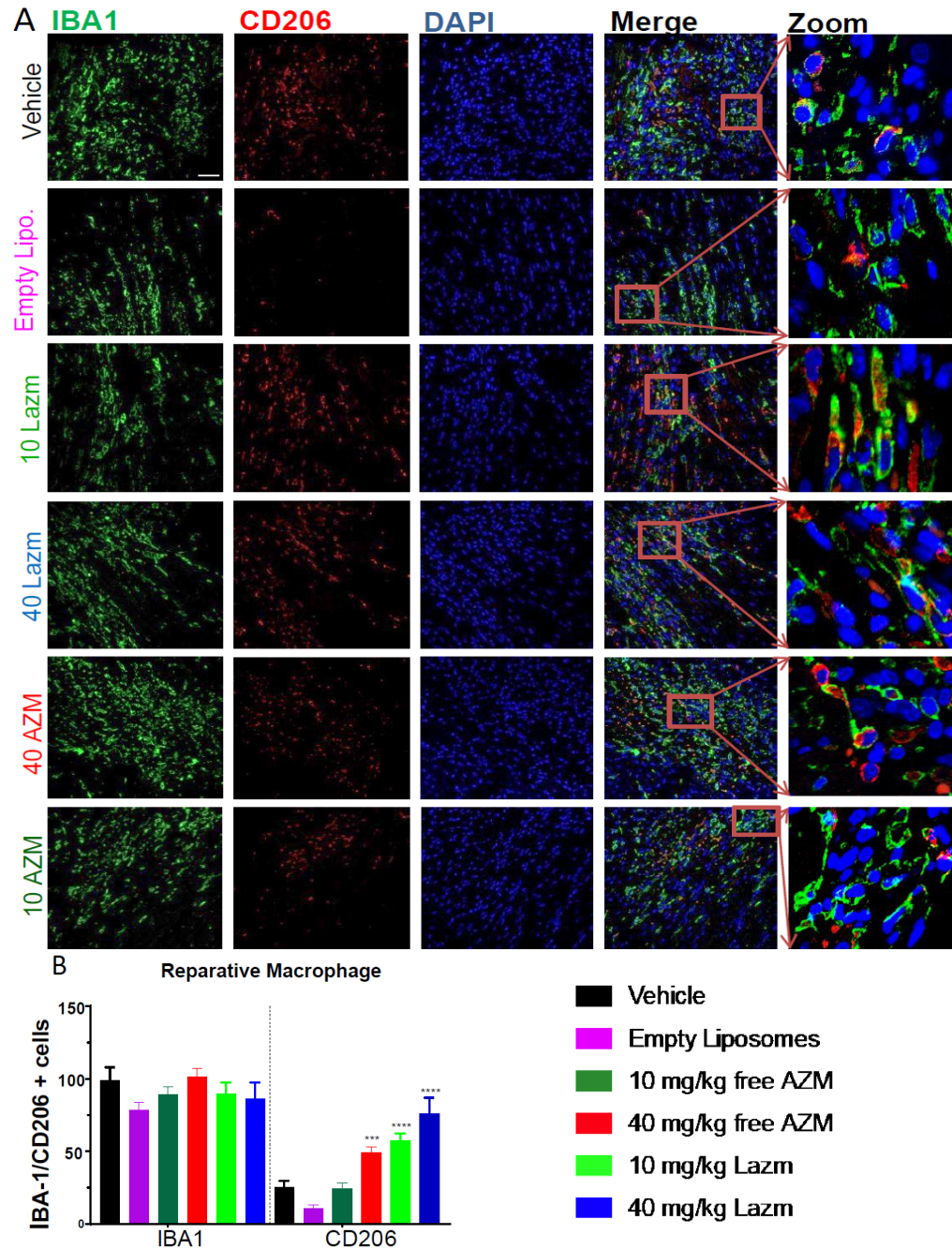


Figure 3.7. Lazm shifts macrophages towards the anti-inflammatory phenotype. Immunohistochemical assessment of general and reparative macrophages (IBA1⁺ and CD206⁺, respectively) 3 days post-MI. **Panel A** shows representative images from controls, free AZM, and Lazm treated mice illustrating IBA1 (green) and CD206 (red) positive macrophages in peri-infarct zones. Images show increased expression of CD206 by macrophages in free and liposomal AZM treated groups compared to controls. **Panel B** represents quantitative assessment of IBA1 and CD206 3-days post-MI (n = 4 animals/group, ***P<0.001 and ****P<0.0001 compared to vehicle control). Scale bars represent 50 μ m. Data presented as mean \pm SEM.

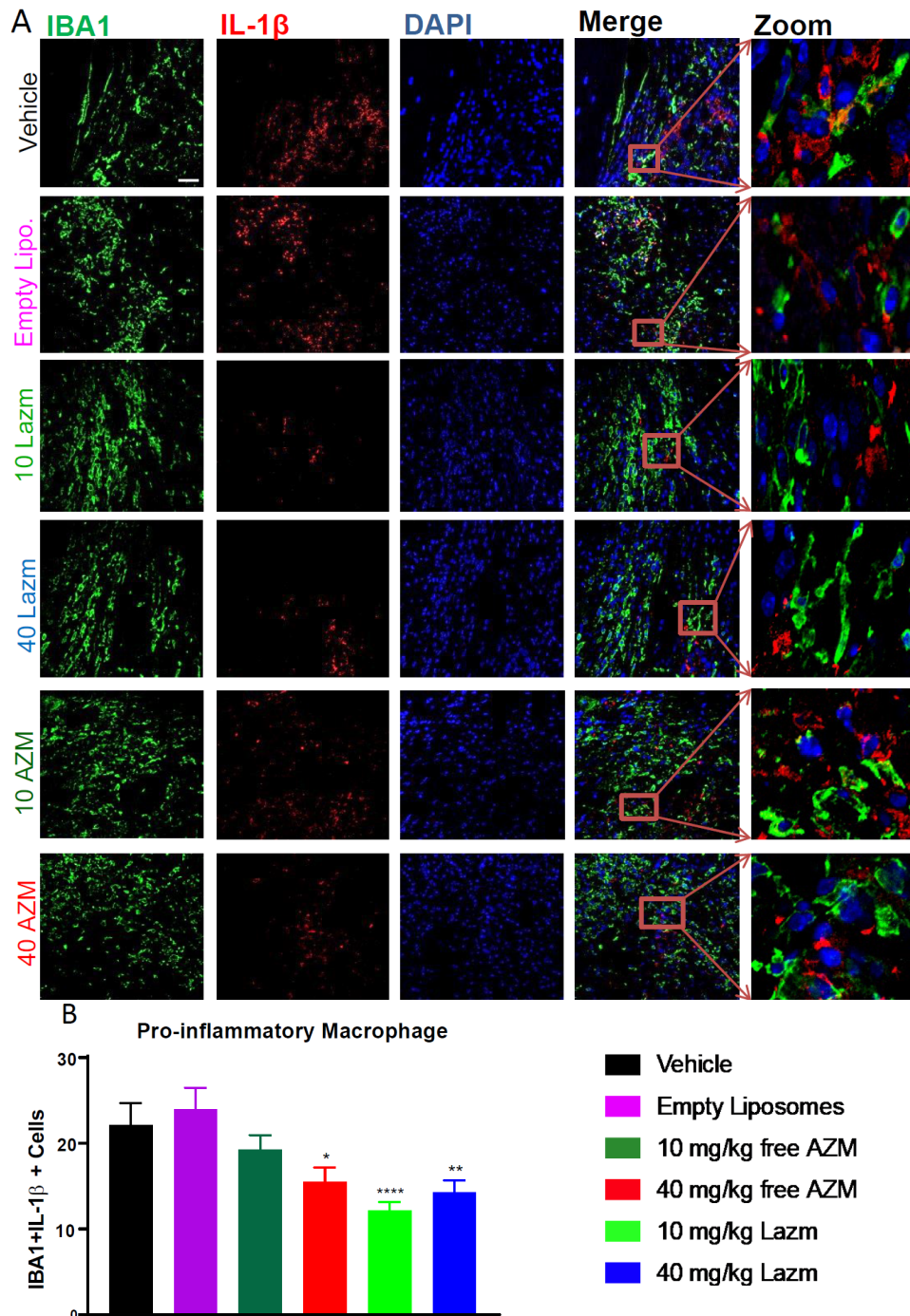


Figure 3.8. Lazm reduces pro-inflammatory macrophages in the ischemic heart. Immunohistochemical assessment of a general macrophage marker (IBA1) co-localized with a pro-inflammatory cytokine (IL-1 β) 3 days post-MI. **Panel A** shows representative images from controls, free AZM, and Lazm treated mice illustrating IBA-1 (green) and IL-1 β (red) positive macrophages in peri-infarct zones. Images indicate a marked reduction in the expression of IL-1 β in macrophages with free and liposomal AZM treatments compared to controls. **Panel B** represents quantitative assessment of IBA-1 and IL-1 β 3-days post-MI (n = 4 animals/group, *P<0.05 **P<0.01, and ****P<0.0001 compared to vehicle control). Scale bars represent 50 μ m. Data presented as mean \pm SEM.

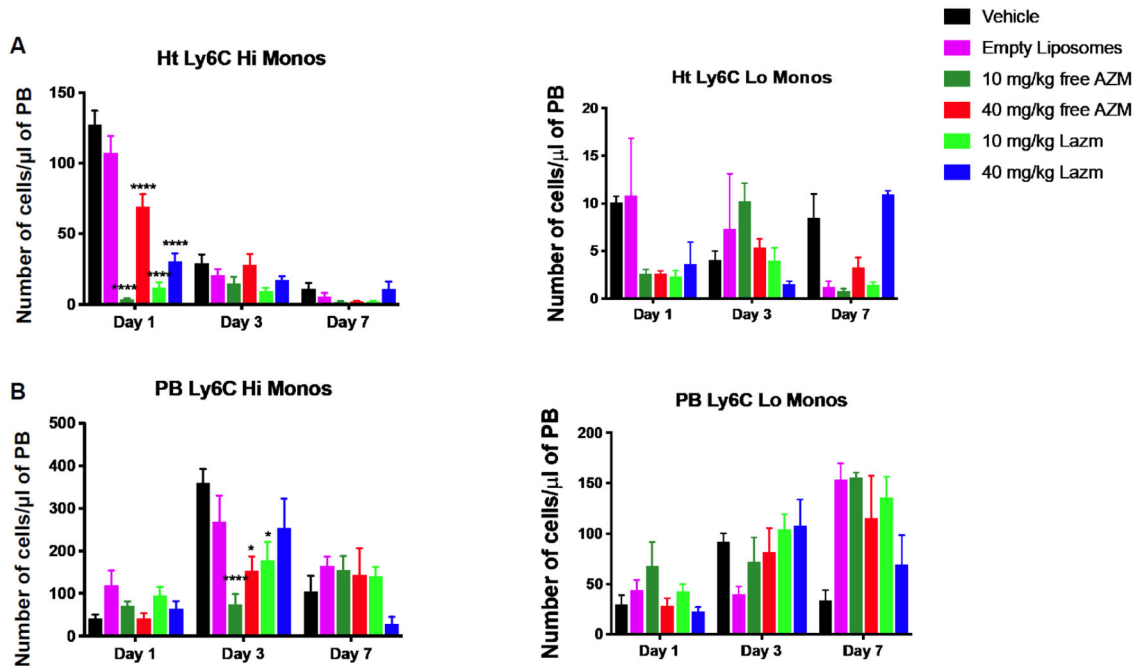


Figure 3.9. Lazm reduces inflammatory monocytes after infarct. Immunomodulation of monocytes by free and liposomal AZM in Ht and blood post-MI. FACS analyses for the relative expression of Ly6C to assess monocyte activation state in controls, free AZM, and Lazm treated groups of mice. Quantitative data indicate that inflammatory monocytes (Ly6C^{Hi}) markedly decrease in liposomal and free AZM treated mice, with a more powerful effect in Lazm groups (**Panel A**). Very little change was noted in the blood (**Panel B**). (N = 4 animals/group/time point, **P<0.01, ***P<0.001, and ****P<0.0001 compared to vehicle control). Data presented as mean ± SEM. AZM, azithromycin; Lazm, liposomal azithromycin; Ht, heart.

