The Macrophage in Cardiac Homeostasis and Disease: *JACC* Macrophage in CVD Series

Kory J. Lavine, MD, PhD[1,2,3,4,*], **Alexander R. Pinto, PhD**[5,*], **Slava Epelman, MD, PhD**[6,7,8,9,10], **Benjamin J Kopecky, MD, PhD**[1,2], **Xavier Clemente-Casares, PhD**[6,9], **James Godwin, PhD**[5,11], **Nadia Rosenthal, PhD**[5,12], and **Jason C. Kovacic, MD, PhD**[13]

[1]Division of Cardiovascular Medicine, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri

[2]Center for Cardiovascular Research, Cardiovascular Division, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri

[3]Department of Developmental Biology Washington University School of Medicine, St. Louis, Missouri

[4]Department of Immunology and Pathology, Washington University School of Medicine, St. Louis, Missouri

[5]The Jackson Laboratory, Bar Harbor, Maine

[6]Toronto General Hospital Research Institute, University Health Network (UHN), Toronto, Ontario, Canada.

[7]Ted Rogers Centre for Heart Research, Toronto, Ontario, Canada

[8]University of Toronto, Department of Laboratory Medicine and Pathobiology, Toronto, Ontario, Canada

[9]University of Toronto, Department of Immunology, Toronto, Ontario, Canada

[10]Peter Munk Cardiac Centre, Toronto, Ontario, Canada

[11]Mt. Desert Island Biological Laboratory, Bar Harbor, Maine

[12]National Heart and Lung Institute, Imperial College London, London, United Kingdom

[13]Zena and Michael A. Wiener Cardiovascular Institute, Icahn School of Medicine at Mount Sinai, New York, New York

Abstract

Corresponding author: Jason C Kovacic, MD, PhD, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1030, New York, New York 10029, Telephone: 646.593.2552, Fax: 212-534-2845, jason.kovacic@mountsinai.org, Twitter: @MountSinaiTwitter | @IcahnMountSinai.
*Co-first Authorship

Macrophages are integral components of cardiac tissue and exert profound effects on the healthy and diseased heart. Paradigm shifting studies employing advanced molecular techniques have revealed significant complexity within these macrophage populations that reside in the heart. In this final of a 4-part review series covering the macrophage in cardiovascular disease, we review the origins, dynamics, cell surface markers, and respective functions of each cardiac macrophage subset identified to date, including in the specific scenarios of myocarditis and after myocardial infarction. Looking ahead, a deeper understanding of the diverse and often dichotomous functions of cardiac macrophages will be essential for the development of targeted therapies to mitigate injury and orchestrate recovery of the diseased heart. Moreover, as macrophages are critical for cardiac healing, they are an emerging focus for therapeutic strategies aimed at minimizing cardiomyocyte death, ameliorating pathological cardiac remodeling, and for treating heart failure and after myocardial infarction.

Condensed Abstract:

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Keywords

Macrophage; Ontogeny; Heart Development; Coronary Angiogenesis; Heart Failure

In this review series, we have covered the basic macrophage biology in Part 1, macrophage biology in atherosclerosis in Part 2, and macrophage biology and imaging at the systemic level in Part 3. Here in Part 4 we address the role of the macrophage in the heart, both in normal homeostasis, and in myocarditis and myocardial infarction (MI).

Cardiac Macrophages in Health and Homeostasis

Introduction

Heart failure is an important cause of morbidity and mortality, and in parallel, it is recognized that innate immune system activation occurs in patients with heart failure, and is associated with adverse clinical outcomes (see review (1)). An important question which then arises is whether the immune system is directly responsible for disease progression, including pathological remodeling of the left ventricle (LV).

To that point, while it is accepted that neutrophils produce robust inflammatory responses and contribute to heart failure after acute ischemic injury (2–4), the exact roles of monocytes and macrophages continue to be debated. The literature is filled with seemingly contradictory reports that macrophages not only trigger damaging inflammatory responses,

but also, that they mediate tissue repair and in some cases cardiac regeneration (5). This paradox is well established in models of ischemic cardiac injury, where macrophages in the post-MI heart drive robust inflammatory responses and pathological remodeling, as well as the resolution of inflammation, tissue repair, and coronary angiogenesis (6). One explanation is that distinct macrophage populations may mediate inflammatory and reparative macrophage behaviors (7). However, until recently, the exact identities of these proposed macrophage subsets were largely undefined.

In this review, we consider recent progress in elucidating macrophage populations resident within the heart, and discuss the functional relevance of these subsets, particularly as relevant to heart development, homeostasis, inflammation, myocarditis and after MI.

Macrophage Heterogeneity and Diversity

As highlighted throughout this review series, macrophages are found in all tissues and play important roles during development, adult physiologic homeostasis, tissue repair and remodeling, and immunity. However, as a challenge faced by the field, prior classification systems and definitions that rely on cell surface markers and inflammatory states (i.e., M1-M2) are increasingly understood to be imperfect, primarily because macrophages display significant plasticity and dynamic expression of cell surface and M1/M2 markers (see Part 1 review article). Furthermore, this heterogeneity has implications for the translation of studies across species. For example, the human equivalent to Ly6c^{high} monocytes was previously considered to be wCD14$^+$CD16$^-$ monocytes, while the human equivalent to Ly6c^{low} monocytes was thought to be CD14^{int}CD16$^+$ monocytes. However, as discussed in Part 1 of this series, studies using advanced profiling techniques have questioned this dogma, and the human equivalents of these populations remain to be clearly defined.

An alternative approach is to define macrophage subsets based on ontogeny or developmental lineage. Thus, recent studies have shown that many tissue-resident macrophages are established during embryonic development and persist into adulthood independent of blood monocyte input (8–11). Specifically, definitive macrophages are subdivided into embryonic (usually fetal liver) and adult-derived subsets, both of which transition through monocyte intermediates and express FLT3 as they differentiate (8,12). Based on these findings, a *Flt3-Cre* mouse was developed to track the progeny of definitive hematopoietic lineages (13). Using this lineage tracking tool, several studies identified primitive (*Flt3-Cre* negative) and definitive (*Flt3-Cre* positive) macrophages in a variety of tissues including the heart (8,12).

In addition to lineage tracking, transcription factors and surface markers may differ between macrophage lineages. For example, yolk sac-derived macrophages have a characteristic CX3CR1^{high}F4/80^{high} CD11b^{low} phenotype, while definitive monocyte-derived macrophages display a CX3CR1^{int}F4/80^{low}CD11b^{high} phenotype (8,11,14). Collectively, these observations suggest that tissue resident macrophages are best defined by a combination of ontological origin, recruitment dynamics, and cell surface marker expression. In the following section we discuss how this approach has elucidated functionally distinct macrophage populations in the heart.

Cardiac Macrophage Populations

Resident cardiac macrophages represent 6–8% of the non-cardiomyocyte population in the healthy adult mouse heart and even larger fraction in the developing heart (15–17). Previously, it was thought that during homeostasis most cardiac macrophages were derived from circulating monocytes and represented a homogeneous population with M2 characteristics (18). Recently, it was shown that unlike other tissues such as the brain which contain a single dominant macrophage population (yolk sac-derived microglia), the heart contains several macrophage populations with discrete ontological origins including primitive yolk sac-derived macrophages, fetal monocyte-derived macrophages, and adult monocyte-derived macrophages (8,9,16). Each of these populations seeds the heart at distinct developmental stages and eventually co-exist within the adult heart.

Developing Heart

Macrophages are first evident in the mouse heart at E11.5 in association with the epicardium. These cells are derived from primitive yolk sac progenitors and are characterized by low surface expression of C-C chemokine receptor 2 (CCR2) and are referred to as CCR2$^-$ (19). They also express low levels of major histocompatibility Complex (MHC) class II. Mechanistically, yolk sac-derived CCR2$^-$ macrophages seed the heart and exist independent of monocyte input. Instead, they rely on instructive cues from the epicardium, with epicardial ablation impeding the recruitment of yolk sac-derived macrophages through an unclear signaling mechanism (20). Beginning at E13.5–14.5, yolk sac-derived CCR2$^-$ macrophages play a critical role in the development and maturation of the coronary system (described below).

