Macrophages are required for neonatal heart regeneration

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Myocardial infarction (MI) leads to cardiomyocyte death, which triggers an immune response that clears debris and restores tissue integrity. In the adult heart, the immune system facilitates scar formation, which repairs the damaged myocardium but compromises cardiac function. In neonatal mice, the heart can regenerate fully without scarring following MI; however, this regenerative capacity is lost by P7. The signals that govern neonatal heart regeneration are unknown. By comparing the immune response to MI in mice at P1 and P14, we identified differences in the magnitude and kinetics of monocyte and macrophage responses to injury. Using a cell-depletion model, we determined that heart regeneration and neoangiogenesis following MI depends on neonatal macrophages. Neonates depleted of macrophages were unable to regenerate myocardia and formed fibrotic scars, resulting in reduced cardiac function and angiogenesis. Immunophenotyping and gene expression profiling of cardiac macrophages from regenerating and nonregenerating hearts indicated that regenerative macrophages have a unique polarization phenotype and secrete numerous soluble factors that may facilitate the formation of new myocardium. Our findings suggest that macrophages provide necessary signals to drive angiogenesis and regeneration of the neonatal mouse heart. Modulating inflammation may provide a key therapeutic strategy to support heart regeneration.

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Inflammation can impede recovery of the adult heart from injury (18). Recent studies demonstrated that disruption of C/EBP signaling in the adult epicardium reduced injury-induced neutrophil infiltration and improved cardiac function (19). However, both positive (20–22) and negative (23–26) correlations between tissue remodeling and monocytes, and their lineage descendant macrophages, have been reported. CCL2, a chemokine that recruits and activates monocytes, seems to play a dual role in MI. Targeted deletion of the CCL2 receptor (CCR2) improved left ventricular dilation and dysfunction (20, 21), while cardiac overexpression of CCL2 also improved outcome (23) by inducing macrophage infiltration, angiogenesis, myocardial IL-6 secretion, and accumulation of cardiac myofibroblasts. Biphasic recruitment of Ly-6C+ and Ly-6C− monocyte subsets from the spleen also promotes infarct healing (26–29).

Multiple observations indicate an inverse relationship between the development of the immune system and epimorphic regeneration in lower vertebrates and support the longstanding hypothesis that the mammalian immune system constrains regenerative capacity (30). Recently, macrophage infiltration was demonstrated to be essential for limb regeneration in newts (31), suggesting that this inflammatory process represents an evolutionarily conserved mechanism for tissue repair. Interestingly, the developmental timing of immune system maturation and terminal differentiation of cardiomyocytes are coupled, suggesting that immunity might influence cardiac regenerative capacity in mammals. We sought to understand the nature of the neonatal immune response to injury in the context of cardiac regeneration. Comparison of the immune response to MI in P1 (regenerative) and P14 (nonregenerative) mice revealed differences in several cell types, including monocytes/macrophages. We focused further on monocytes/macrophages, given that they are central to outcome after MI in adults and can promote mammalian regeneration of skeletal muscle and the nervous system (32, 33). By utilizing a model of mono-
cyte/macrophage depletion in P1 mice, we show that monocytes/macrophages are required for neonatal heart regeneration and efficient neoangiogenesis. Our findings suggest that therapeutic modulation of monocytes/macrophages will facilitate regeneration of the mammalian heart.