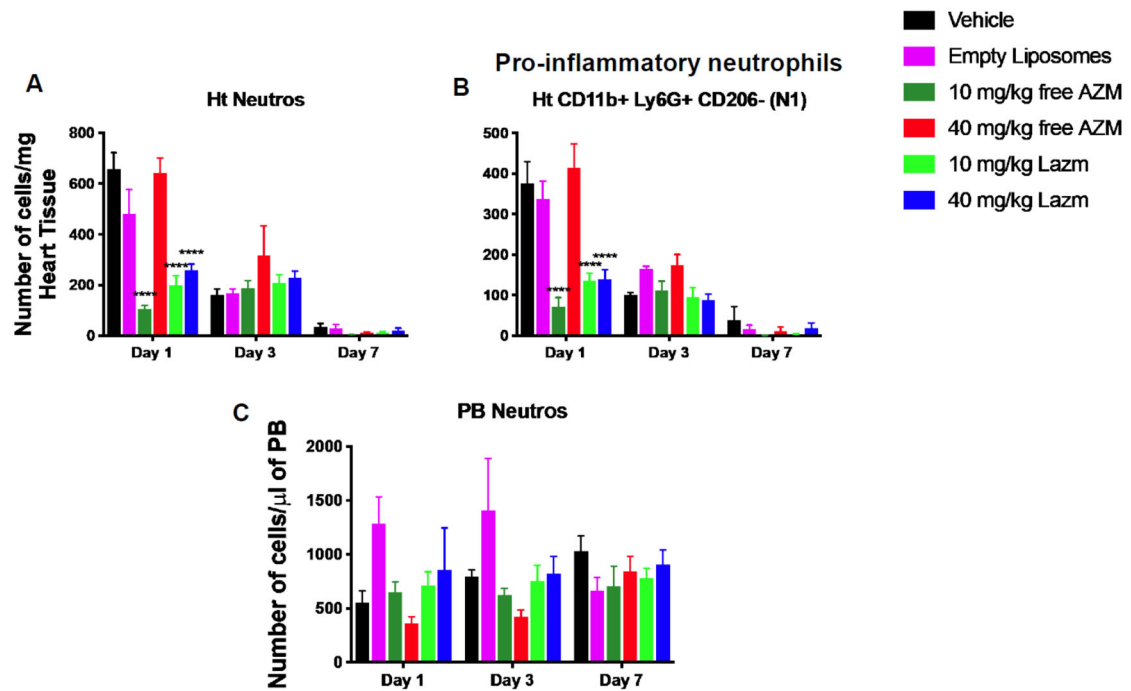


Figure 3.10. Lazm treatment reduces neutrophil counts, in particular N1 neutrophils. FACS analyses suggest that neutrophil counts are significantly decreased in the Ht (**Panel A**), specifically N1 (CD11b+Ly6G+CD206-) neutrophils in Lazm treated mice on day 1 post-MI (**Panel C**). No changes in neutrophils were noted with free AZM treatment. Neutrophils were reduced on day 7 in Lazm groups in blood (**Panel C**) (N = 4 animals/group/time point, **P<0.01 and ***P<0.001 compared to vehicle control). Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; Ht, heart; PB, peripheral blood.

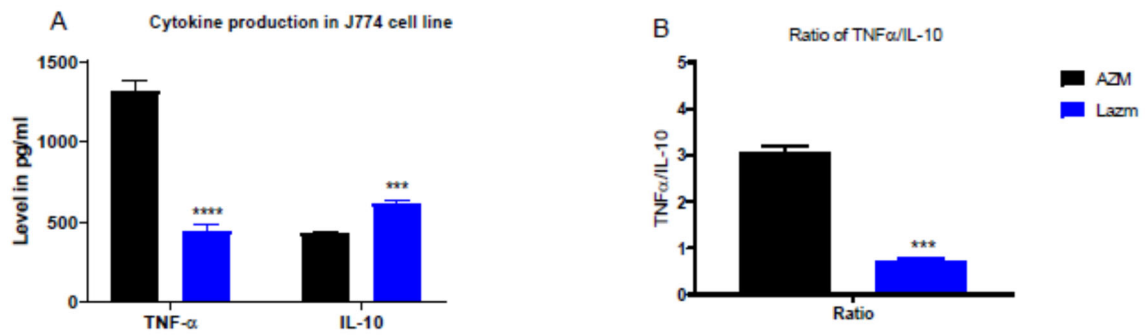


Figure 3.11. Liposomal azithromycin produces a more significant shift in macrophage response towards the anti-inflammatory state.

This figure shows quantitative analyses of pro-inflammatory cytokine, TNF- α , and anti-inflammatory cytokine, IL-10, production from J774 macrophages subjected to LPS stimulation for 48 hours. The analyses demonstrate a more significant reduction of TNF- α with Lazm compared to free AZM at 48 hours post-MI. Moreover, IL-10 production is additionally enhanced with Lazm. Treatment (two independent experiments and 4 replicates/time point, *** $P < 0.001$ and **** $P < 0.0001$ compared to free AZM). Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; IL-10, interleukin 10; TNF- α , tumor necrosis factor-alpha.

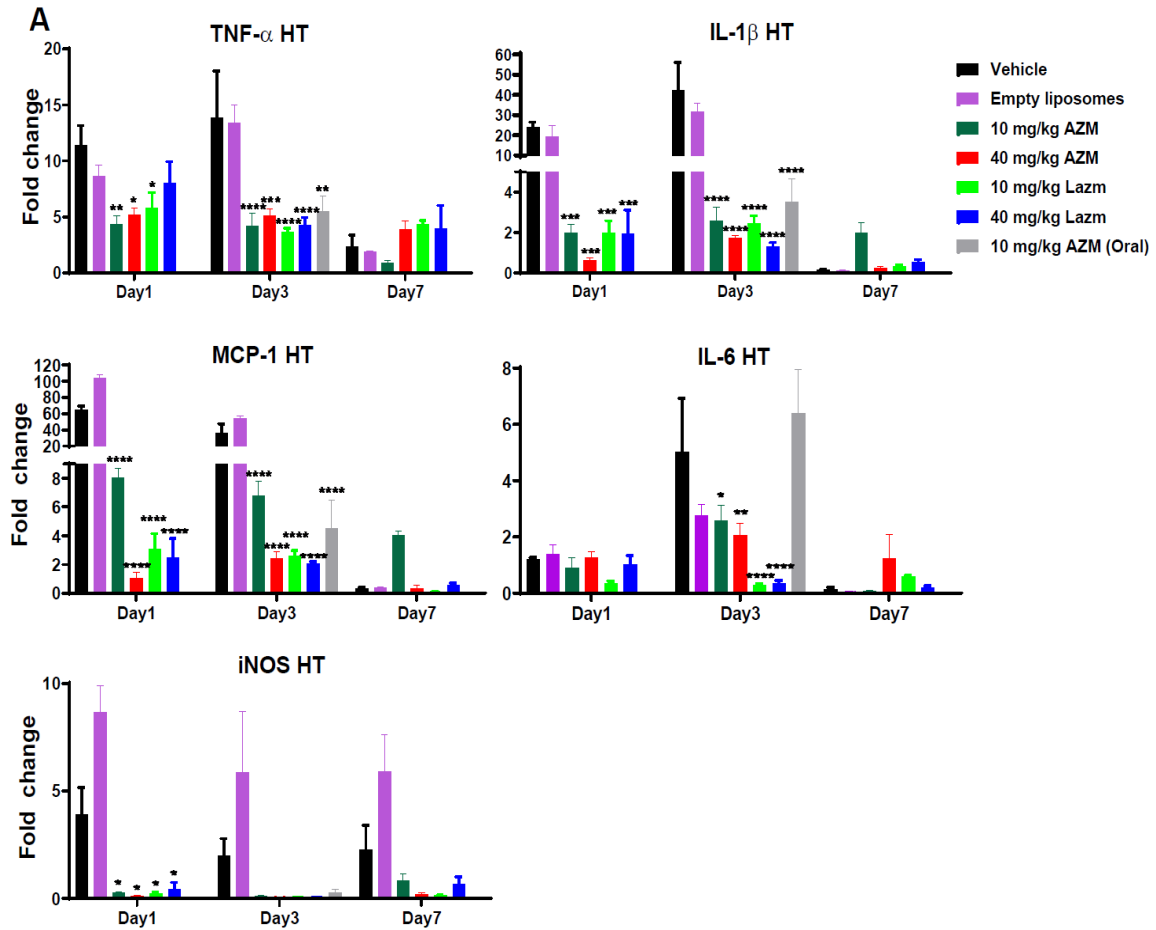


Figure 3.12A. Lazm modulates pro- and anti-inflammatory cytokine expression. Heart homogenate (Ht) cells were used to quantify pro-inflammatory cytokine secretion via RT-PCR. Pro-inflammatory cytokine expression, including iNOS, TNF- α , MCP-1, IL-1 β and IL-6c(**A**). Data shows significant reduction in inflammatory cytokines at days 1 and 3 with Lazm treatment in Ht ($n = 4$ animals/group/time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ compared to the vehicle control). Fold change relative to sham control. Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; Ht, heart; IL-1 β , interleukin 1 beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor-alpha.

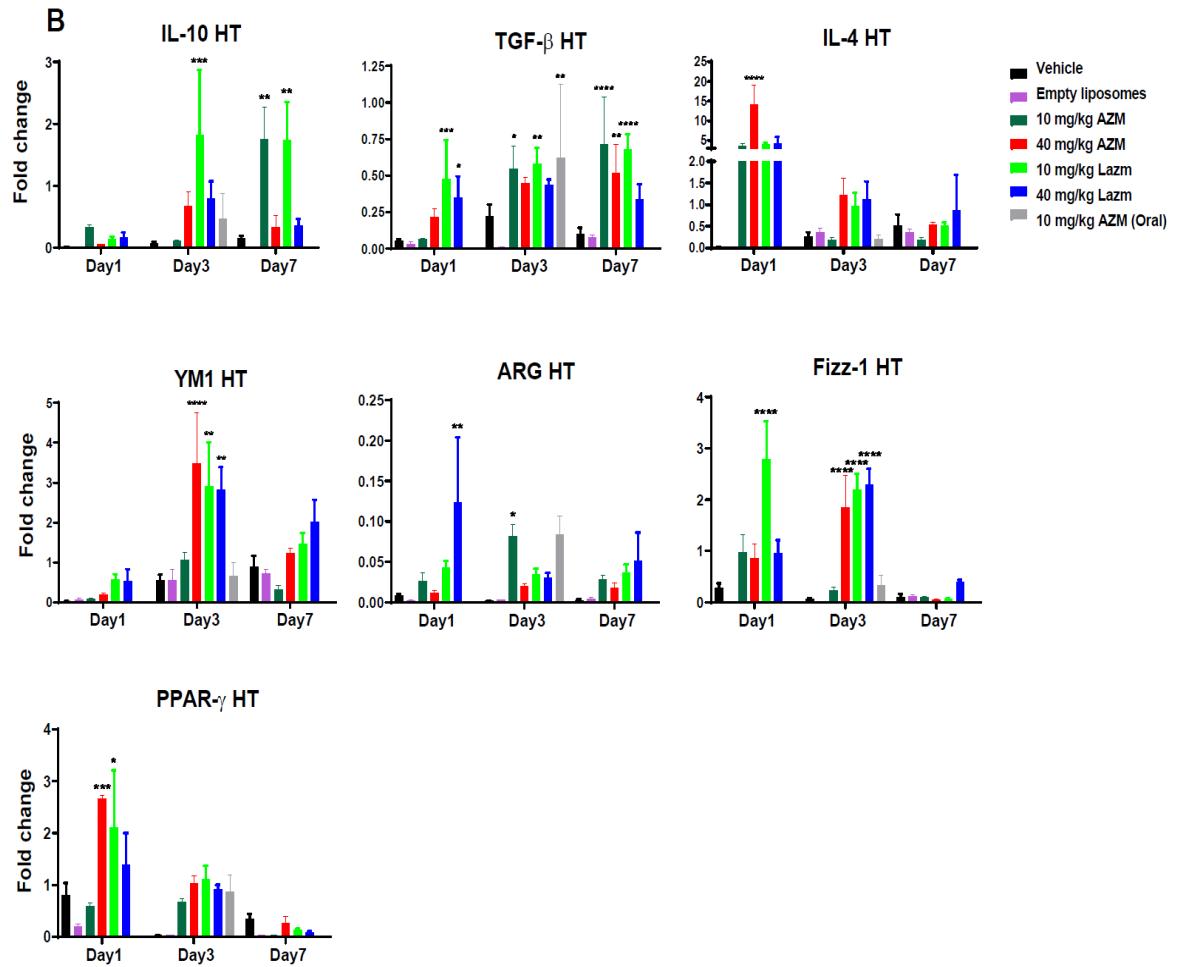


Figure 3.12B. Lazm modulates pro- and anti-inflammatory cytokine expression. Heart homogenate (HT) cells were used to quantify anti-inflammatory cytokine secretion via RT-PCR. Anti-inflammatory IL-10, TGF- β , Fizz1, and YM1, ARG, PPAR γ , IL-4 expression (**B**). Data shows significant increase in anti-inflammatory cytokines on days 1, 3 and, 7 with Lazm treatment in HT (n = 4 animals/group/time point, *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 compared to the vehicle control). Fold change relative to sham control. Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; HT, heart; IL-4, interleukin 4; ARG, arginase; Fizz1, found in inflammatory zone 1; PPAR γ , peroxisome proliferator-activated receptor gamma; TGF-1 β , tissue growth factor 1 beta; YM1 (Chil3), chitinase-like 3; IL-10, interleukin-10.