Also beginning at E14.5, a population of CCR2$^+$MHC-II^{low} macrophages is recruited to the heart, and becomes associated with the endocardial surface (19). These cells are derived from definitive hematopoiesis (predominately fetal monocyte progenitors) and require monocyte input for their maintenance. The function of these cells is unknown as they appear to be dispensable for proper cardiac development.

Neonatal Heart

During the first week of life, the mouse heart contains a single yolk sac-derived CCR2$^-$ macrophage population that expands via local proliferation (21). Beginning 2 weeks after birth, a second population of CCR2$^-$ macrophages that are *Flt3-Cre$^+$* (definitive hematopoietic origin) enter the heart. This latter population is presumably derived from fetal monocytes based on their timing of entry, cell surface characteristics (CX3CR1^{int}), and developmental origin (8,9). At this time-point, both primitive and definitive CCR2$^-$ macrophages are MHC-II^{low}.

Adult Heart

During homeostasis, the adult mouse heart contains at least 3 macrophage subsets: CCR2MHC-II^{low}, CCR2$^-$MHC-II^{high}, and CCR2$^+$MHC-II^{high}. Monocytes display a CCR2$^+$MHC-II^{low} cell surface phenotype (8,9,16,21). This classification system is supported by single-cell RNA sequencing data indicating that CCR2 and MHC-II expression

are sufficient to define the major monocyte and macrophage populations within the naïve adult mouse heart (22).

CCR2⁻MHC-II^{low} and CCR2⁻MHC-II^{high} macrophages are long-lived, derived from embryonic origins including primitive yolk sac and fetal monocyte progenitors, and are maintained independent of monocyte input through local proliferation (Figure 1). The mechanisms responsible for acquisition of MHC-II^{high} expression first observed at 3–4 weeks of age are unclear. Interestingly, during aging, more substantial contributions from circulating monocytes are observed, suggesting that monocytes may differentiate into CCR2⁻ macrophages (21,23). CCR2⁺MHC-II^{high} macrophages are derived exclusively from blood monocytes and require ongoing monocyte input through a CCR2-dependent mechanism. CCR2⁺MHC-II^{high} macrophages are first evident in the heart at 3–4 weeks of age. The signaling events responsible for their initial recruitment remain unexplored.

Following acute tissue injury such as MI, a dramatic shift occurs and resident macrophages are largely replaced by infiltrating CCR2⁺ monocytes and CCR2⁺ monocytederived macrophages (Central Illustration) (7,8,16,21,23). In general, infiltrating CCR2⁺ macrophages are thought to be pro-inflammatory and contribute to adverse remodeling through collateral myocardial injury, neutrophil recruitment, and inflammatory cytokine production (21,24). However, infiltrating monocytes likely assume a variety of cell fates that are influenced by environmental cues (covered in greater detail later in this article).

Cardiac Macrophages Functions

Shared Functions

Adult cardiac macrophages share a number of typical macrophage functions including the ability to sample the local microenvironment and engulf debris through micropinocytosis, phagocytosis, and efferocytosis (8,9,16,18). In addition, a recent report has implicated cardiac macrophages in atrial-ventricular (AV) conduction (22). The authors reported abundant CCR2⁻ and CCR2⁺ macrophages within the AV node of the mouse and human heart. Within the AV node, resident cardiac macrophages appear to make direct contact with cardiomyocytes via gap junctions. Furthermore, cardiac macrophages in vitro were able to form spontaneous gap junctions with cultured cardiomyocytes and facilitated electrical conduction. In vivo, removal of cardiac resident macrophages or conditional deletion of connexin 43 in macrophages resulted in impaired AV conduction and in some instances heart block.

Functions of CCR2⁻ macrophages

Yolk sac-derived CCR2⁻ macrophages are essential mediators of coronary development (19). They are closely associated with the epicardium, epicardial-derived cells (precursors of coronary interstitial cells: pericytes and smooth muscle cells), and coronary endothelium during heart development. Removal of CCR2⁻ macrophages in the embryo results in aberrant remodeling and maturation of the coronary arterial vasculature. Within this context, primitive CCR2⁻ macrophages are selectively recruited to perfused coronary vasculature where they stimulate vessel growth and remodeling, at the expense of other vessels receiving

minimal blood supply. Mechanistically, insulin-like growth factor (IGF) 1 and IGF2 are potential mediators by which primitive CCR2− macrophages regulate coronary remodeling (19).

Yolk sac-derived CCR2− macrophages are also critical regulators of cardiac tissue repair (21). Particularly in the neonatal heart and in certain species such as zebrafish, this function may be of major importance (15,25,26), although, it remains to be elucidated whether macrophage populations necessary for tissue regeneration in zebrafish and amphibians are derived from embryonic progenitors.

The functions of CCR2− macrophage subsets within the adult heart are less clear. Compared to CCR2+MHC-IIhigh macrophages, CCR2−MHC-IIlow and CCR2−MHC-IIhigh macrophages express fewer inflammatory mediators and have less capacity for inflammatory chemokine and cytokine expression (8,9,21). These observations suggest that CCR2−MHC-IIlow and CCR2−MHC-IIhigh subsets may retain a reparative phenotype. Consistent with this, CCR2MHC-IIlow and CCR2−MHC-IIhigh macrophages express IGF1 and are pro-angiogenic (19). Furthermore, compared to CCR2+ macrophages, resident CCR2− macrophages show enhanced capacity to phagocytose cardiomyocyte content (27), likely contributing to tissue homeostasis. To date, no differences between the functions of CCR2− macrophages derived from primitive versus definitive hematopoiesis have been observed. However, among the CCR2− subsets, only CCR2−MHC-IIhigh macrophages have the capacity to display antigen and stimulate T-cell responses (8,9).

Functions of CCR2+ Macrophages

CCR2+ cardiac macrophages are primarily derived from circulating monocytes and are enriched in proinflammatory genes, in particular the NLRP3 pathway, which leads to delivery of interleukin 1β (IL-1β) (21) - a proinflammatory cytokine and therapeutic target (28). While the exact functions of resident CCR2+MHC-IIhigh macrophages under steady state conditions are incompletely defined, their activation likely represents a proximate mechanism driving inflammation in the failing adult heart. This is supported by the observation that resident CCR2+MHC-IIhigh macrophages regulate myocardial neutrophil infiltration following ischemia-reperfusion injury. Mechanistically, resident CCR2+MHC-IIhigh macrophages are activated by mitochondrial DNA (presumably released from dying cardiomyocytes) through a toll-like receptor (TLR) 9-dependent pathway that results in the release of the neutrophil chemokines, chemokine (C-X-C motif) ligand (CXCL) 2 and CXCL5 (29). Whether resident CCR2+MHC-IIhigh macrophages similarly govern monocyte recruitment and macrophage ontogeny through a shared molecular pathway is not yet clear, but represents an attractive hypothesis. Similar to CCR2−MHC-IIhigh macrophages, CCR2+MHC-IIhigh macrophages have the capacity to display antigen and stimulate T-cell responses in vitro (8,9). Whether CCR2+MHC-IIhigh macrophages do so in vivo and whether this population displays differential capacity to stimulate effector and regulatory T-cell subsets remains to be seen.

The Macrophage, Myocardial Infarction and Cardiac Regeneration

In models of mammalian wound healing, immune cells are a major force in determining the ultimate quality of repair (30). Recent research has uncovered a central role for the innate immune system in the response to cardiac tissue damage, where successful tissue regeneration depends on cellular and signaling components of local inflammatory reactions. In the uninjured heart, tissue–resident immune cell populations participate in routine immune-surveillance, contribute to angiogenesis, and participate in signaling networks that modulate the local stromal environment to dampen tissue inflammation. Why then are mammalian hearts so prone to perturbed immune reactions that lead to detrimental inflammation and scarring after injury?

Clues can be gained from the central role played by macrophages in the resolution of cardiac injury in highly regenerative scenarios. The regenerative capacity of a tissue is influenced by regulatory networks orchestrated by local immune responses to tissue damage, with macrophages being a central component of the injury response, promoting healing and tissue remodeling through secretion of anti-inflammatory and angiogenic cytokines. Furthermore, as mentioned, the capacity for full cardiac regeneration of immature mammalian hearts is critically dependent on macrophage actions: the mouse neonatal heart harbors resident cardiac macrophages that generate minimal inflammation and promote post-MI cardiac recovery, where they influence neo-vascularization (15,21).