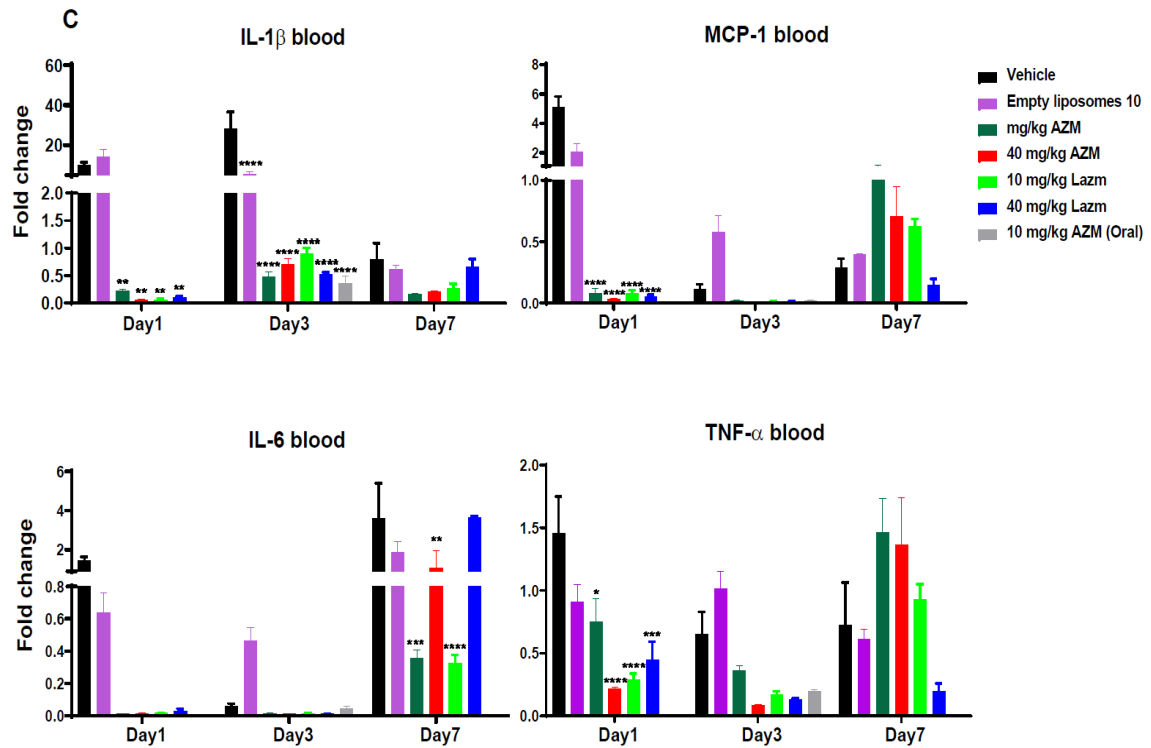


Figure 3.12C. Lazm modulates pro- and anti-inflammatory cytokine expression. Peripheral blood cells were used to quantify pro-inflammatory cytokine secretion via RT-PCR. Pro-inflammatory cytokine expression, including TNF- α , MCP-1, IL-1 β and IL-6(**C**). Data show significant reduction in inflammatory cytokines at days 1 and 3, and 7 with Lazm treatment in blood ($n = 4$ animals/group/time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ compared to the vehicle control). Fold change relative to sham control. Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; HT, heart; IL-1 β , interleukin 1 beta; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; PB, peripheral blood; TNF- α , tumor necrosis factor-alpha.

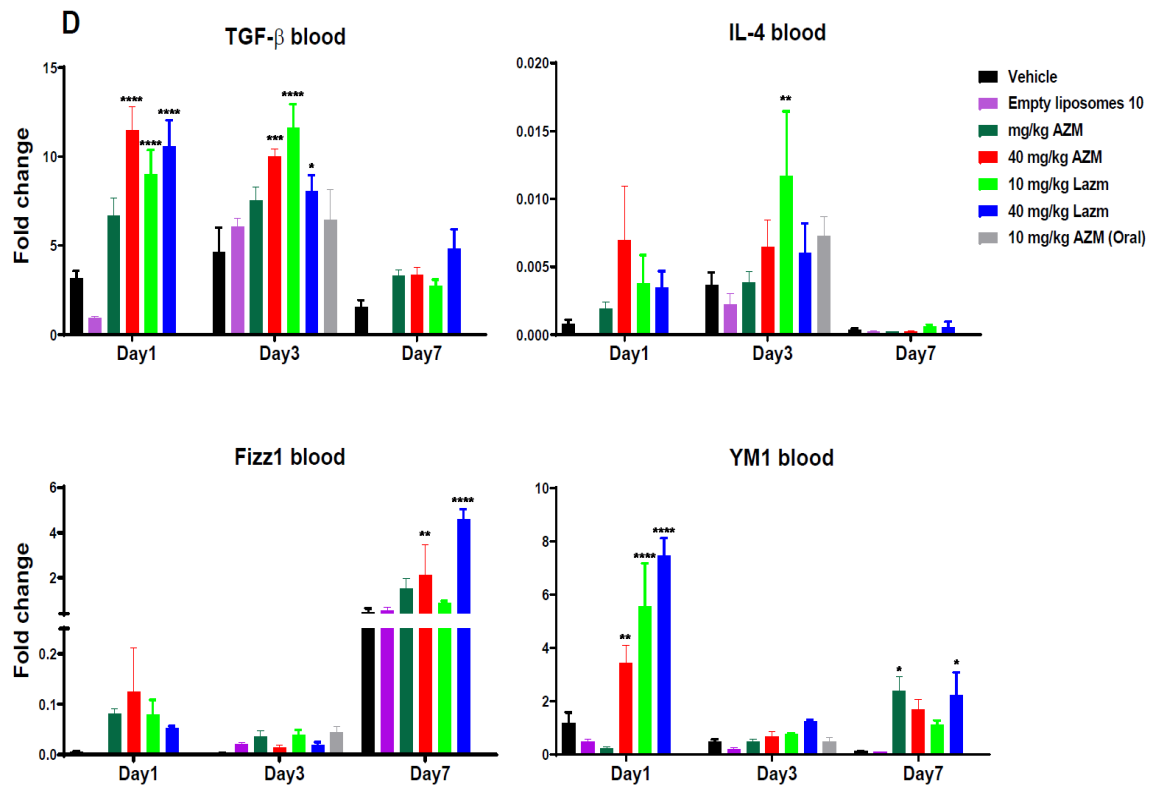


Figure 3.12D. Lazm modulates pro- and anti-inflammatory cytokines expression. Peripheral blood cells were used to quantify anti-inflammatory cytokine secretion via RT-PCR. Anti-inflammatory TGF- β , Fizz1, and YM1 expression (**D**). Data show significant increase in anti-inflammatory cytokines in days 1, 3 and, 7 with Lazm treatment in blood ($n = 4$ animals/group/time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ compared to the vehicle control). Fold change relative to sham control. Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; HT, heart; IL-4, interleukin 4; Fizz1, found in inflammatory zone 1; PB; TGF-1 β , tissue growth factor 1 beta; YM1 (Chil3), chitinase-like 3.

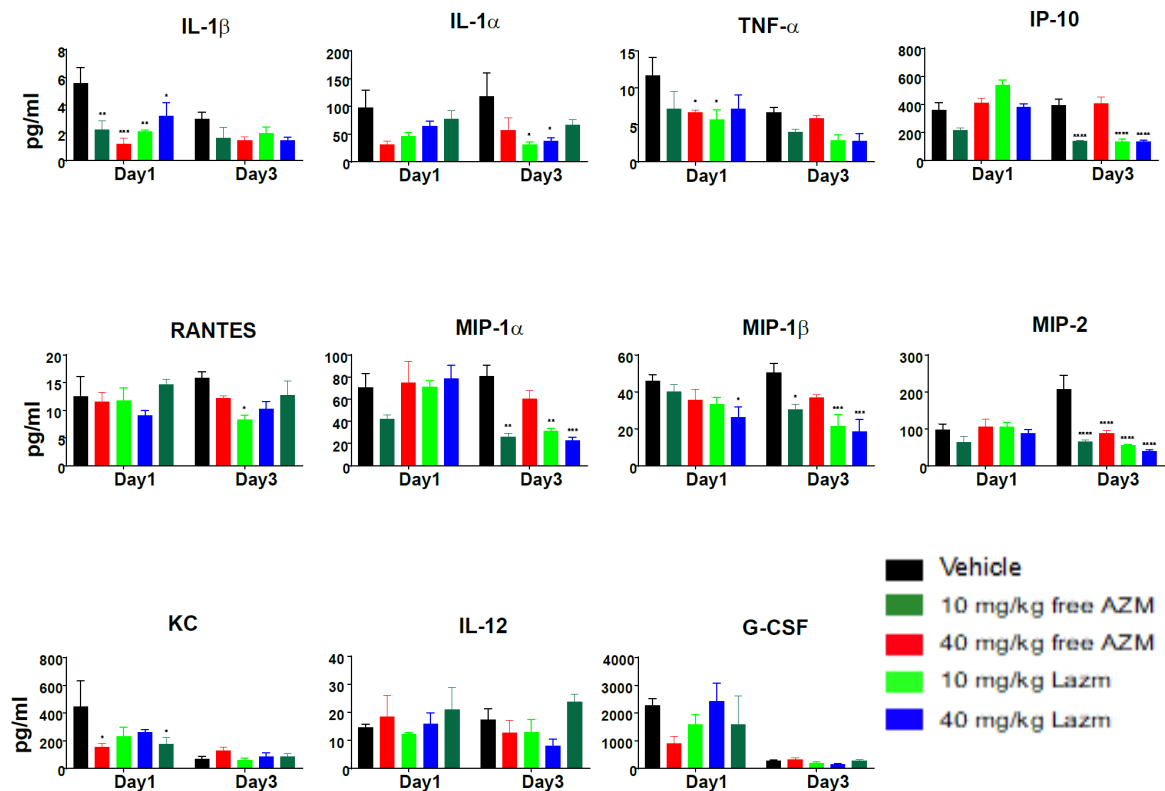


Figure 3.13. Lazm modulates pro-inflammatory cytokine and chemokine production. Plasma quantification of pro-inflammatory cytokine and chemokine secretion via Luminex assay. Data shows significant reduction in inflammatory cytokines and chemokines at days 1 and 3 with Lazm therapy (n = 4 animals/group/time point, *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 compared to the vehicle control). Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin; IL-1, interleukin 1; TNF- α , tumor necrosis factor-alpha; RANTES, regulated on activation, normal T cell expressed and secreted; IL-12, interleukin 12; MIP, macrophage inflammatory protein; KC, Keratinocyte chemoattractant; IP, Inducible protein; LIX, Lipopolysaccharide-induced CXC chemokine ; G-MCSF, Granulocyte-Macrophage Colony-Stimulating Factor.

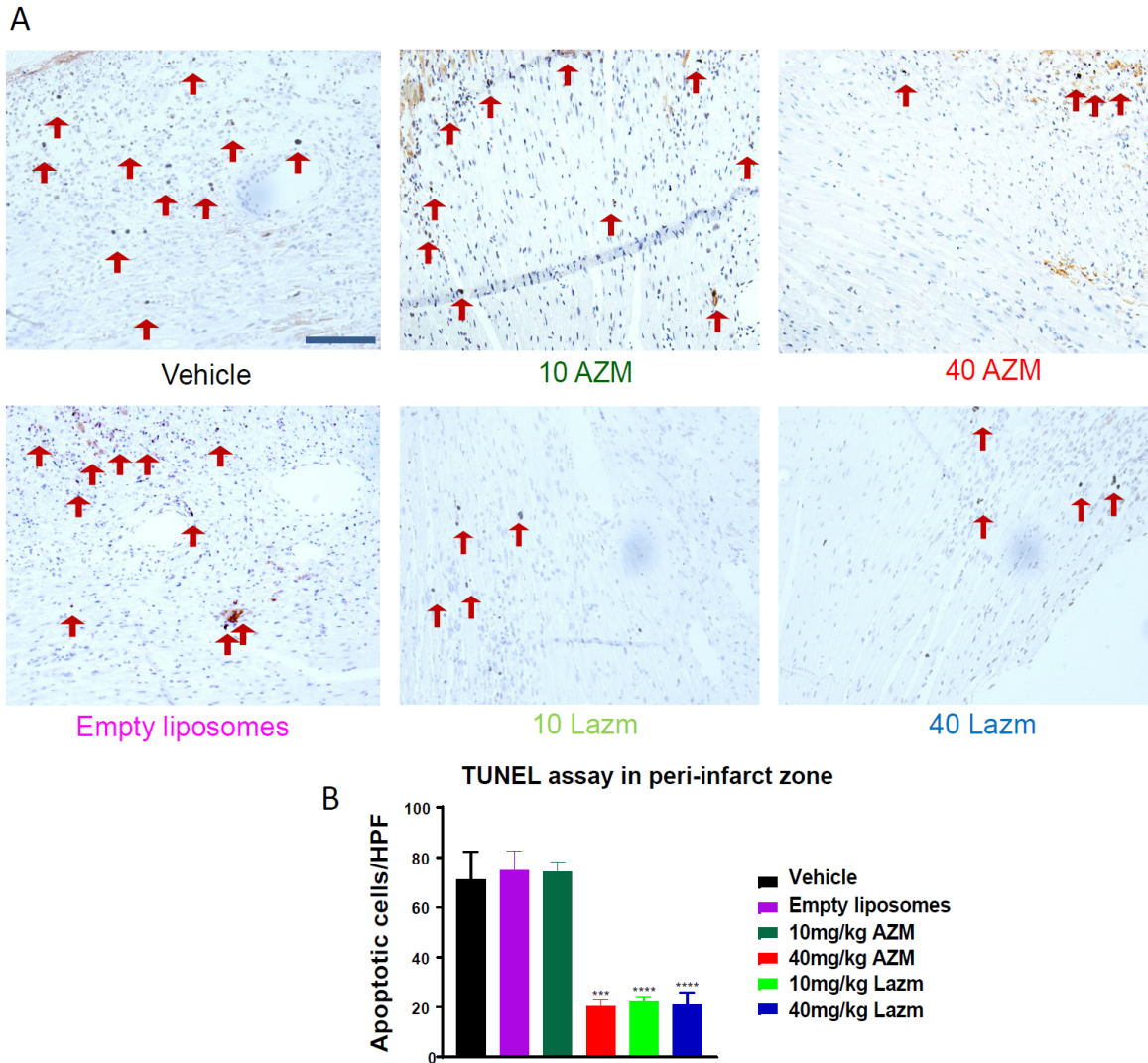


Figure 3.14. AZM reduces apoptosis in the heart post-infarction.

Panel A shows representative light microscope images of TUNEL staining of peri-infarct regions in controls, free AZM, and Lazm treated mice 3 days post-MI. **Panel B** represents quantitative analysis of apoptosis which reveals a remarkable reduction of TUNEL positive cells in free and liposomal AZM treated groups compared to the vehicle control group ($n = 4$ animals/group, $**P < 0.01$ and $***P < 0.001$ compared to vehicle control). Scale bars represent 100 μm . Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin

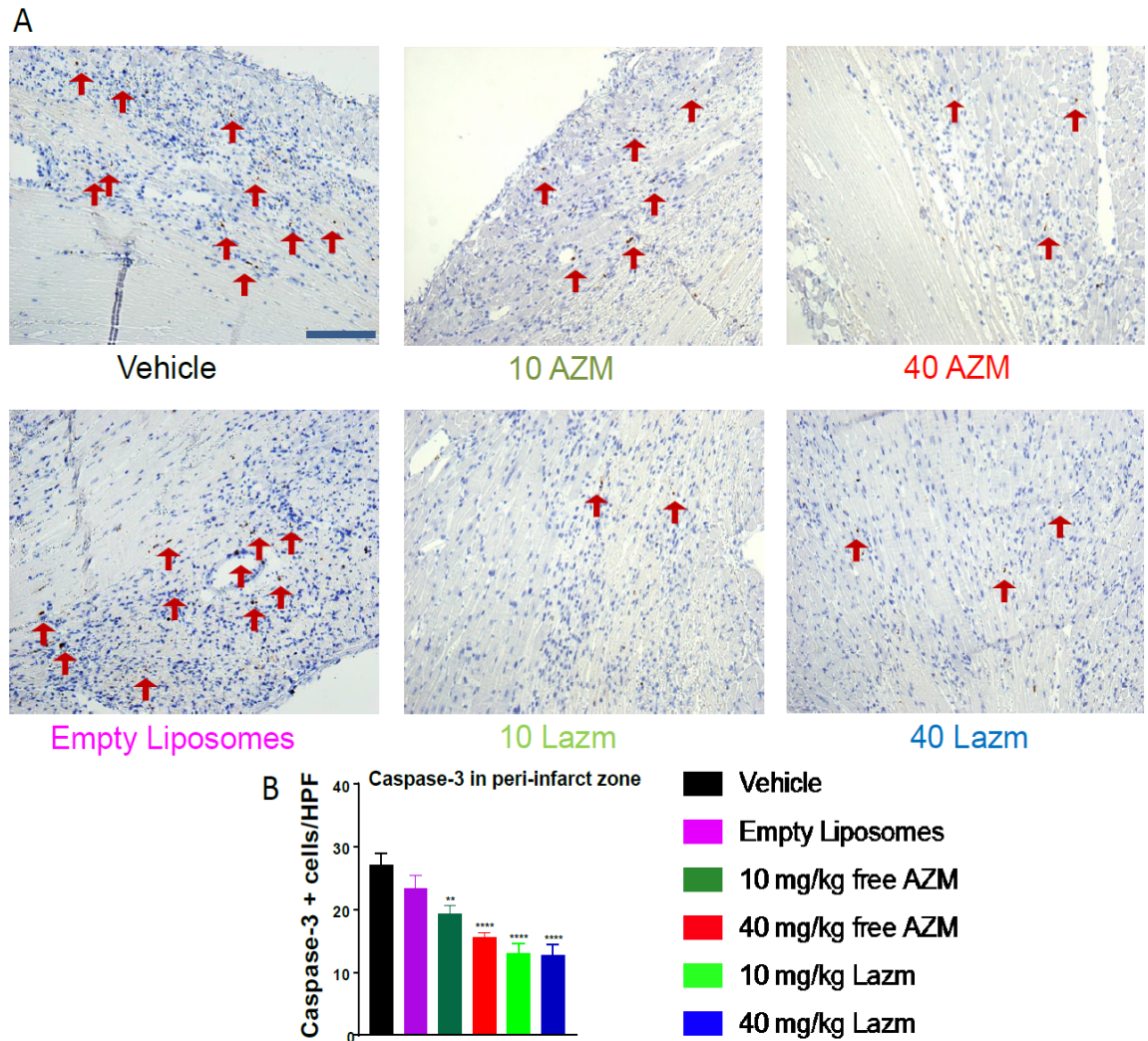


Figure 3.15. AZM reduces apoptosis in the heart post-infarct.

Panel A shows representative light microscope images of caspase-3 staining for peri-infarct regions in controls, free AZM, and Lazm treated mice 3 days post-MI. **Panel B** represents quantitative analyses of apoptosis which reveal a remarkable reduction of caspase-3 positive cells in free and liposomal AZM treated groups compared to the vehicle control group (n = 4 animals/group, **P<0.01 and ****P<0.0001 compared to vehicle control). Scale bars represent 100 μ m. Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin.

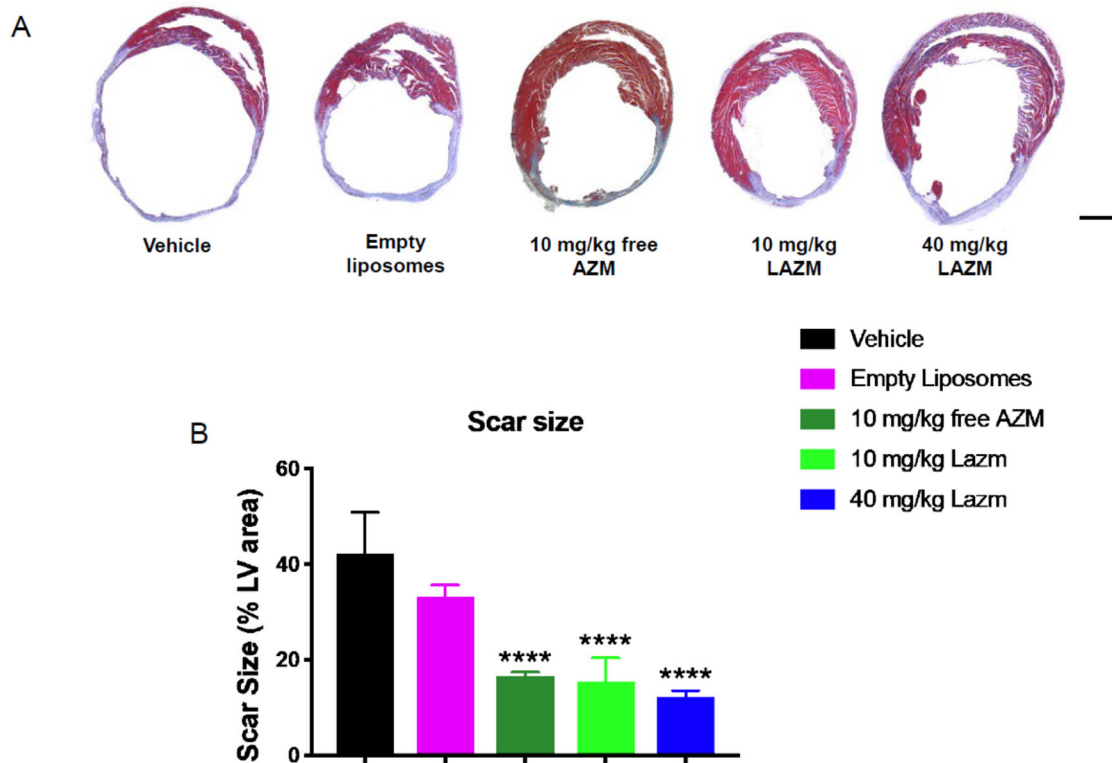


Figure 3.16. Lazm therapy reduces scar size after infarct.

Panel A shows representative Masson's trichrome staining at 30 days post-MI in control, free AZM, and Lazm treated groups, indicating a marked reduction in scar size with liposomal and free AZM. **Panel B** represents quantitative analysis of scar as a percentage of LV area, which shows significant reduction in liposomal and free AZM treated groups relative to the vehicle control group ($n = 6-10$ animals/group, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$ compared to the vehicle control). Scale bars represent $1000 \mu\text{m}$. Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin.

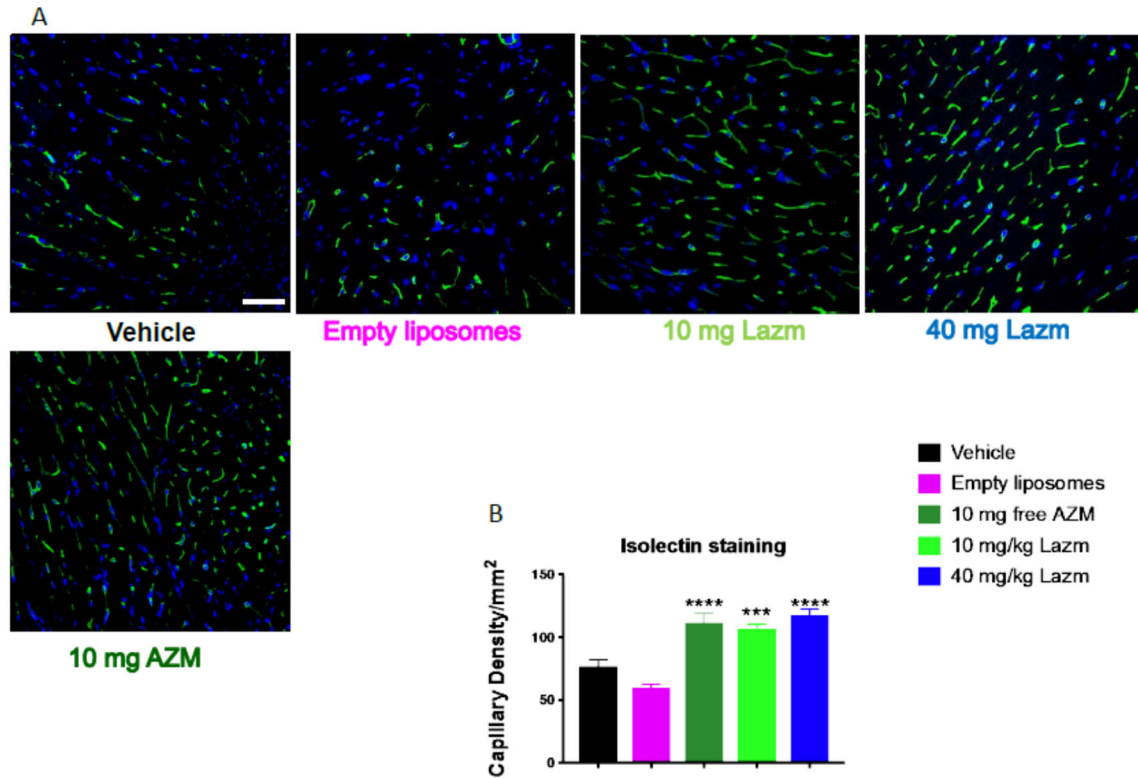


Figure 3.17. Lazm therapy promotes angiogenesis after infarct.

Panel A shows representative isoelectin staining (Green) at 30 days post-MI for capillary density in the peri-infarct region in control, free AZM, and Lazm treated animals. This data demonstrates higher capillary density in Lazm groups compared to vehicle control.

Panel B is quantitative analysis of capillary density, which confirms a higher rate of angiogenesis and capillary density in Lazm treated groups (n = 5-10 animals/group, ****P<0.0001 compared to the vehicle control). Scale bars represent 50 μ m. Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin.