Variation in the interaction of macrophages with damaged tissues is a likely factor in the range of regenerative potential across the animal kingdom (26). Curiously, some closely related species exhibit robust cardiac regeneration, while others undergo regenerative failure. Among adult amphibians, several divergent salamander species are capable of cardiac regeneration, while more distantly related frogs fail to regenerate and heal cardiac injury with non-functional scar tissue (31). Among bony fishes, some teleost fish including the Zebrafish, Goldfish, and Giant danio feature robust regenerative potential (32–34), but those in the closely related Medaka group fail to regenerate (35).

While the immune landscape in many of these species remains to be fully characterized, studies of macrophage profiles in models of compromised versus effective repair suggest that retaining a balanced immune response is imperative for regenerative therapies to meet their full potential in the adult human heart.

Macrophage Functions in Damaged Cardiac Tissue

Resident tissue macrophages play distinct roles in stress responses, accumulating at sites of cardiac injury within five minutes (36), likely due to movement toward areas of damage. A diverse range of additional leukocytes is subsequently recruited (21,37–39). Following infiltration of neutrophils, which peak at the injury site after one day and release reactive oxidative species, monocytes/macrophages are the predominant leukocyte type within the injured heart from days 2–5 post injury (Figure 2) (7,39,40), with cell numbers gradually reaching basal levels thereafter (7,40). Sager et al (41) shed light on the pathways underlying this recruitment, showing that IL-1β is produced by the infarcted heart and leads to bone

marrow production and release of neutrophils and Ly6c^{high} monocytes, which are then recruited to the injured heart.

In response to cardiac injury, recruited macrophages produce a sequential spectrum of cytokines, chemokines, matrix metalloproteinases, and growth factors exerting both pro- and anti- inflammatory effects. In addition, resident and monocyte-derived macrophages are decorated with an array of pattern recognition receptors that detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). DAMPs are comprised of exogenous molecular signatures such as PAMPs, or alarmins which are endogenous molecular signatures arising from tissue damage. To detect these DAMPs, macrophages express TLRs, mannose receptors, purinergic receptors and surface molecules that mediate phagocytosis, such as CD36. Following MI, macrophage pattern recognition receptors sense key alarmins such as HMGB1, ATP and other nucleotides, extracellular matrix components such as hyaluronon and fibronectin fragments, and dead cells (42–44). While much of resident macrophage activity likely involves non-phlogistic phagocytosis (phagocytosis that does not signal inflammation), the alarmins generated in the infarcted heart from apoptotic and necrotic cardiomyocytes elicit a pro-inflammatory response from both resident macrophages and infiltrating monocytes. As an example of this regulatory system and how DAMPs are critical following cardiac injury, King et al (45) have shown that MI causes the release of danger signals including double stranded DNA from dying cells. Infiltrating cells including macrophages sense the DNA, leading to activation of the transcription factor IRF3 (interferon regulatory factor 3) and subsequent induction of type I interferon production. Attenuated post-MI IRF3-dependent signaling reduced cardiac inflammation and improved cardiac function and survival, suggesting this may be a target for therapeutic cardioprotection (45).

Post-MI macrophage depletion in both neonatal and adult hearts results in impaired tissue remodeling, with decreased cardiac fibrosis and functional decrement, including cardiac thrombus formation (15,46). A key function of macrophages in the cardiac lesion is clearance of tissue debris, and in particular efferocytosis—the phagocytosis of dead cells. Recent work has demonstrated that while macrophage function goes beyond phagocytosis, efferocytosisdependent signaling is important for almost all aspects of macrophage activity in the injured heart. In multiple cardiac injury models, macrophage depletion results in the inability to clear tissue debris and dead cells including necrotic cardiomyocytes (15,46,47). The targeting of genes that support macrophage phagocytosis such as *MerTK* and *Mfge8* results in impaired tissue healing and functional decline after cardiac injury, demonstrating that tissue debris clearance is an essential aspect of healing following cardiac injury (27,48,49).

Efferocytosis after MI also stabilizes the myocardial wall to prevent wall rupture, with the loss of *SR-A*, another gene implicated in macrophage tissue clearance activity (50), increasing post-MI cardiac rupture (51). This is at least partly due to increased proteolytic activity through MMP9, which is induced by increased levels of post-MI tumor necrosis factor (TNF)-α (51,52). Indeed, MMP9 deficiency decreases the incidence of cardiac rupture (53), and also increases CD36-dependent macrophage phagocytosis (54). Macrophages also express transforming growth factor (TGF)-β, a key factor inducing

myofibroblast differentiation and myocardial fibrosis (55), which is enhanced by MerTK-dependent efferocytosis following ischemic injury (27).

Macrophages also participate in post-MI vascular bed regeneration. In this context, macrophages may physically mediate vascular anastomosis or regulate angiogenesis by paracrine means (56–58). Macrophage depletion following cardiac injury leads to a reduction in vascular endothelial growth factor A (VEGFa), and a coinciding reduction in cardiac capillary density (47). Loss of MerTK and Mfge8 results in blunted VEGFa production (48). Moreover, macrophage-restricted genetic ablation of VEGFa, in *LysM-Cre:VEGFa^{fl/fl}* mice, leads to impaired heart function and angiogenesis (48). Furthermore, VEGFa is likely to also act on nonendothelial cells. In non-cardiac models of tissue injury, VEGFa overexpression augments the recruitment of circulating monocytes to injured tissues and promotes a pro-angiogenic macrophage phenotype (59).

The regulation of transient extracellular matrix and matrix-derived signals is important in mediating cardiomyocyte proliferation in regenerative species such as salamander and zebrafish (see review (60)). In macrophage-depleted salamanders, expression of extracellular matrix components is suppressed, correlating with regenerative failure (25,61). Thus, evidence is mounting that macrophages play a critical role in regulation of the extracellular matrix microenvironment in regeneration by secretion of matrix degrading enzymes, and through interactions with local mesenchymal cells (62). However in the salamander cardiac cryo-injury model, macrophage depletion derails regeneration despite the persistence of cardiomyocyte proliferation (25). In the mouse neonate, major transcriptional shifts in the leukocyte population following MI may provide clues to the local inflammatory responses providing a permissive environment (63), however macrophage-depleted neonatal mouse hearts fail to regenerate despite normal cardiomyocyte proliferation (15). These findings indicate that current clinical efforts into promoting cardiomyocyte proliferation (reviewed in (64)) may be necessary but not sufficient, and that modulation of the macrophage-dependent stromal environment is critical for promoting effective scar-free cardiac repair.

Monocyte and Macrophage Dynamics Following Cardiac Injury

Findings in regenerative organisms such as the zebrafish and salamander implicate the timing and profile of innate immune cell recruitment to injured tissues as a critical factor in regenerative success. Salamanders require macrophages to regenerate limbs and hearts after injury (25,61) and a similar requirement is observed in zebrafish fins and heart (65,66). The timing of macrophage invasion post injury is accelerated relative to mammalian injury, featuring a rapid switch to anti-inflammatory signaling (61). Liposomal clodronate depletion of macrophages in regenerating salamanders or zebrafish structures has identified an early requirement for macrophage infiltration. When macrophage engagement is delayed, scar formation prevents tissue regeneration (25,61,65). Studies comparing the response to cardiac damage in zebrafish with Medaka, a related teleost lacking cardiac regenerative capacity (35), have revealed that the timing of inflammatory resolution may be critical for successful regeneration. These studies demonstrated that the infiltration kinetics of Medaka neutrophils, and their lack of early and effective clearance and resolution by macrophages, led to poor regeneration that could be enhanced by macrophage stimulation (65). In addition, as

reviewed in Part 2 of this review series, failure of resolution of inflammation is a key aspect leading to atherosclerosis progression. In further support of this concept, additional studies have pinpointed the regulation of inflammation via macrophages and TNFα as a key factor for zebrafish fin regeneration (67). Furthermore, yet another study highlighted the critical role of macrophages in dampening the IL-1β response to injury from epithelial cells that leads to apoptosis of regenerative progenitors (68).

While the injured mammalian heart accumulates high numbers of monocytes and macrophages, these cells are only present transiently (Figure 2). Within two hours of injury, resident macrophages begin to die (16). In contrast, infiltrating monocytes and macrophages have a cardiac residence period of ~20 hours (69). After MI, mammalian monocytes and macrophages follow a bi-phasic response to injury, transiting from a pro-inflammatory toward an anti-inflammatory phenotype at later stages (7,40) (Figure 3). In the inflammatory phase (days 1–4 post-MI), CCR2^{+}Ly6c^{high} monocytes are the dominant monocytic population and exhibit a pro-inflammatory phenotype, secreting factors such as IL-1β, IL-6 and TNFα (40). These monocytes also produce proteolytic factors and undertake extensive phagocytosis. In the reparative phase (days 5–14), monocytes differentiate to F4/80[high] macrophages, exhibit an antiinflammatory phenotype by secretion of factors such as IL-10 and TGF-β (40), and promote angiogenesis and scar formation by secretion of factors such as VEGFa in addition to TGF-β (40). Although macrophage transition from proinflammatory to a reparative phentoype has been classically defined as M1-to-M2 polarization, macrophages in the post-MI heart produce mediators that lie outside the M1/M2 spectrum, again highlighting the complexity of macrophage subtypes and their dependence on the specific stimulus/environment (70).

Origins of Monocytes and Macrophages at Cardiac Injury Sites

In the injured neonatal heart, an embryonic lineage of resident macrophages induces minimal inflammation, instead promoting cardiomyocyte proliferation and coronary angiogenesis (15,21). Conversely in the adult, in addition to local immune cell activation, cardiac stress induces a robust systemic inflammatory response (71,72), leading to an increased number of circulating monocytes that support the monocyte/macrophage demands of the injured myocardium (Figure 3). In steady state and depending upon the subset, mouse monocytes have a lifespan of 1–2 days (11,73,74). In contrast, the major human monocyte subset to infiltrate sites of injury—classical monocytes (CD14^{+}CD16^{−})—have a lifespan of ~3 days (75). The increased demand for monocytes and macrophages in adult MI is associated with increased monocyte production in the bone marrow, likely stimulated by circulating DAMPs arising from the injured myocardium (76). While other pathways are also of importance (45), a key stimulus for emigration of monocytes from the bone marrow is CCR2 signaling (77). After tissue injury or pathogen-induced inflammation, CCL2 (monocyte chemoattractant protein-1) is produced locally by a wide range of cells including endothelial cells, fibroblasts and smooth muscle cells (78). In addition, bone marrow endothelial cells produce CCL2 following MI (79), while CCR2^{+} hematopoietic progenitors proliferate and increase the production of CCR2^{+} monocytes.

The spleen also serves as an 'emergency reservoir' that may contribute up to half of all monocytes that enter the cardiac lesion. In the spleen, monocytes reside in clusters of 20–50 cells (80) and respond to angiotensin II, which increases in circulation after MI (81). Angiotensin II induces splenic monocytes to undergo chemokinesis and enter the circulation where they eventually migrate to the injured heart (81). Splenic monocytopoiesis is an important factor in maintaining this monocyte reservoir. In response to MI, splenic progenitors undergo proliferation which is dependent on IL-1β signaling (69). Inhibition of sympathetic nervous system activity by β3-aderoceptor antagonists leads to a decrease in circulating hematopoietic progenitors and splenic monocytopoiesis (71), supporting the concept that splenic monocytopoiesis is enabled by colonization of the spleen by bone marrow-derived hematopoietic progenitors (71). Following MI, sympathetic nervous system activity inhibits bone marrow expression of stem cell retention factors CXCL12, angiopoietin and stem cell factor, permitting medullary egression of progenitors (71). In contrast, there is a concomitant increase in expression of stem cell factor and vascular cell adhesion molecule (VCAM)-1 (an additional stem cell retention factor) in the spleen, to accommodate splenic engraftment and retention of stem cells (71,82). Intriguingly, retention of hematopoietic progenitors through regulation of the bone marrow stem cell niche is dependent upon macrophages (82,83).

Targeting Monocytes and Macrophages for Treating MI

A large body of experimental research has demonstrated that modulation of monocytes is beneficial for cardiac recovery (Online Table 1). Beneficial outcomes have been achieved by interdicting CCR2 signaling, either by ablating *Ccr2* or *Ccl2* genes or by targeting key inflammatory mediators supporting monocyte recruitment. Conversely, mice that have increased levels of circulating monocytes, such as in *ApoE*$^{-/-}$ mutants, have impaired cardiac recovery after MI (84). In the clinical setting, increased levels of classical monocytes (CD14$^+$CD16$^-$ monocytes) correlate with adverse outcomes following acute MI (85–91).

While no clinical trials have addressed the specific issue of whether targeting monocytes is beneficial post-MI, approaches that adventitiously impair monocyte function and their mobilization are now in routine use, specifically, the administration of angiotensin converting enzyme (ACE) inhibitors and beta-blockers. Indeed, administration of these antagonists within the first 24 hours following MI forms part of the recommended treatment guidelines for STelevation MI (92). Moreover, while many clinical trials dampening inflammation have yielded negative results (93,94), the IL-1β inhibitor Canakinumab was recently shown to reduce cardiovascular events in high risk patients with atherosclerosis (28).

These examples notwithstanding, reducing or depleting monocytes post-MI may have unintended consequences, including risk of prolonging cardiac inflammation due to insufficient clearance of tissue debris (i.e., failed resolution of inflammation). While it has not been directly proven that monocyte depletion compromises cardiovascular repair, a study of veterans who lost their spleens due to injury found that this group suffered increased mortality from ischemic heart-disease (95), suggesting that significant reduction in monocytes during the course of MI may be deleterious. Notably, monocyte depletion would

also reduce salutary macrophages that are differentiated from monocytes at later injury phases (40) and impairment of tissue macrophage phagocytic activity negatively correlates with healing after MI (96).

A more compelling case can be made for boosting anti-inflammatory macrophage numbers and activity as a potential treatment following MI, primarily because this should enhance tissue stabilization, clearance of tissue debris and cardiac healing. However, expanding the anti-inflammatory cardiac macrophage population without affecting circulating monocytes may be difficult, since increased macrophage numbers after MI are dependent on infiltrating Ly6c^{high} monocyte precursors. One approach may be to exploit the intrinsic capacity of cardiac resident macrophages to divide, both in a resting state (8,16,97) or after MI (98). An alternate approach would involve accelerating the transition of infiltrating monocytes to an antiinflammatory phenotype, using key mediators such as Nr4a1 (40) or the transcription factor IRF5 (99). The validity of this approach is supported by a recent study of neutrophil depletion in mice, which unexpectedly perturbed the functionality of M2-like macrophages in the post-MI heart, leading to increased fibrosis, worsened cardiac function and heart failure (3). Furthermore, experimental bone marrow transplantation in mice elevated levels of reparative M2-like macrophages in the heart, with enhanced cardiomyocyte survival, neovascularization and improved post-MI remodeling (100). Future therapeutic strategies will benefit from advancing our understanding of the molecular networks underpinning cardiac macrophage phenotypes and dynamics, using single-cell transcriptomic analyses, and gene editing of macrophages and their progenitors for promoting a regenerative response to cardiac injury.

Monocytes, Macrophages and Myocarditis

Myocarditis – Background

The evolutionary role of inflammation is to defend tissue against pathogens while retaining the ability to promote beneficial, reparative programs after injury. Defensive mechanisms (e.g., leukocyte recruitment, cytotoxic cell death) and changes in environmental cues (cytokine production, metabolic signals, etc.) can disrupt basic homeostatic mechanisms that govern specialized organ functions, such as within the heart, and in turn, set in motion a cascade that leads to pathological tissue remodeling and heart failure. Here we review the pathogenesis of myocarditis and the role of the cardiac macrophage in modulating heart failure development. Given that loss of all macrophages during myocarditis can lead to significant mortality (101), understanding their precise role and gaps in the literature is critical to move the field forward (Figure 4).

Infectious Myocarditis—The most common cause of myocarditis is viral infection. Indeed, several studies suggest that we are underestimating the impact of viral myocarditis and development of heart failureassociated with viral infections (102,103). For example, viral genomes can be detected within the myocardium of 35–70% of patients with dilated or chronic cardiomyopathy, suggesting that chronic cardiac disease may develop from previous myocarditis (104,105). Interestingly, while the presence of viral genomes within the myocardium associates with cardiac dysfunction, the presence of virus does not predict

progression to cardiomyopathy (104–106). Since a precise diagnosis is rarely achieved in viral myocarditis, our current clinical knowledge is largely based on the association of a prior infectious exposure (i.e. viral prodrome), unexplained LV dysfunction and correlative clinical investigations (systemic markers of inflammation and cardiac imaging).