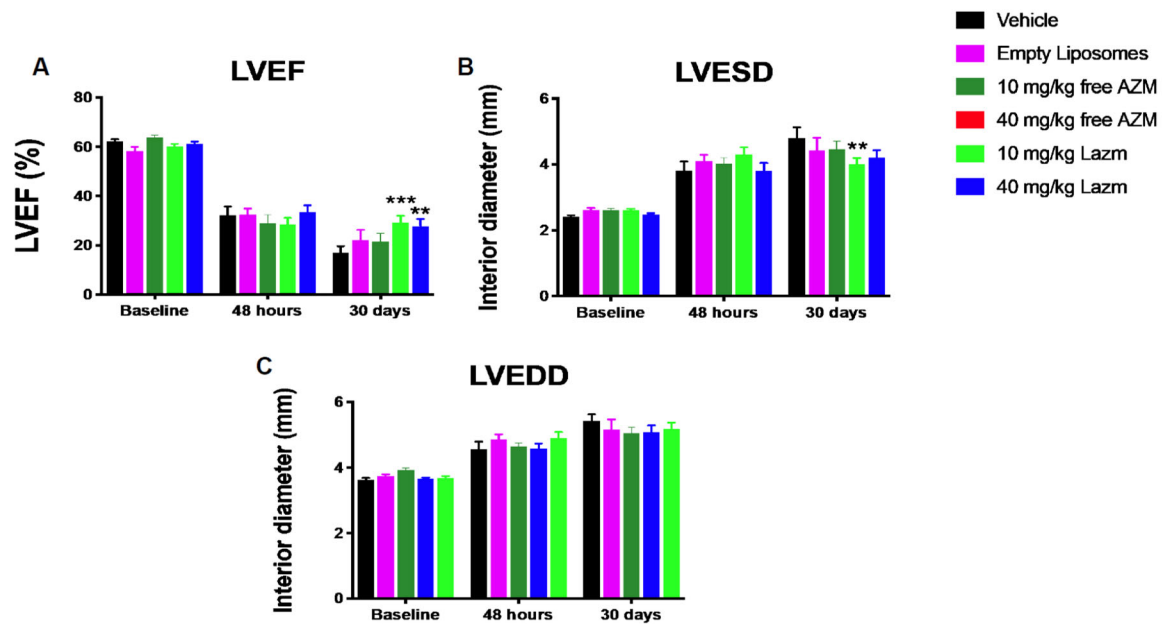


Figure 3.18. Lazm therapy improves adverse cardiac remodeling.

30 days following MI, echocardiography was performed on control, free AZM, and Lazm treated animals to evaluate left ventricular function and remodeling parameters. Quantitative analyses demonstrate significant recovery in LV function as assessed by ejection fraction (LVEF) (**Panel A**), which significantly improved in Lazm groups. Data also show significant improvements in LV adverse remodeling parameters such as end-systolic diameter (LVESD) (**Panel B**) and end-diastolic diameter (LVEDD) (**Panel C**). (n = 6-10 animals/group, *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 compared to the vehicle control). Data presented as mean \pm SEM. AZM, azithromycin; Lazm, liposomal azithromycin.

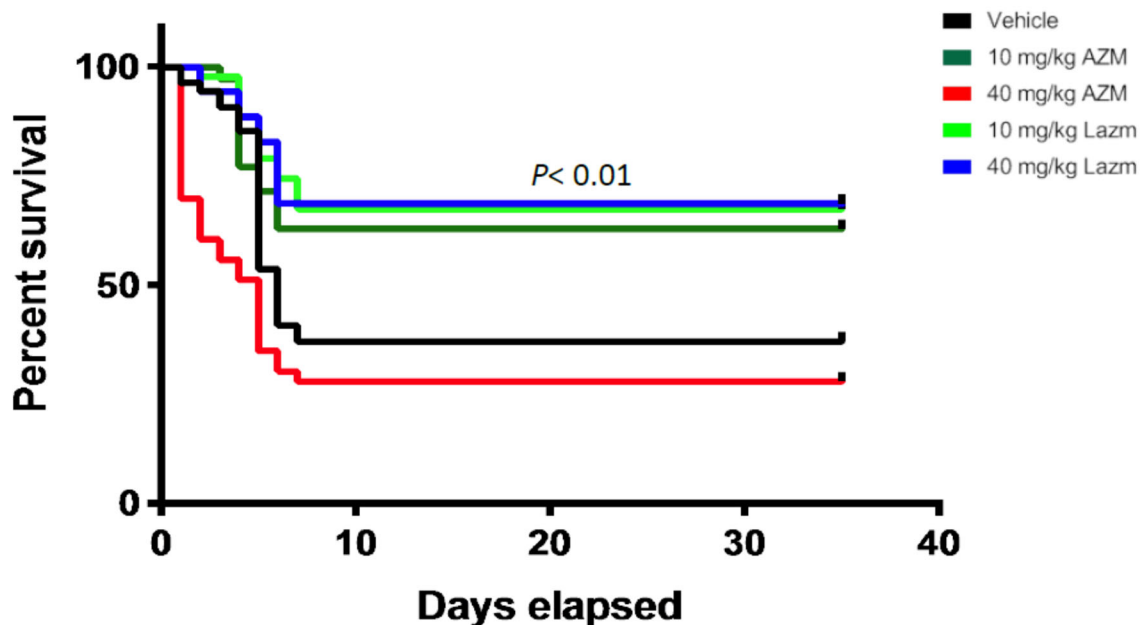


Figure 3.19. AZM therapies enhance survival.

Survival curves of free/liposomal AZM and vehicle-treated mice 35 days post-MI demonstrate a marked improvement in survival with Lazm and low-dose free AZM treatment. Mortality in 40 mg/kg IV AZM group occurs during the drug administration due to severe changes in cardiac conduction and hemodynamic parameters. While mice in other groups died of cardiac rupture, a common complication after MI surgery. (vehicle group, n =34, 40 mg/kg IV AZM group, n=33, 40 mg/kg Lazm group, n=35, 10 mg/kg Lazm group, n=31, 10 mg/kg IV AZM, n=35). AZM, azithromycin; Lazm, liposomal azithromycin.

CHAPTER IV. General discussion

4.1 Summary

The purpose of the work in this dissertation is to determine the role of azithromycin (AZM) as an immunomodulatory therapeutic agent in reducing the inflammatory response and related adverse cardiac remodeling after infarction. The ultimate goal of our research is to provide a clinically translatable approach with rapid onset of action and low off-target effects by using a liposomal formulation. To achieve our goals, we adopted a comprehensive approach utilizing various *in vivo* and *in vitro* models to examine the effects of free and liposomal encapsulation formulations of AZM in an established murine MI model.

First, we sought to investigate the efficiency of AZM as an immunomodulatory and anti-inflammatory drug in switching macrophage phenotypes towards the reparative state, and thus, alleviating the exaggerated post-MI inflammation. These studies are based on a growing body of evidence showing that altering the inflammatory response by repolarizing macrophages to the reparative phenotype is protective against acute ischemic injury and improves cardiac recovery. Research has shown that AZM, a macrolide antibiotic, alters inflammation and polarizes macrophages to the alternatively activated state in inflammatory conditions. Therefore, we hypothesized that AZM therapy reduces cardiac inflammation and mitigates adverse cardiac remodeling after MI. We treated mice with either AZM (160 mg/kg/day, oral) or vehicle, starting 3 days prior to MI. We selected the timing of AZM therapy to ensure appropriate steady state levels at the time of injury. We found that AZM treatment significantly decreased inflammatory macrophages while increasing reparative ones. Neutrophils also decreased with AZM treatment via enhanced apoptosis. Furthermore, AZM treatment significantly reduced infiltration of inflammatory monocytes to the heart, while anti-inflammatory monocytes were increased. Immune cell modulation was associated with significant downregulation of pro-inflammatory genes, and upregulation of anti-inflammatory genes. Taken together, AZM treatment resulted in a systemic and local shift in the post-MI inflammatory response. The

immunomodulatory effects of AZM translated into reduced cardiac cell death and scar size, and enhanced angiogenesis. Finally, these protective effects led to improved cardiac functional recovery and reduced adverse remodeling (Figure 4.1).

Due to limited clinical translational perspective (pre-MI treatment and relatively high dose of AZM) of our initial proof-of-concept study, we executed a follow-up study using a liposomal formulation of AZM (Lazm) to achieve rapid onset of action via targeted accumulation in the damaged myocardium. Liposomes are a valuable tool in producing a faster mode of action since they extensively localize in the injured myocardium and are specifically taken up by phagocytes. Furthermore, liposomal formulation is an efficient tool to enhance on-target and reduce off-target effects. Based on our preliminary studies with Lazm, we hypothesized that low dose of liposomal AZM (Lazm) treatment, started after MI, yields enhanced immunomodulatory potential and cardiac recovery with low risk of adverse effects. Therefore, we treated mice with low doses of encapsulated AZM immediately after infarction. We observed that Lazm induces early resolution of the post-MI inflammatory response as evidenced by switching of the activation state of monocytes/macrophages towards the reparatory phenotype. Neutrophils were substantially decreased, particularly pro-inflammatory neutrophils. Cytokine profiles also shifted to the anti-inflammatory status with Lazm therapy. Inflammation-resolving effects of Lazm were associated with reduced apoptosis in the injured heart, as well as improved scar size, angiogenesis, and LV function in our long-term studies. These findings imply that low dose, post-MI treatment with Lazm improves outcomes post-MI through early resolution of inflammation and initiation of the repair phase (Figure 4.1). Besides the immunomodulatory effects of Lazm, we observed that encapsulation of AZM substantially reduces the risk of off-target effects as well.

This work is early evidence demonstrating the efficacy of free and liposomal AZM as an immunomodulatory pharmacological agent after MI. Our results indicate that the anti-inflammatory effects of AZM relate to its direct effects on immune cells, which lead to alleviation of post-MI inflammation. Assessments of

cardiac and systemic changes demonstrate that the beneficial effects of AZM result in significant improvements in scar size, adverse cardiac remodeling, cardiac function, and survival post cardiac ischemia. Additionally, this is the first study demonstrating the immune modulation properties of liposomal AZM, which have potential wide therapeutic applications beyond the cardiovascular field. In addition, this work is the first to demonstrate that liposomal encapsulation is significantly protective from adverse drug effects such as hemodynamic changes, which are previously unexamined. These data introduce a novel and clinically relevant immunomodulatory therapy for ischemic heart and other sterile inflammatory diseases. AZM has a wide therapeutic window, allows oral administration, has a low side effect profile, and has clinical approval in humans. These criteria make it an ideal candidate for drug repurposing to improve survival and reduce heart failure following MI in humans.

4.2 Inflammation after infarction

Coronary artery occlusion of prolonged duration results in a time-dependent increase in cardiomyocyte death, particularly in the peri-infarct zones of the injured myocardium, and thus, restoration of blood flow plays a crucial role in cardiomyocyte protection and prevention of further deterioration in heart function (Lucchesi, Werns et al. 1989). With progression of cardiomyocyte death and tissue degradation, there is increased activity of the complement system, production of chemotactic factors, and a pro-inflammatory cytokine storm. All these events mediate propagation of post-MI inflammation, which is an essential element to replace the nonfunctional injured myocardium with scar of fibrotic nature to maintain the integrity of the heart (Romson, Hook et al. 1983). Following the initial heightened inflammatory response, a phase characterized by inflammation resolution starts. This phase includes removal of damaged tissue and dead cells, inhibition of inflammatory mediators, activation of healing mechanisms, and induction matrix turnover (Frangogiannis 2012). However, the detrimental effects of the initial inflammatory phase may persist (days) even when inflammation has been resolved (Lucchesi, Werns et al. 1989).

Data from clinical studies suggests that elevated levels of inflammatory cells and chemokines in MI patients increase the likelihood of developing impaired cardiac recovery and poor clinical outcome on long-term follow up. The profound recruitment of inflammatory cells to the ischemic heart forms the background to conclude that post-MI inflammation contributes to further infarct expansion after the initial insult (Frangogiannis 2014). Moreover, prolonged presence of pro-inflammatory macrophages (M1) in the damaged myocardium may result in infarct expansion (ter Horst, Hakimzadeh et al. 2015). Indeed, high numbers of inflammatory cells in the heart negatively relate to the degree of myocardial salvage (Maekawa, Anzai et al. 2002, Tsujioka, Imanishi et al. 2009). Therefore, timely resolution of inflammation is critical in infarct expansion prevention and reduction of adverse cardiac remodeling. We note that AZM mediates an earlier resolution of post-MI inflammation and enhanced healing, as demonstrated by phenotypic cell analyses of immune cells and gene expression of inflammatory mediators, inducing better protection against post-MI complications.