Autoimmune Myocarditis—Autoimmune myocarditis results from dysregulated activation of innate or adaptive immunity. Some autoimmune syndromes, such as systemic lupus erythematosus or inflammatory bowel disease, can present with a cardiac component (107). Giant cell myocarditis and cardiac sarcoidosis are rare forms of inflammation-driven myocarditis with specific histological features, including the formation of multinucleated giant cells within the myocardium presumed to be macrophages (108–111).

Animal models have been developed to mimic autoimmune myocarditis, referred to as Experimental Autoimmune Myocarditis (EAM). These models are typically based on immunization with cardiac antigens and a strong adjuvant or antigen presenting cells, such as dendritic cells (DCs) loaded with cardiac antigens in order to promote an autoreactive adaptive immune response (112).

Some evidence suggests that under certain circumstances infectious agents may lead to the activation of autoreactive T cell clones that promote heart failure after full clearance of the primary infection in animal models (113,114). In clinical studies, it has been difficult to demonstrate an autoimmune component, with recent trials in viral myocarditis showing little beneficial effect of corticosteroids in the prevention of heart failure and dilated cardiomyopathy (115).

Toxic Myocarditis—The increasing clinical use of disease modifying pharmaceuticals to treat recalcitrant diseases, such as cancer or autoimmunity, has been associated with an increasing number of patients with cardiotoxicity (116,117). A wide range of substances, including heavy metals and several immunomodulatory agents, can promote cardiac injury through a variety of mechanisms (107), including direct cardiomyocyte injury (e.g. doxorubicin) and indirect cardiac tissue damage secondary to inflammation. More recently, immune check-point inhibitors, which release inhibitory signals on activated cytotoxic CD8$^+$ T cells, have been associated with potentially lethal fulminant myocarditis in a small subset of patients secondary to massive myocardial infiltration of CD8$^+$ T cells (118).

Macrophages and Myocarditis – Direct Evidence Versus Inference

The majority of published studies have focused on viral and autoimmune myocarditis and, to a lesser extent, doxorubicin-induced myocarditis. It will be in this experimental context that we will examine macrophage dynamics and functions. In all myocarditis models, there is a clear expansion of macrophage numbers within the myocardium (119–121), presumably driven by monocyte influx. Genetic fate mapping has, within the context of viral myocarditis in mouse models, confirmed the massive infiltration of circulating monocytes, and also identified a loss of resident CCR2$^-$ reparative macrophages during the acute infectious period (119). While embryonic-derived cardiac macrophages have been tracked during viral myocarditis, their exact functions remain elusive. It is known that those cardiac macrophage subsets that express high levels of MHC-II are capable of processing and presenting antigen

to CD4$^+$ T cells ex vivo (8). However, the role of antigen presentation by resident immune cells to recruited T cells in vivo is not clear. By extrapolation, neonatal cardiac macrophages, which lack MHC-II expression, are less proinflammatory (produce less TNF-α and IL-1β), and promote more reparative functions (cardiomyocyte and endothelial proliferation) as compared to cardiac macrophages isolated from injured adult animals (15,21).

Broad differences exist in the character of the innate immune response to cardiac stress when comparing different physiological stressors. For example, while the resident macrophage population is lost following viral myocarditis, it increases in number during hypertensive challenge (8). MI and hypertensive stress both promote significant neutrophil infiltration, while there is a surprising lack of neutrophil infiltration in viral myocarditis (7,119,121). Thus, conclusions about macrophage functions made in one model may not necessarily apply to other systems.

Cardiac Macrophages and the Confounding Role of Cardiac Dendritic Cells— Any discussion about the role of tissue macrophages is incomplete without understanding whether macrophages and DCs can be efficiently delineated, in particular during inflammation. Recent studies have developed a framework to identify and separate out cardiac DCs from cardiac macrophages, both of which can express DC markers such as CD11c and MHC-II (119,122). Conventional DCs can be distinguished from macrophages through their expression of the conventional DC lineage transcription factor ZBTB46 (123,124), and a grouping of additional cell surface markers (most importantly CD26) (119,122,125).

Until recently, the ability to specifically deplete conventional DCs was lacking, and the main system to deplete DCs relied on the *Cd11c-DTR* strain. Depletion of DCs during viral myocarditis (using encephalomyocarditis virus [EMCV-D]) leads to significant mortality, while use of the more specific *Zbtb46-DTR* does not, but leads to impaired generation of antigen specific CD8$^+$ T cells and cardiac dysfunction, suggesting that additional cell types, such as macrophages, could also be targeted by CD11c-dependent cell depletion (101,119). Thus, the appropriate selection of cell surface markers, transcription factors and targeting strategies that utilize a contemporary immunologic understanding are required to study the individual roles of macrophages and DCs.

Monocyte versus Macrophage Functions in Myocarditis— Studies have attempted to ascertain the role of macrophages in myocarditis through the use of clodronate liposomes, which deplete circulating blood monocytes and differentially deplete resident cardiac macrophages (depending on the formulation). Using the EMCV viral myocarditis model, clodronate-mediated depletion was associated with a pronounced increase in mortality (101), while CVB3 infection led to increased viral titres, a variable increase in mortality, and reduced late cardiac fibrosis in surviving animals by depletion of cardiac macrophages that produced the pro-fibrotic cytokine galectin-3 (101,126). In contrast to viral infection where the immune system presumably promotes a combination of immune-protective and immune-damaging effects, the immune response in EAM appears to be entirely pathologic as it is directed at self-antigens. Clodronate-liposome administration in the chronic phase of EAM, and not in the acute phase, led to improved cardiac function and decreased adverse

remodeling (127). As an important consideration, the use of clodronate-liposomes leads to difficulties in data interpretation, since depleting blood monocytes prior to their recruitment versus depletion of all monocytes/macrophages would likely have very different effects. Nevertheless, the data appear to suggest that in viral myocarditis, while monocytes and macrophages may promote cardiac fibrosis their net effect is protective; however, in autoimmune models, activation promotes chronic heart failure. Additional studies using targeted approaches are required to fully understand the role of recruited monocytes versus resident macrophages in these settings.

Monocytes can differentiate into either macrophages or DCs, which are an essential part of protective immunity. Deletion of the receptor of CX3CL1 (fractalkine) or CX3CR1, worsens acute CVB3-induced myocarditis (128). CX3CR1 appears to act on monocyte differentiation into macrophages, macrophage survival, and is also important in vascular patrolling behavior of Ly6c^{low} monocytes, which are processes that may be activated following viral infection (129,130). Interfering with CCR5 or its ligand CCL5, led to increased cardiac tissue injury (131); whereas interfering with CCL2 or CCL3-mediated recruitment ameliorated acute CVB3 infection (132–134), suggesting that CCL2, CCL3 and CCL5, despite their known ability to target monocytes, possess non-overlapping roles. In autoimmune myocarditis, preferential monocyte targeting appears to be beneficial – a finding supported by siRNA-mediated inhibition of CCR2 on peripheral monocytes (121). Preventing the recruitment of monocytes by chemokine neutralization (CCL2, CCL3) or deletion of chemokine receptors (CCR2-deficient mice which are blood monocyte deficient; or CCR5) has been shown to ameliorate EAM (121,135). While CCR2 inhibition is classically associated with inhibiting monocyte influx, CCR2 is expressed on cardiac DC subsets and is specifically required for DC homing to cardiac tissue, but not other tissue beds (119). Thus, care must be used when assigning cellular targets when chemokines are targeted.

Associative Studies of Macrophage Activation and Myocarditis Outcomes— Anti-viral immune responses are initiated and modulated by sensing systems able to detect PAMPs and DAMPs in a variety of cell types, including macrophages. Enteroviral (singlestranded RNA Picornaviruses) infection, such as with EMCV or CVB3, is associated with the release of double-stranded RNA, a common intermediate of viral replication and a TLR3 ligand. Hence, deficiency of TLR3 or its molecular adaptor TRIF is associated with fulminant myocarditis (136–141). Interestingly, TLR3 polymorphisms may also confer susceptibility to myocarditis and dilated cardiomyopathy in patients (142). In contrast, whole-body deletion of the TLR adaptor molecule MyD88, and its downstream signaling molecule IRAK4, ameliorates myocarditis and inflammation (131,143). IRAK4 limits the expression of CCR5 and its ligands preventing the recruitment of protective CCR5$^+$ monocytes – a pathway active in macrophages but not conventional or plasmacytoid DCs. TLR4 signaling seems to play a role in controlling the induction of deleterious pro-inflammatory cytokines, such as IL-1β or IL-18 (144). TIM-3, an inhibitory receptor found on macrophages and DCs, prevents exaggerated inflammatory responses, with blockade leading to worsened CVB3 myocarditis associated with an expansion of cardiac macrophage and neutrophil populations (145).

Progression from acute CVB3 myocarditis to chronic inflammation is dependent on viral persistence (146,147). Hence, an effective, but limited, immune response is important to prevent chronic myocarditis. Several studies have shown that in resistant mouse strains, early type I interferon or interferon-γ production promotes viral clearance via nitric oxide production (148–150) and concurrent early induction of antigen-presentation machinery (151). In contrast, in susceptible strains, delayed and exaggerated proinflammatory cytokine and inducible-nitric oxide synthase production by macrophages was associated with increased cardiac damage (152). Hence, persistent infection and an ineffectual, delayed and deleterious inflammatory response could contribute to macrophage mediated cardiac injury.

Macrophage Activation in Doxorubicin-Induced Myocarditis—The cardio-toxic effects of doxorubicin are well recognized. In correlative studies, TLR4 and TLR2 deficiency attenuated doxorubicin cardiotoxicity in mice, suggesting a role for innate immune cells in the disease (153,154). In contrast, TLR2 blockade with neutralizing antibodies reduced the inflammatory response and subsequent adverse cardiac remodeling, while TLR4 blockade exacerbated cardiac tissue damage (155). Importantly, these studies did not differentiate between TLRs expressed in cardiomyocytes, macrophages or other immune cells. Kobayashi et al. demonstrated that NLRP3-induced production of IL-10 by macrophages is an innate mechanism that protects against severe doxorubicin cardiotoxicity (120). Nevertheless, knowledge of the precise role of macrophages in chemotherapy-induced myocarditis is limited.

Conclusions and Future Directions on Cardiac Macrophages

A new paradigm has emerged in which various populations of cardiac macrophages, which include recruited and resident cells with distinct lineages/subsets, have specific functions in cardiac homeostasis and disease. However, numerous challenges remain. A critical hurdle will be to determine whether the human heart contains similar macrophage populations as observed in the mouse. Furthermore, while it is clear that the human heart contains an abundance of macrophages that are involved in myocarditis and MI, many questions remain. Indeed, a recent report provided initial evidence that the human failing heart contains CCR2$^-$ and CCR2$^+$ macrophage subsets that are functionally analogous to mouse CCR2$^-$ and CCR2$^+$ macrophages. Intriguingly, CCR2$^+$ macrophage abundance was associated with persistent LV systolic dysfunction and LV remodeling following mechanical unloading, suggesting that CCR2$^+$ macrophages contribute to heart failure pathogenesis (156). Nevertheless, the exact roles for these cells in myocarditis, MI, and chronic heart failure remain to be determined.

As an exciting development, contemporary immunological techniques have provided a framework to separately investigate the roles of recruited monocytes, resident macrophage subsets and cardiac DCs in cardiac homeostasis and disease. By utilizing lineage-specific genetic tools that can separate the roles of these subsets, the ability to perform a precise immunologic dissection of the innate and adaptive immune systems is likely to yield major mechanistic progress. In turn, a deeper understanding of the functions of each macrophage subset should facilitate the development of strategies to target inflammatory responses and

improve the heart's intrinsic capacity for tissue repair. The future of cardiac immunology is indeed bright, and rich with new possibilities for therapeutic innovation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

Abbreviations

CCL	CC chemokine ligand
CCR	CC chemokine Receptor
CD11b	Cluster of Differentiation 11b
CD206	Cluster of Differentiation 206
CVB3	Coxsackievirus B3
CXCL	chemokine (C-X-C motif) ligand
CXCR1	CXC chemokine Receptor 1
DAMP	damage-associated molecular patterns
DC	Dendritic cell
EAM	Experimental Autoimmune Myocarditis
EMCV-D	Encephalomyocarditis virus
IGF	Insulin-like growth factor
IL	Interleukin
IRF3	Interferon regulatory factor 3
MHC	Major Histocompatibility Complex
PAMP	pathogen-associated molecular patterns
TGF-β	transforming growth factor-β

TLR	Toll-like receptor
TNFα	tumor necrosis factor -α
VEGF	vascular endothelial growth factor

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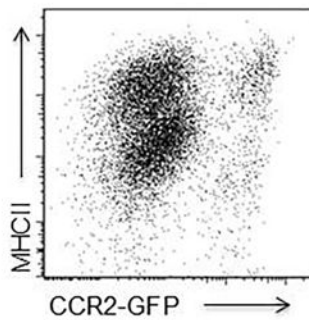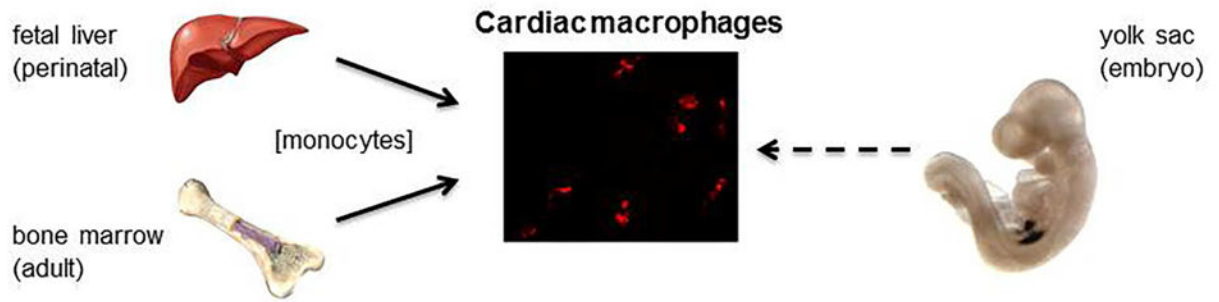
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Figure 1: Macrophage heterogeneity and functions in the adult mouse heart.
Top, Schematic depicting the developmental origins of cardiac macrophages. Bottom, Flow cytometry showing macrophage populations within the adult heart during homeostasis and the mechanisms by which each population is maintained. CCR2 expression was examined by measuring GFP fluorescence in *Ccr2*-GFP reporter mice. Adapted from (21).
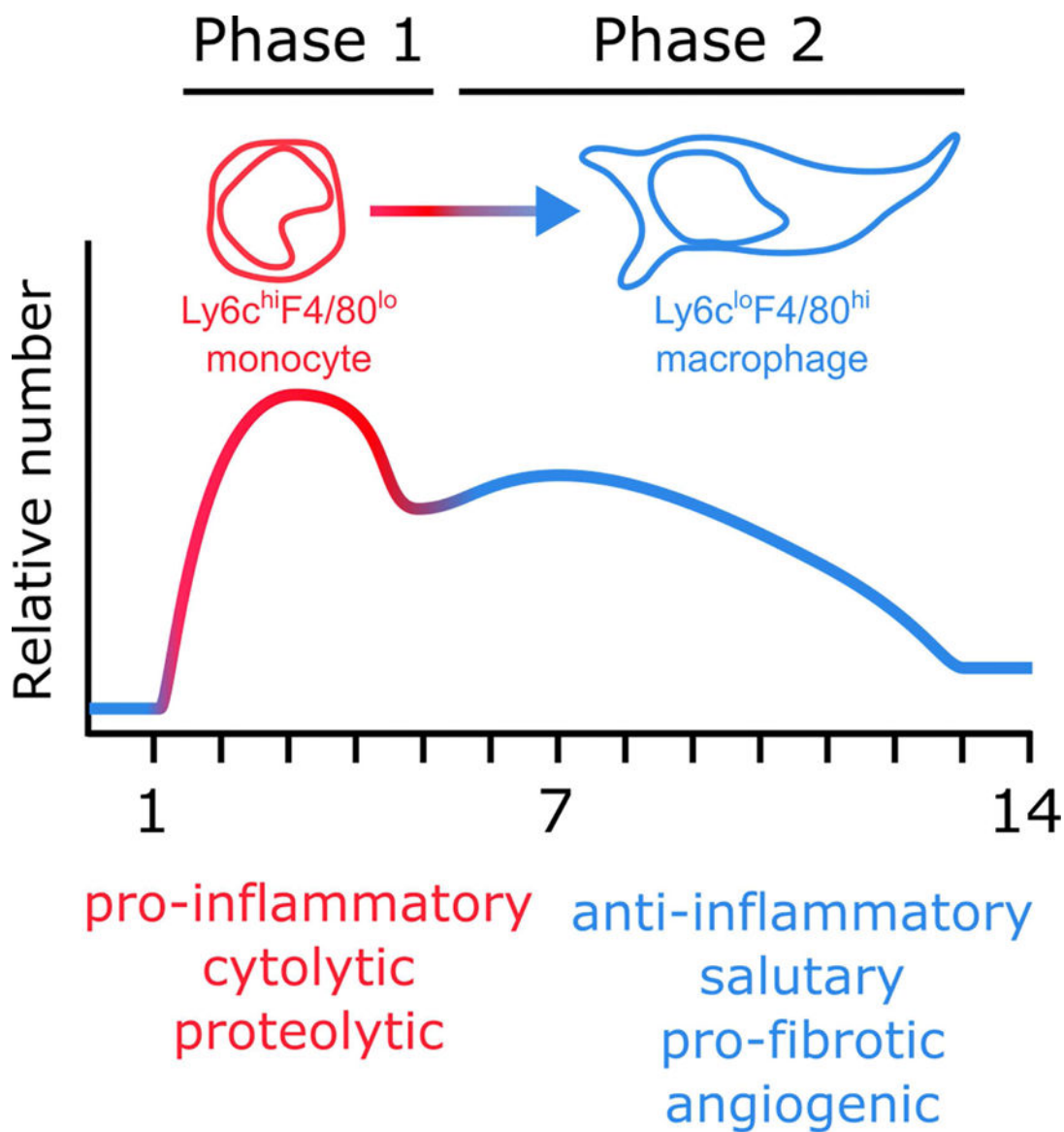
Figure 2. Macrophage dynamics and phenotype during cardiac injury.