4.2.1 Anti-inflammatory therapies in MI

The major contributing mechanism to the pathophysiology of MI is the inflammatory response (Frangogiannis 2014). The inflammatory pathways primarily regulate heart healing and remodeling after infarction. Literature suggests that the involvement of the inflammatory response in the pathogenesis of cardiac remodeling may include the following mechanisms: a) damaged matrix proteins via cytokine-induced MMP activation diminish tensile strength of the injured myocardium, accompanied with slippage of myocytes and loss of stimuli that supports cardiomyocyte function; b) a prolonged uncontrolled inflammatory reaction could activate apoptosis in surviving cardiomyocytes, forming new jeopardized regions (Frangogiannis 2014). Therefore, therapeutic interventions targeting inflammation represent attractive strategies after infarction (Frangogiannis 2014). However, multiple clinical trials fail to demonstrate the effectiveness of traditional anti-inflammatory drugs such as corticosteroids, cyclosporine, and non-steroidal anti-inflammatory drugs in treating patients with

MI. (Bulkley and Roberts 1974, Akgur, Brown et al. 2000, Schjerning Olsen, Fosbol et al. 2011).

Furthermore, inhibition of other inflammatory mediators (integrin, PI3K, and C5) was not successful in clinical trials, despite success in animal experiments (Frangogiannis 2014, Seropian, Toldo et al. 2014). More selective targeting of inflammatory mediators such as IL-1 β showed some promise in improving clinical outcomes in high-risk patients with ischemic heart disease, albeit with high cost and modest benefits (Seropian, Toldo et al. 2014). Blocking of pro-inflammatory signals such as IL-1 and MCP-1 could add benefits in patients who have prolonged post-MI inflammation (Christia and Frangogiannis 2013). However, these venues have not been tested in large clinical trials of post-MI patients (Hartman, Groot et al. 2018). Heart failure has a complex pathophysiology; hence it is unlikely to be reversed by the manipulation of one receptor, cytokine or pathway (Hartman, Groot et al. 2018). It is important to mention that some studies targeting cytokines and/or their receptors yielded contradictory results due to limitations in these kinds of studies (Hartman, Groot et al. 2018). Limitations in translating pre-clinical studies are discussed in more detail later.

Taken together, targeting inflammation after MI has proven an elusive goal in clinical trials and there is unmet need for clinically relevant therapies. The exacerbated inflammatory response after myocardial injury creates difficult challenges to develop therapies that govern this response without altering its beneficial effects, mainly clearing dead cells and tissues and preparing the myocardium for the healing phase. Modulation of the inflammatory response at the cellular level, instead of its complete suppression, may represent a promising therapeutic avenue in treating MI as demonstrated by multiple studies (de Couto, Liu et al. 2015, Kain, Liu et al. 2017) (will be discussed in more detail in modulation of the inflammatory response). However, limited data exists on similar approaches with proven safety in humans. Here, we observed that AZM is successful in reducing the intensity and duration of the inflammatory phase and inducing an earlier transition to the reparatory phase through reprogramming of, rather than by a profound suppression of, post-MI inflammation. Our findings show that AZM

reduces the expression of pro-inflammatory cytokines and M1 markers and simultaneously raises anti-inflammatory genes and M2 markers. Changes in pro-inflammatory and anti-inflammatory genes are consistent between heart and blood, suggesting systemic immunomodulatory actions. Interestingly, AZM substantially reduces the expression of IL-1 and MCP-1, which is an attractive therapeutic aim in clinical practice. In addition, repurposing AZM is more clinically relevant and can be a safe and cost-effective immunotherapy for post-MI patients.

In another direction, it could be an interesting avenue to examine the effect of AZM on immune response post-MI in MCP-1 and/or IL-1 knock out mice in order to explore mechanisms of these modified immune cells' recruitment to the injured myocardium. These experiments can be potentially extended to include more chemokines and cytokines such as IL-6 knock out mice, since IL-6 is involved in trafficking of inflammatory cells in the first day after infarction (de Couto, Liu et al. 2015).

Studies confirm a second inflammation phase peaking 8 days after the initial inflammatory response but limited data has been published about the specifics of this phase (Moro, Jouan et al. 2007). The etiology of the second wave of inflammatory cells and cytokines has not been studied but could be related, at least in part, to wall stress caused by adverse remodeling of the left ventricle. Therefore, the therapeutic utility of AZM in the second inflammatory wave after MI warrants further examination.

4.2.2 Modulation of the post-MI inflammatory response

Early activation of inflammatory cells and production of cytokines/chemokines are important for removing tissue debris and dead cells, as well as for successful initiation of the subsequent reparative response (Frangogiannis 2014). Uncontrolled and/or extended pro-inflammatory cascades are associated with more severe damage, worsened cardiac remodeling, and impaired cardiac recovery (Frangogiannis 2014). Even though the role of inflammation in the progression of cardiomyocyte death is controversial,

involvement of the inflammatory and fibrotic cascades in adverse cardiac remodeling and the ensuing heart failure is well-established (Frangogiannis 2014).

Macrophages are essential players in inflammation and fibrotic processes post-MI (Chen and Frangogiannis 2017). Macrophages mediate cardioprotection through their phagocytic properties, suppression of pro-inflammatory cascades, promotion of cytoprotective signaling, activation of reparative fibroblasts and angiogenic cells, and stimulation of regenerative pathways (Chen and Frangogiannis 2016). Furthermore, macrophages may enhance cardiomyocyte survival through production of myeloid-derived growth factor (MYDGF) (Korf-Klingebiel, Reboll et al. 2015). Considering the activation state of macrophages, the reparative phenotype (M2) is the most abundant phenotype in the heart during post-MI healing. Animal experiments show that early intervention aimed at altering the macrophage activation state or shifting the balance towards the anti-inflammatory phenotype can substantially reduce the risk of heart failure development (de Couto, Liu et al. 2015, Kain, Liu et al. 2017). Kain et al. (need year) initiated a phenotypic switch in macrophages after myocardial injury by using either free or encapsulated 15-epi-Lipoxin A4. They demonstrated upregulation of reparative macrophages, as well as anti-inflammatory/macrophage modulatory genes (Mrc-1, Ym-1, Arg-1). Furthermore, cardiac function improved in 15-epi-Lipoxin A4 treated mice compared to controls (Kain, Liu et al. 2017). Multiple studies confirmed these findings using different therapeutic approaches, such as cardiosphere-derived cells (de Couto, Liu et al. 2015) and endogenous Annexin-A1 (Qin, Finlayson et al. 2017). However, previous studies on alternative macrophage activation in MI utilized experimental therapeutics that had limited safety data in humans. Conversely, our strategy of using AZM is more clinically relevant and translatable to clinical trials since AZM is already approved and widely used in humans.

In this work, we used AZM for the first time in the setting of MI to enhance inflammation resolution via repolarization of macrophages towards the reparative phenotype. We demonstrated that inflammatory macrophages significantly decrease with AZM treatments, while reparatory cells increase. Inflammatory

neutrophils also decrease with AZM treatments. Furthermore, infiltration of inflammatory monocytes to the heart are significantly reduced with AZM treatment, while anti-inflammatory monocytes are increased. Immune cell modulation is associated with significant downregulation in pro-inflammatory genes (TNF- α , IL-1 β , MCP-1, and IL-6), and upregulation of anti-inflammatory genes (TGF β and IL-10). Therefore, AZM treatment is associated with a global trend of shifting immune cells to the reparatory state. These results are in agreement with the observed immunomodulatory effects of AZM in spinal cord injury and lung infection (Feola, Garvy et al. 2010, Zhang, Bailey et al. 2015). The immunomodulatory effects of AZM translate into reduced cardiac cell death and scar size, and enhanced angiogenesis. Finally, these outcomes reduce adverse cardiac remodeling and, thus, improve cardiac function. This is early evidence of a promising and clinically-relevant approach to modulate post-MI inflammation via the repolarization of macrophage phenotypes. In addition, AZM has an excellent safety profile and is approved for use in humans. All these specifics make it an ideal agent for novel and effective therapeutic interventions to improve survival and reduce heart failure following MI in humans.

4.2.3 Resolution of inflammation through phagocytosis of apoptotic cells

Timely resolution of inflammatory signals is a prerequisite for successful initiation of healing and cardiac recovery. Phagocytosis of apoptotic cells is an essential component in suppressing these signals (Frangogiannis 2015). It has been shown that cardiomyocyte efferocytosis markedly reduces post-MI inflammation (Wan, Yeap et al. 2013), and inhibition of this process produces an extended inflammatory response and more severe adverse cardiac remodeling (Frangogiannis 2015). It is also reported that macrophage myeloid-epithelial-reproductive tyrosine kinase (MERTK) in cardiac macrophages is an important mediator for the efficient removal of apoptotic cardiomyocytes (Maekawa, Anzai et al. 2002).

Neutrophils are extensively recruited to the injured myocardium, and are then cleared by apoptosis. There is a well-established relationship between

reduced myocardial neutrophil infiltration and rescue of the heart from ischemic injury (Hansen 1995). However, the use of neutrophils as a therapeutic target to reduce adverse cardiac remodeling after MI is controversial. On one hand, inefficient clearance of neutrophils post-MI leads to matrix breakdown, collagen malposition, and increased risk of cardiac rupture (Lorchner, Poling et al. 2015). On the other hand, complete or near complete neutrophil depletion after MI is detrimental and is associated with poor healing and cardiac rupture (Horckmans, Ring et al. 2017). Apoptosis is much less significant than necrosis post-MI as shown in clinical studies, which is the proposed mechanism of uncontrolled post-MI inflammation, causing further tissue destruction (Garlichs, Eskafi et al. 2004). Although the effect of apoptotic neutrophil clearance on initiating the anti-inflammatory phase was not investigated, timely removal of infiltrated immune cells is necessary for post-MI inflammation resolution (Frangogiannis 2012).

Here, we show that AZM treatment results in a statistically significant decrease in neutrophil counts. We postulate that this reduction is related to a reduced neutrophil lifespan since macrolides can stimulate programmed cell death in activated neutrophils (Inamura, Ohta et al. 2000). Interestingly, we find that AZM enhances apoptosis in neutrophils after infarction. These findings suggest a potential mechanism to modulate macrophages and to alter the inflammatory response after MI. Engulfment of apoptotic neutrophils by macrophages stimulates the anti-inflammatory machinery by blockade of pro-inflammatory cytokine production and generation of IL-10, TGF- β , and pro-resolving lipid mediators (Horckmans, Ring et al. 2017).

We are planning future *in vitro* studies to explore the interaction between neutrophils and macrophage polarization. These studies include:

a) Treating stimulated macrophages, pre-exposed to AZM or vehicle, with conditional media from stimulated neutrophils, also pre-exposed to AZM or vehicle, then examining M1 and M2 macrophage markers. These experiments explore whether neutrophils produce certain mediators that influence the activation state of macrophages.

b) Co-culturing macrophages with neutrophils pre-exposed to AZM to examine neutrophil apoptosis and macrophage phenotype. This approach could determine whether AZM directly affects promotion of neutrophil apoptosis, and whether the expected apoptotic effect drives macrophage polarization to the alternative activation state.

Additionally, we can test the relationship between neutrophils and macrophages *in vivo*. These experiments could include:

a) Depleting neutrophils in groups of mice, except a group treated with AZM, then resupplying them with neutrophils treated with AZM after MI induction to examine macrophage polarization. This offers a tool to test whether resorption of neutrophils, either apoptotic or not (control), regulates macrophage polarization and cardiac recovery. There are limitations to this study since neutrophil depletion is associated with undesirable effects on cardiac recovery, as mentioned earlier, which could complicate any analysis.

b) We can test the role of AZM-induced neutrophil apoptosis by examining the effect of AZM in MPO knock out mice since these mice have a depleted ability for induction of programmed cell death in infiltrated neutrophils (Milla, Yang et al. 2004). It would be of interest if these studies were repeated in an I/R model since the role of neutrophils in MI pathogenesis in this model is more profound (Baxter 2002).

Research in an ischemic stroke model shows that preferentially increased numbers of N2 neutrophils induced by using the PPAR γ agonist, rosiglitazone, decrease the volume of brain infarction (Cuartero, Ballesteros et al. 2013). Increment of N2 and/or decrement of N1 neutrophil count may be a promising therapeutic strategy in MI patients (Ma, Yabluchanskiy et al. 2016). We find that AZM profoundly reduces N1 (pro-inflammatory) neutrophils, which could represent a novel therapeutic avenue in treatment of MI patients who experience prolonged

neutrophilia after infarction. This feature is likely to have wider therapeutic applications in other inflammatory diseases in which neutrophils are key players. Moreover, N1 neutrophil and M1 macrophage states could be activated by linked mechanisms. It is also likely that reduction in N1 neutrophils relates to increased protection against cardiomyocyte death. It is also highly possible that the enhanced apoptosis by AZM is specific for N1 neutrophils, suggesting potential differences in the metabolism/fate between the N1 and N2 neutrophils, which needs more investigation.