During phase 1 or the 'inflammatory' phase of the injury response, Ly6c^{high} monocytes are the predominant monocyte/macrophage population. During phase 2, or the 'reparative' phase, Ly6c^{low} macrophages are the most prevalent. Figure based on data from (7,39,40) and adapted from (40).
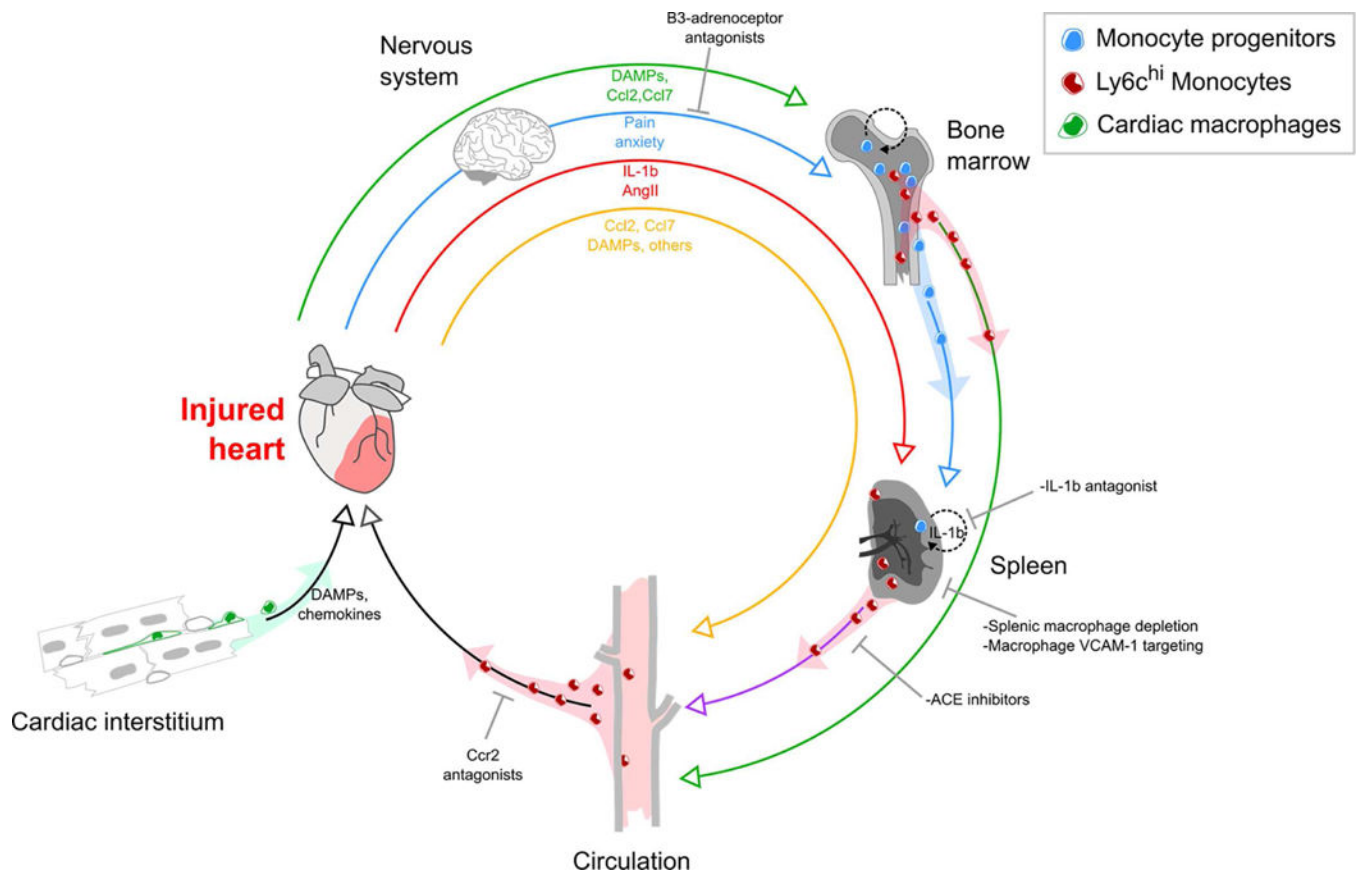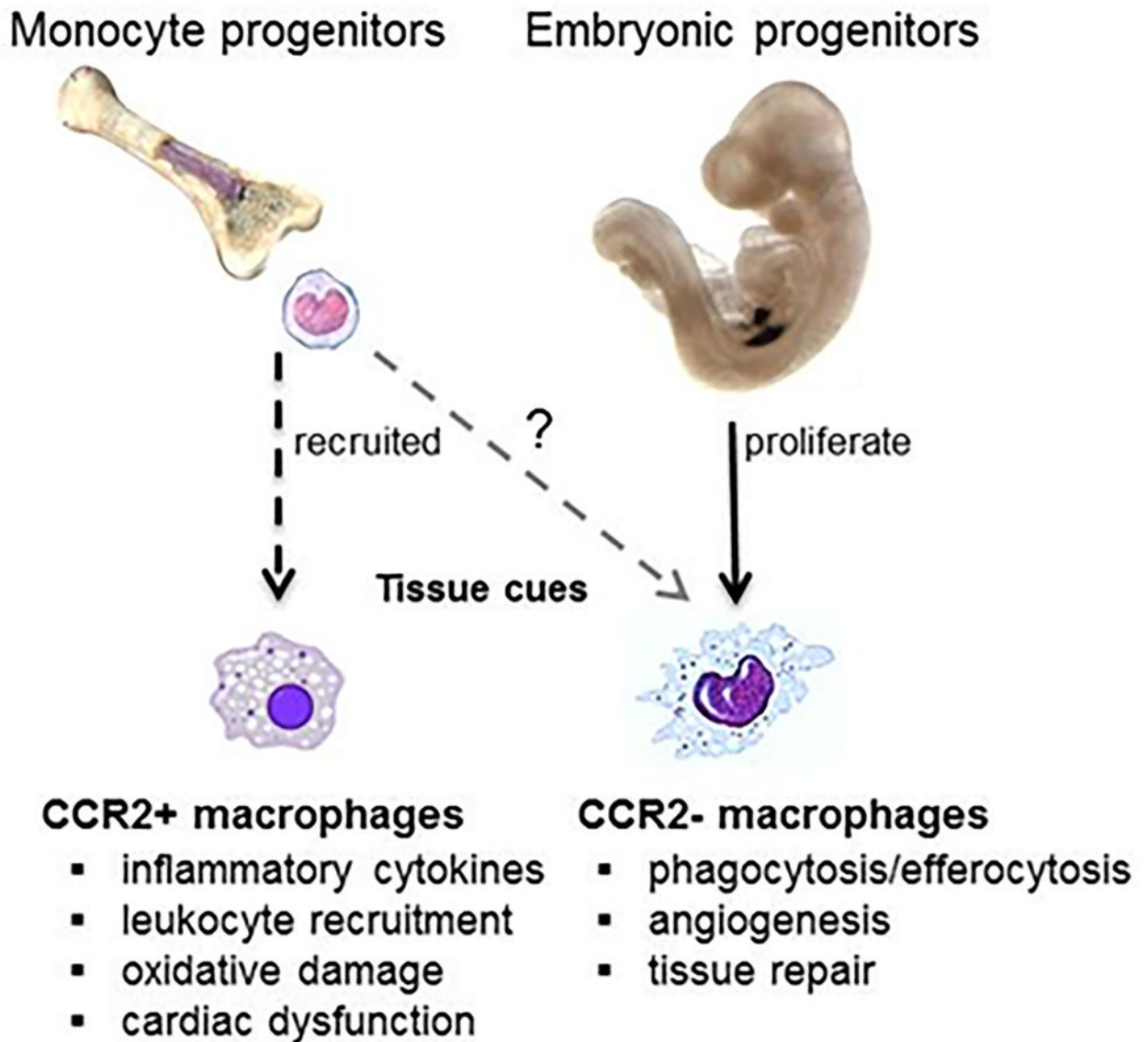
Figure 3. Signaling circuitry promoting monocyte and macrophage accumulation in the injured heart.