4.3 Studies to explore the immunotherapeutic mechanisms of AZM in MI

It is important to study the underlying mechanisms of the immunotherapeutic effects of AZM. They likely represent cornerstones for a more complete understanding of MI immunopathology and roles of immune cells in this process, as well as for ultimate development of successful immunomodulatory therapies for ischemic heart disease. These potential studies include:

a) Tribbles homolog 1 (Trib1) knock out mice: Trib1 protein modulates the MAPK pathway through binding to MAPKK. These mice are unable to generate M2 macrophages after infarction, with a preserved ability to produce M1 macrophages (Mahbub, Deburghgraeve et al. 2012). We can use this model to explore whether M2 macrophages are principle mediators of the cardioprotective effects of AZM in MI. We hypothesize that AZM cardioprotection will attenuate or disappear in these mice. We can extend these experiments to examine M2 macrophage source (bone marrow derived and/or derived from cardiac resident macrophages) by generating Trib1 cardiac or bone marrow derived specific knock out mice. These experiments will help to elucidate the local, systemic, or combinatorial nature of AZM's effects.

b) Inhibition of colony stimulating factor 1 (CSF-1): CSF-1 stimulates monocyte and macrophage survival and enhances macrophage activation towards the M2 phenotype, which can be blocked by using an inhibitor (GW2580) (Leblond,

Klinkert et al. 2015). Similar to the above experiments, if the immunotherapeutic effects of AZM are modified by this antagonist, it reinforces the importance of M2 macrophages in mediating cardioprotective effects of AZM in MI. This experiment would provide additional insights into the protective mechanisms of AZM, specifically its effects on alternative macrophage polarization.

c) *Ex vivo* polarization of macrophages: Using adoptive transfer of macrophages for phenotype reconstitution can define the role of shifted macrophages by AZM and exclude the contribution of various endogenous factors like other immune cells to certain levels. This experiment can be achieved by treating bone marrow derived macrophages with AZM then transfusing them intravenously into mice, which were previously treated with clodronate-containing liposomes to deplete endogenous phagocytes and macrophages (Weisser, van Rooijen et al. 2012). Depletion of systemic macrophages could also be achieved by crossing back mice onto a CD11b-inducible diphtheria toxin (DTR) background. Although this approach is useful, it is expensive and time consuming (Weisser, van Rooijen et al. 2012).

4.4 Potential signaling pathways related to AZM effects in MI

The current understanding of molecular mechanisms mediating macrophage polarization to a specific activation state remains inadequate and requires further study. Such studies would enable us to reprogram macrophages efficiently to the reparative phenotype, prevent harmful interventions, and improve outcome after infarction. Production of cytokines, chemokines, and adhesion molecules is primarily regulated by NF- κ B and I κ B α complex (Lenardo and Baltimore 1989). Stimuli like cytokines (TNF- α , IL-1 β), ROSs, and TLR-stimulated ligands can activate the expression of NF- κ B (Frangogiannis 2006). In the steady state, NF- κ B dimers bind to an inhibitory molecule, I κ B α , and reside in the cytoplasm. In the disease state, I κ B α is phosphorylated, ubiquitinated, and then degraded by proteolytic cleavage in the proteasome. Cleavage of NF- κ B from I κ B α leads to NF- κ B activation and translocation to the nucleus, inducing transcription

of effector genes (Figure 1.2). It is suggested that NF- κ B has a primary role in initiating and maintaining the inflammatory response in an experimental model of MI (Kupatt, Habazettl et al. 1999, Frangogiannis 2006). Reduction of infarction results from *in vivo* transfer of NF- κ B decoy oligodeoxynucleotides to inhibit its activity, which suggests an important role for NF- κ B in regulating post-MI inflammation (Morishita, Sugimoto et al. 1997). It is also shown that NF- κ B depletion reduces adverse cardiac remodeling post-MI by decreasing the severity of post-MI inflammation and collagen deposition (Frantz, Hu et al. 2006). Interestingly, NF- κ B has a dual role in MI since mice with mutated I κ B α , that inhibits NF- κ B translocation to the nucleus in cardiomyocytes, had larger infarction with increased cardiomyocyte apoptosis (Misra, Haudek et al. 2003).

Unpublished data from Dr. Feola's lab clearly demonstrates that AZM inhibits the translocation of p65 to the nucleus and elevates IKK β kinase levels. Inhibited translocation is associated with suppression of negative feedback mechanisms which block IKK β kinase expression. They also show that using IKK β kinase inhibitor blocks Arg-1 expression, which infers that IKK β kinase is an important effector molecule in the signaling pathway modulated by AZM. Moreover, this study suggests that AZM inhibits STAT-1 phosphorylation, which is also dependent on IKK β kinase. Collectively, AZM modulates NF- κ B and STAT-1 pathways, connected by cross talk including IKK β kinase, which could explain the immunomodulatory and anti-inflammatory effects of AZM.

We conducted *in vitro* studies to examine the effect of hypoxia/reperfusion injury on the phenotype of J774 macrophages in the presence or absence of AZM. Following 24 hours of hypoxia and reperfusion, we noted high levels of TNF- α compared to IL-10 levels. AZM treatment shifted the cytokine production back towards the anti-inflammatory pattern with a significant increase in IL-10/TNF- α ratio. These findings demonstrate results from Dr. Feola's lab.

Based on the findings mentioned above, we can test key decision nodes that may be involved in inflammation resolution and macrophage polarization *in vivo*. The potential molecular targets in the immunomodulatory effects of AZM include:

a) NF- κ B: We can examine the effects of NF- κ B by using mice with targeted disruption of NF- κ B signaling. If the immunomodulatory properties of AZM reduce or are completely lost in these mice, it would suggest that NF- κ B is an important regulator in the molecular events induced by AZM. Moreover, a study like this also addresses whether NF- κ B mediates macrophage phenotype transition. We can extend these experiments to include adoptive transfer of immune cells from NF- κ B knock out mice to WT or vice versa to restore the immunomodulatory effects and exclude the diverse effects of NF- κ B in different cells, as discussed earlier.

b) IKK β and I κ B α : After testing the effect of NF- κ B, we can test upstream molecules in the signaling cascade of NF- κ B like IKK β and I κ B α by using mice with targeted disruption of these molecules.

d) STAT1: Based on our observation that AZM upregulates IKK β which inhibits phosphorylation of STAT1, we can determine the effect of STAT1 in the immunomodulatory properties of AZM using STAT1 knock out mice.

By the end of these studies, we can clarify roles of these molecules in repolarizing macrophages to the anti-inflammatory state and determine if they are potential mediators of AZM effects.

4.5 Liposomal AZM

Liposomal research bridges urgent intellectual gaps between clinical targeting of post-MI inflammation and the optimal drug delivery system in MI. We are the first to demonstrate the immunomodulatory and anti-inflammatory properties of liposomal AZM, which has wide therapeutic potential beyond the cardiovascular field. Our work opens doors for more advanced studies to improve outcomes with AZM or other therapeutic agents post-MI using encapsulated formulation.

In addition to the cardioprotective effects of AZM, we observe that liposomal packaging of AZM is indeed protective against the drug's conduction changes. These findings clearly imply that the liposomal dosage form of AZM is indeed protective against the drug's hemodynamic modifications, which is more likely attributed to the specific delivery of AZM to the immune cells, avoiding cardiomyocytes. It is very likely that encapsulation of AZM reduces the risk of other adverse effects related to this drug, which is something that needs to be earnestly considered in clinical practices of high risk patients.

Liposomes are modifiable in terms of the surface attached groups. PEGylation of liposomes stabilizes them and prevents their opsonization by serum proteins and phagocytosis by Kupffer cells and hepatocytes. This results in liposomes with longer half-life, and thus, more accumulation in the injured myocardium caused by blood vessel leakage in the infarct areas (Takahama, Minamino et al. 2009). Due to the dynamic and progressive nature of the injured myocardium, this strategy may exhibit protective benefits with fewer doses or even a single dose following MI, improving patient tolerance. We observe potent immunomodulatory effects with a low dose of AZM encapsulated in liposomes, so examination of the relationship between immunomodulatory properties and the dosing interval of AZM using this approach could be an interesting avenue for future studies.

Additional modulation can be achieved by attaching specific antibodies onto the surface of the liposomes (immunoliposomes), which is a potential therapeutic strategy for efficiently targeting certain kinds of cells. For instance, research shows that incorporating TLR ligands into liposomes promotes the efficacy of vaccines and the targeting of drugs (Ramirez, Iyer et al. 2014). With liposomal AZM, we can incorporate a macrophage marker (F4/80), or neutrophil marker (Ly6G) to enhance the targeted delivery of AZM, and therefore its efficacy. As a broader target, we can also use fluorescent immunoliposomes to enable monocyte/macrophage tracking to identify their distribution, function and kinetics in tissue injury. Expansion of liposomal work can include other therapeutic agents such as gene

therapy or small interfering RNA (siRNA) for targeting innovative biological pathways in resolving post-MI inflammation.

AZM was prepared previously in liposomal formulations for antibiotic purposes. It has been shown that liposomal encapsulation promotes the antibacterial activities of AZM (Oh, Nix et al. 1995). When comparing the antibacterial effects of free AZM and liposomal AZM against intracellular *M. avium* growth in macrophages, it was found that the encapsulated formulation is 41-fold more potent than free AZM. Although there is a lack of efficient assays to precisely determine intracellular concentrations of AZM with liposomal formulation, these liposomes enhance the intracellular deposition of other antibiotics such as ciprofloxacin (Oh, Nix et al. 1995). Encapsulated formulations of antibiotics promote the antibacterial effectiveness both *in vivo* and *in vitro* (Cynamon, Klemens et al. 1992, Oh, Nix et al. 1995). Our data from *in vitro* experiments implies a similar trend regarding the immunomodulatory and anti-inflammatory effects of encapsulated dosage form. We find that liposomal AZM induces a more significant shift in macrophage response towards the anti-inflammatory state than free AZM. These findings are significant because they strongly suggest that liposomal AZM not only favorably modifies pharmacokinetic characteristics, but also augments pharmacodynamic effects, which are of great importance and require further exploration. We are currently working with our collaborators to prepare biotinylated AZM that can be traced both in *in vivo* and *in vitro*.

4.6 Clinical applications

Experimental animal models do not recapitulate the heterogeneity of pathology of complicated diseases like MI and heart failure. Much less is known about the macrophage phenotypes and functions in humans under normal physiological conditions and disease states. More studies are needed to understand human macrophage function, gene expression, and other properties to improve translation from bench to the bedside (Chen and Frangogiannis 2016). Therefore, targeting monocytes as precursors of macrophages (ter Horst, Hakimzadeh et al. 2015) may be more feasible to modulate macrophage

phenotypes. Harnessing isolated monocytes from MI patients can investigate the effectiveness of immunotherapies such as AZM and determine the destiny of monocytes after infiltration to the injured myocardium.

We demonstrate that AZM is a potent immunomodulatory agent of the exaggerated post-MI inflammatory response. We find that AZM alters monocyte response in the injured heart via shifting the balance towards anti-inflammatory monocytes. Similar findings are simultaneously observed in peripheral blood. We also find that AZM treatment reduces neutrophil counts. Moreover, AZM significantly suppresses pro-inflammatory cytokine and chemokine levels in plasma. These effects are important from a clinical prospective as they provide an effective tool to track the immuno-dynamic characteristics of AZM and its related cardioprotective properties in humans. Besides these effects, we observe a robust decrease in M1 macrophages with AZM therapy, suggesting additional advantage for considering AZM as immunotherapy in MI patients, who are mostly elderly. Research shows that M1 macrophages increase with age, while M2 macrophages show a decrease. Reducing M1 activation state may be a promising therapeutic target for MI patients.

4.7 AZM and oxidative stress in MI

ROS generation is increased in cardiovascular diseases such as MI, in particular after reperfusion, through enhanced expression of cytokines and growth factors. ROSs promote production and activity of proteases that control ECM breakdown and turnover and induce cardiomyocyte and blood vessel cell death (Frangogiannis 2018). Free radical scavengers which reduce ROSs, such as enzymes superoxide dismutase and catalase, show beneficial effects in reducing scar size (Jolly, Kane et al. 1984). Cytokine-related MAPK activation and stress-responsive protein kinases, at least partially induced by redox, participate in activation master transcription factors like NF- κ B and AP-1, which induce MMP transcription (Siwik and Colucci 2004).

Macrolides, in general, reduce production of free radicals, in addition to their anti-inflammatory effects. AZM downregulates the expression and activity of GST

by up to 40% in cystic fibrosis cell lines (IB3-1 and 2CFSMEo cells). The anti-inflammatory mediator, IL-10, also decreases GST activity to a similar extent, indicating a possible link between antioxidant and anti-inflammatory pathways (Zarogoulidis, Papanas et al. 2012). Studies show that AZM modifies MAPK and AP-1 cascades (Kano and Rubin 2010). Moreover, unpublished data from Dr. Feola's lab shows that AZM reduces NF- κ B activation through decrease of the transcription factor translocation to the nucleus, which could indicate that AZM inhibits NF- κ B activity via ROS suppression. We found that AZM significantly upregulates IL-10 expression after infarction. This evidence implies that in addition to its immunomodulatory effects, AZM could exhibit novel antioxidant properties contributing to its cardioprotective effects. As such, these effects can be assessed in an I/R injury model where ROSs play a central role in the pathophysiology of MI. Using AZM alone or in combination with ROS scavengers is an important approach for future myocardial preservation studies.