(Green pathway) Circulating DAMPs and inflammatory mediators mobilize bone marrow hematopoietic progenitors and monocytes. Monocytes enter the circulation, whereas progenitors migrate to the spleen and support splenic monocytopoiesis. Monocytes from the bone marrow and spleen eventually arrive at the injured heart. (Blue pathway) Pain and anxiety activate sympathetic pathways suppressing hematopoietic progenitor retention factors in the bone marrow leading to further progenitor cell release. (Red pathway) The injured heart directly signals to the spleen and induces splenic monocytopoiesis and hematopoietic progenitor cell proliferation by IL-1β and AngII dependent mechanisms. (Yellow pathway) Inflammatory mediators and DAMPs signal directly to recruit monocytes. Adapted from (157).
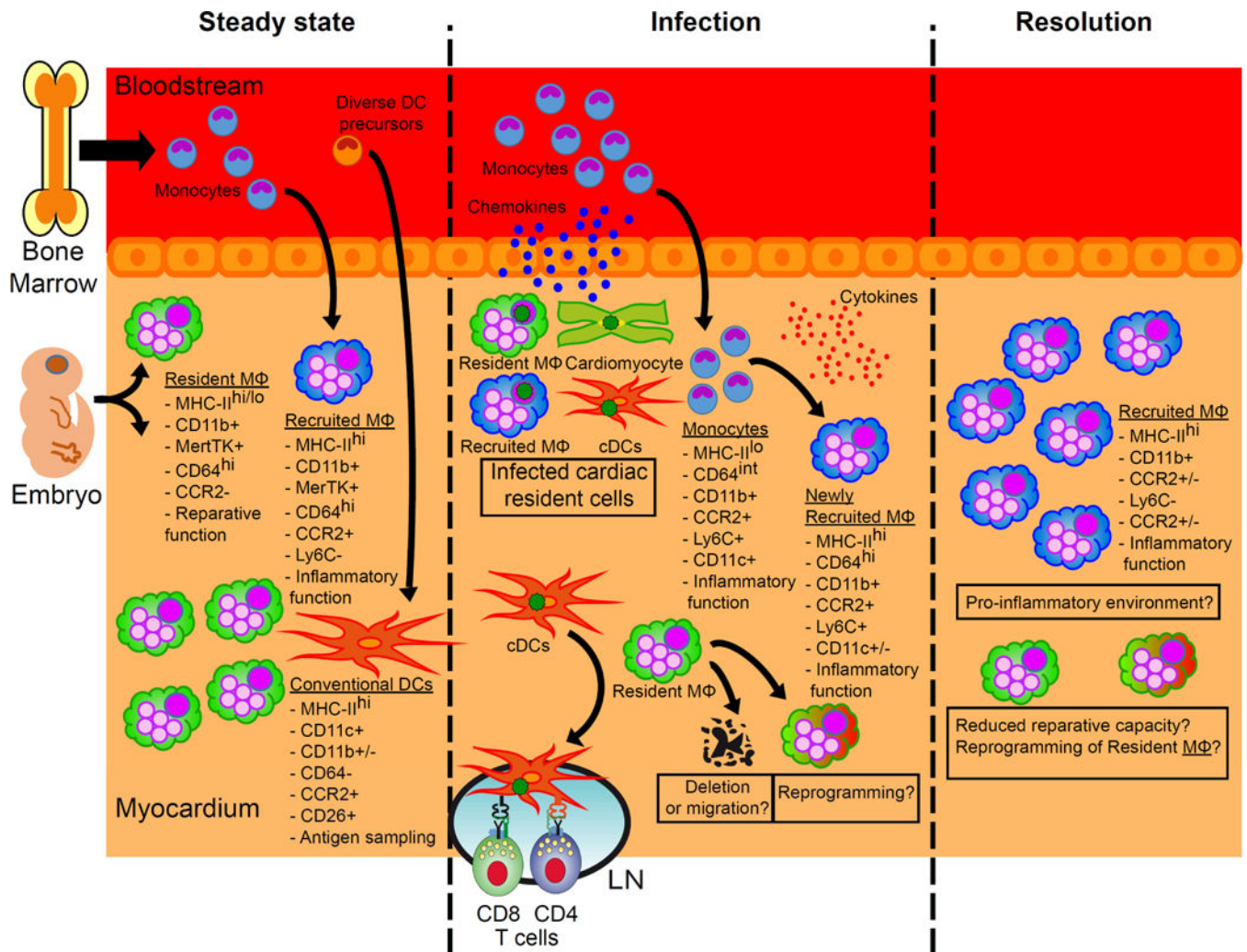
Figure 4. The role of macrophages in viral myocarditis.
In homeostasis, two ontologically different populations of macrophages (MΦ), with different functions, reside in the heart. Upon viral infection, the resident cells of the myocardium produce chemokines that promote the recruitment of monocytes, which in turn differentiate into macrophages with proinflammatory functions. However, besides the fact that their numbers recede, little is known about the role of resident macrophages during and after infection, and many questions remain (see boxes). The difficulty in distinguishing these different populations using (significantly overlapping) surface markers adds to the challenges faced in addressing these questions.

Monocyte progenitors

Embryonic progenitors

recruited ? proliferate

Tissue cues

CCR2+ macrophages
- inflammatory cytokines
- leukocyte recruitment
- oxidative damage
- cardiac dysfunction

CCR2- macrophages
- phagocytosis/efferocytosis
- angiogenesis
- tissue repair

Central Illustration. Myocardial injury triggers a dramatic shift in macrophage composition.
While tissue resident CCR2$^-$ macrophages expand through local proliferation, resident
macrophage subsets are vastly outnumbered by recruited monocytes. Upon entering the
heart, monocytes predominantly differentiate into CCR2$^+$ macrophages with smaller
contributions to other macrophage subsets (i.e., CCR2$^-$ macrophages). CCR2$^+$ macrophages
display a predominantly pro-inflammatory phenotype, while CCR2$^-$ macrophages exhibit a
reparative/regenerative phenotype.