4.8 AZM and age impact on ischemic heart disease

A linear elevation in M1 macrophages and reduction in M2 macrophages in the heart is reported with age (Ma, Chiao et al. 2015). It seems that there are multiple factors mediating this transition, since stimulating bone marrow derived macrophages with different stimuli *in vitro* produces both phenotypes, suggesting no defect in macrophage polarization with age (Mahbub, Deburghgraeve et al. 2012). Proposed theories as to why the M1 macrophage phenotype dominates with age include over-recruitment of monocytes to the heart and alterations in cardiac resident macrophage behavior (Ma, Mouton et al. 2018). We observe that M2 macrophages dominate the heart after infarction with AZM treatment, as justified by the significant reduction of M1/M2 macrophage ratio. Therefore, AZM may be more effective in aged mice due to the decrement of M2 and the abundance of M1 macrophages. This expected benefit is especially important clinically since most MI patients are elderly and they experience exaggerated cardiac damage with MI. Thus, future experiments should test the

immunomodulatory efficacy of AZM in aged animals with MI injury to increase the clinical relevance of this work.

4.9 AZM and regeneration post-MI

There is a well-recognized regenerative capability in the heart during the neonatal life stage, which ends 7 days after birth (Inamura, Ohta et al. 2000, Wan, Yeap et al. 2013). This regenerative capacity is completely disturbed in the absence of macrophages (Aurora, Porrello et al. 2014). Important questions that must be addressed are as follows: (a) Are macrophages solely responsible cell effectors or are other immune cells involved? (b) What is the optimal activation state of macrophages to induce regeneration? (c) What is the best way to modulate macrophage activation to enhance regeneration considering the plasticity of macrophages? AZM administration could address these questions through design of *in vivo* and *in vitro* experiments. Examining changes in the progenitor cells with AZM administration in multiple organs such as BM, blood, and heart may be the first step in determining whether AZM influences the cardiac regenerative capacity post-MI. Markers such as c-kit and Ki-67 can be used to identify stem cells or regeneration, respectively. *In vivo* experiments with AZM in neonatal mice may be useful in examining the mechanisms involved in heart renewal. Additionally, *in vitro* experiments such as treating cardiomyocytes with AZM, then challenging them may highlight whether AZM has a direct effect in enhancing cardiomyocyte survival. In this regard, treating cardiomyocytes with conditional media from macrophages incubated with AZM, or co-culturing them with these macrophages may also be elucidating experiments.

4.10 Advanced research techniques

State-of-the-art technology can provide answers to important questions related to the immunopathology post-MI and to the role of AZM in MI. These are some of these technologies:

a) Histo-cytometry: This technique unites immunohistochemistry with cytometry software to determine spatially different cell populations in tissues (Deniset and Kubes 2016). Although we used Celigo in our experiments to determine the co-localization of immune cells with liposomes, it is likely that this technique could provide more insights about this interaction such as whether these liposomes are located inside or outside the cells, or whether they remain separate.

b) Next-generation sequencing: This technique depends on high-throughput sequencing platforms able to generate information about cell-specific transcriptome and gene regulation profiles (Deniset and Kubes 2016). Relative contribution of various immune cells in heart-injury-triggered inflammation requires further exploration. We can isolate inflammatory cells (macrophages, monocytes, and neutrophils) from the heart post-MI and determine the gene profile for each cell type and the effect of AZM on the cell activation state. These approaches will address novel roles for these cells in the pathogenesis of adverse cardiac remodeling and heart failure, possible cardioprotective mechanisms, and potential therapeutic targets for myocardial preservation after MI.

c) Mass cytometry: In this technique, cells are labeled with heavy metal conjugated antibodies and then run through a mass spectrometer. There is no need for compensation and it can deal with larger panel sizes of antibodies compared to conventional flow cytometry techniques (Deniset and Kubes 2016). This technique could be beneficial in determining the multiple activation states of immune cells (macrophages, monocytes, and neutrophils) with and without AZM therapy by adding more markers. It is also possible to track whether AZM influences the activity of progenitor cells, which could explain changes in the infiltration of immune cells to the heart post-MI.

d) Fate-mapping and cell-specific loss-of-function approaches: These studies could be useful in examining whether AZM-induces repolarization of macrophages from one state to another or whether its actions are due to

downregulation of one activation state (M1) and/or upregulation of other activation states (M2).

4.11 Repurposing AZM

The National Center for Advancing Translational Sciences defines the repurposing process for drugs equates to discovering new clinical benefits of drugs that are already approved to treat other diseases or conditions with proven safety record in humans. It is estimated that 30% of new drugs and vaccines approved by the US FDA in recent years are attributed to the process of drug repurposing, repositioning, and rescuing (Agranat, Caner et al. 2002). This process offers many advantages including cost and time saving from circumventing the extensive testing typically required for newly developed drugs. Since these drugs are approved for human use, the well-characterized properties (pharmacology, toxicology, and formulation) of these candidates allow for a quick therapeutic switch to novel clinical use. The estimated cost associated with the development of a new drug is over \$2 billion, and the timeline typically involves more than 12 - 16 years. In contrast, a repurposed drug can be approved in a shorter time frame, 3- 12 years, with an average cost of \$300 million (DiMasi and Grabowski 2007). Due to extensive financial and time commitments required for new drug development, repurposing an old drug to a new market with a relatively low cost is very attractive. In addition, approval rates for new drugs are extremely low, typically close to 10% while the approval of therapeutically repurposed drugs is typically triple that figure. Finally, the process of repurposing drugs represents substantial assistance for low-income patients. This increases the significance of our work since AZM has a proven safety record in humans and could be beneficial in the management of millions of patients with acute myocardial infarction worldwide.

4.12 Limitations in preclinical animal studies

In preclinical studies, enthusiasm for the role of inflammation in various pathological conditions, and the large body of positive evidence of animal studies could be misguided (Frangogiannis 2014). Negative experiments are often unpublished, creating selection bias, which results in unrealistic expectations from new therapies. Targeting the inflammatory pathways using animals with certain genetic modifications or specific inhibitors has examined the role of inflammation in the progression of myocyte death. However, there is still controversy since positive studies are contradicted by others that fail to demonstrate reduction in infarct expansion and disappointing clinical trials. These opposite observations could be attributed to an overstated role for the inflammatory response in MI, or methodological variability in study design and data analysis/interpretation. For instance, MI in young mice is often associated with a profound inflammatory response that is not particularly observed in older mice (Frangogiannis 2014). Furthermore, variability in collateral blood flow and post-MI metabolic rate significantly impacts scar size (Ito, Tate et al. 1987). It is also important to note that the inconsistency observed in these studies is related to the different MI experimental designs (permanent ligation vs. I/R). It is assumed that I/R provokes a stronger inflammatory response (Moro, Jouan et al. 2007). Using large animals like pigs or dogs could reduce this variability in experimental MI models compared to rodents but one must consider the cost and logistic demands as well (Verdouw, van den Doel et al. 1998).

Patients with MI are a heterogeneous population, adding another layer of complexity for direct clinical translation of successful strategies in animal studies (Frangogiannis 2014). Most MI patients suffer comorbidities (diabetes, hypertension, and hyperlipidemia), are typically older and exhibit a wide range of pre-existing pharmacological treatments. All these factors could markedly change the inflammatory and reparative stages post-MI. These variable factors make the clinical relevancy of animal models challenging and problematic (Frangogiannis 2014).

However, experimental MI models in mice remain tremendously useful to study cellular and molecular mechanisms controlling the pathophysiology of MI (Frangogiannis 2014), and to explore new therapeutic interventions. This model provides a way to assess both acute and long-term benefits of novel therapeutic approaches after MI. Recent advancements in genetic and surgical interventions of murine models of cardiovascular disease have significantly reduced variability and provide a more complete understanding of cardiovascular response to ischemic injury, making it a beneficial tool in predicting the influence of various factors in human heart function. Furthermore, principal similarities of the immune response initiated by MI between mice and humans (Tahto, Jadric et al. 2017) make animal studies extremely helpful in appropriately determining the role of inflammation in acute injury and its subsequent complications.

In this work, we used AZM in a murine MI model as a means to modulate intense inflammation and improve cardiac recovery after infarction. The finding that free/targeted liposomal AZM therapy is effective in moderating cardiac immunopathology of ischemic cardiac injury may be very important to clinical practice. Our work with AZM in mice allowed us to test multiple protocols, doses, controls, and dosage forms to identify immunomodulatory and cardioprotective effects of the drug in MI. To enhance clinical translation of our work, it would be quite interesting to explore the cardioprotective effect of AZM in ApoE^{-/-} mice that show a prolonged inflammatory phase and a trend towards hyperlipidemia similar to that of humans (Panizzi, Swirski et al. 2010). Additionally, we can treat these mice with high fat diet, to represent the pathogenesis of human atherosclerotic patients, and then subject them to MI injury. In another but rather complementary direction, these initial results in small animals can be confirmed in a large animal model that more closely resembles the human heart function, anatomy, and genetic conservativeness (Heidt, Courties et al. 2014). Nevertheless, it is also important to consider the economic, ethical and logistic limitations with large animals as they are more expensive to buy and maintain compared to smaller rodents (Chen and Frangogiannis 2017).

4.13 Concluding remarks

The work of this dissertation reveals novel findings, which are summarized in the following points:

- a)** We found that AZM (free and liposomal formulations) reduces cardiac inflammation and improves adverse cardiac remodeling after infarction via shifting immune cell response towards the reparative state.
- b)** This work is early evidence of a novel and clinically relevant immunotherapy using AZM for ischemic heart disease, which could be applicable for other sterile inflammatory diseases.
- c)** This is the first study to demonstrate the immune modulatory properties of liposomal AZM, which are previously unexamined and have potential wide therapeutic applications beyond the cardiovascular field.
- d)** This the first study to clearly demonstrate that the liposomal dosage form of AZM is indeed protective against the drug adverse effects such as hemodynamic modifications, which is a truly important consideration in clinical practices of high risk patients.

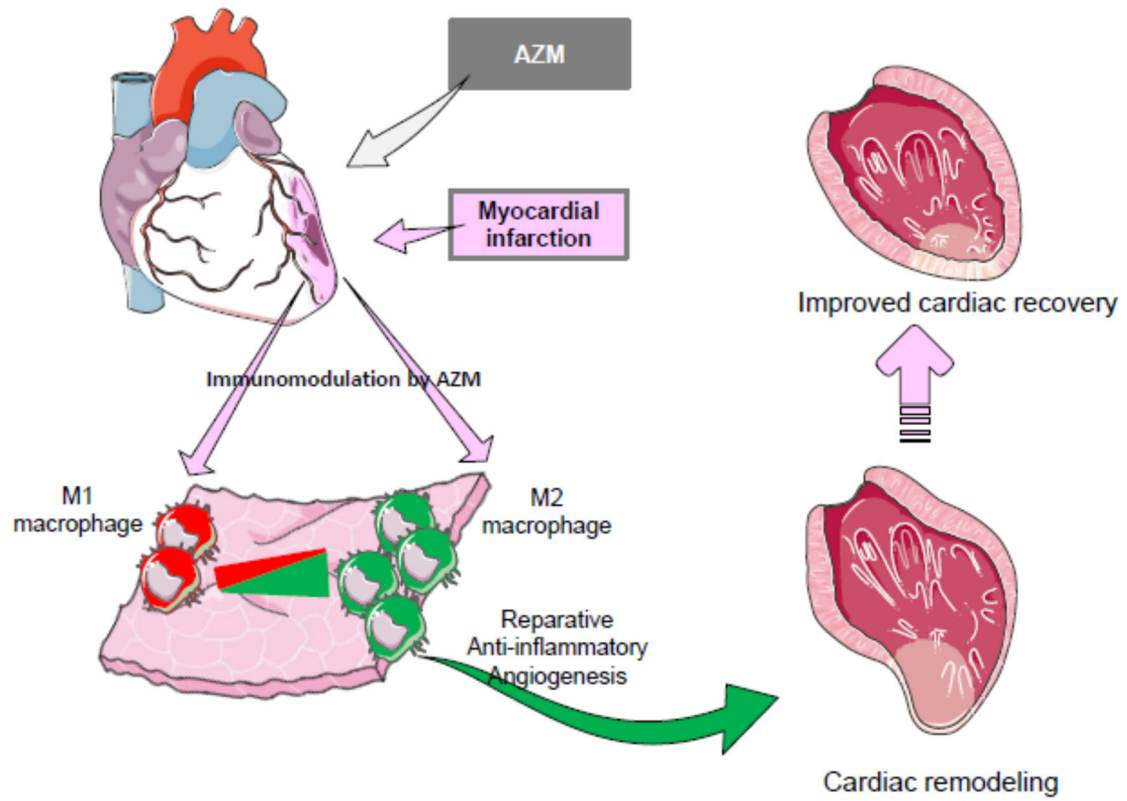


Figure 4.1 Illustration of the immunotherapeutic effects of AZM in MI.

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