**Results**

The immune response to MI differs between P1 and P14 mice. To understand differences between the immune response during and beyond the regenerative period, we profiled the cellular response to MI in P1 and P14 mice over time by FACS. MI was induced in mice by permanent ligation of the left anterior descending coronary artery. Before MI or at 1, 4, 7, or 12 days after MI, pooled hearts or spleens were enzymatically digested or mechanically disrupted, respectively, to single cell suspensions and stained with antibodies to distinguish neutrophils (CD11b+Ly-6G+) and monocytes/macrophages (CD11b+Ly-6G–) (top panels). Within the mononuclear phagocyte population, macrophages/DCs are classified as (F4/80/CD11c–I-Ab)hiLy-6Clo and monocytes are depicted as (F4/80/CD11c–I-Ab)loLy-6Chi or (F4/80/CD11c–I-Ab)loLy-6Clo (bottom panels). Percentages of cells are indicated for the representative dot plots. (B) Quantification at the indicated time points to compare the percentage of all mononuclear phagocytes or macrophages/DCs (MΦ/DCs) relative to the leukocyte-enriched gate or mononuclear phagocyte population, respectively, in mice undergoing MI at P1 (blue) or P14 (red) (n = 3–5 per time point). (C) Total number of mononuclear phagocytes per milligram of heart tissue 7 days after MI at P1 or P14. (D) Relative percentages of Ly-6Chi and Ly-6Clo monocytes in the heart were quantified over time following MI of mice at P1 or P14 (n = 3–5 per time point). (E) Real-time RT-PCR analysis of cardiac chemokine expression at 3 days following MI of P1 or P14 mice. Expression is relative to that in P1 sham-operated mice (P1 Sham) (n = 3). Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
following MI at P1 or P14 (Figure 1A), and dynamic alterations over time were quantified as a percentage of the leukocyte-enriched live gate, determined by characteristic forward/side scatters (Figure 1B and Suppamental Figure 1A). Additional staining of the mononuclear phagocyte population allowed us to further delineate Ly6C\textsuperscript{hi}(F4/80/I-Ab/CD11c)\textsuperscript{hi} macrophages/DCs, Ly6C\textsuperscript{hi}(F4/80/I-A\textsuperscript{b}/ CD11c)\textsuperscript{hi} monocytes, and Ly6C\textsuperscript{lo}(F4/80/I-A\textsuperscript{b}/CD11c)\textsuperscript{lo} monocytes (Figure 1A) according to established methods (28).

Mononuclear phagocyte abundance was relatively increased in the heart in the absence of injury and at 7 days following MI at P1 compared with P14 (Figure 1B). Absolute numbers of mononuclear phagocytes in the myocardium at 7 days following MI confirmed there was an increase of mononuclear phagocytes in the P1 group (Figure 1C). Furthermore, the quantity of macrophages/DCs was higher prior to MI and at 7 days after MI (Figure 1B), a period during which cardiomyocyte proliferation and the regenerative process are robustly active (3). We also quantified the relative percentage of Ly-6C\textsuperscript{hi} or Ly-6C\textsuperscript{lo} monocytes infiltrating the heart. While P1 hearts showed an increased percentage of Ly-6C\textsuperscript{hi} monocytes compared with P14 hearts before and at 1 day following MI, both groups had a biphasic, sequential infiltration of Ly-6C\textsuperscript{hi} and Ly-6C\textsuperscript{lo} monocytes into the heart in response to infarction (Figure 1D), as previously described for the adult mouse (28). Comparisons were also made in the spleen, a major source of monocyte reserves following myocardial injury. While baseline percentages of all mononuclear phagocytes and Ly-6C\textsuperscript{hi} monocytes were higher in P1 mice, the abundance was similar between groups at multiple time points following MI at P1 or P14 (Supplemental Figure 2, A and B). Given the differences in the magnitude of mononuclear phagocytes, we compared the expression level of monocytic chemokines in the heart 3 days following sham surgery or MI at P1 or P14 to examine possible differences in recruitment signals. Interestingly, the robust upregulation of expression that occurs following MI at P14 was not present for most chemokines following MI at P1, suggesting that these signals are not the mechanism for cell recruitment or activation (Figure 1E).

Neutrophil numbers peaked at day 1 following MI at P1 and P14 (Supplemental Figure 1A), consistent with established knowledge of neutrophil responses. While neutrophil abundance was relatively higher in the heart at many time points following MI at P1 compared with that at P14 (Supplemental Figure 1A), we did not initially focus on this cell type due to its transient presence at the injury site. Given that the lymphocyte response is relatively weak and underdeveloped at birth, FACS was not a reproducible method to quantify the low abundance of T and B lymphocytes in the heart following MI at P1. Therefore, we compared lymphocyte responses to MI at P1 and P14 by quantifying them in the spleen, a secondary lymphoid organ and major site of lymphocyte accumulation and activation. Representative FACS plots from spleens before injury or at 7 days following MI at P1 or P14 illustrate the gating used to differentiate B cells, T cells, and T cell subsets (Supplemental Figure 3, A and B). B cells and T cells were generally present at higher percentages in P14 spleens before and after injury (Supplemental Figure 3C) compared with P1 spleens. Furthermore, within the TCR\textsuperscript{+} population of the spleen, P1 mice had a significantly lower percentage of CD4\textsuperscript{+} T cells and CD25\textsuperscript{+} CD4\textsuperscript{+} regulatory T cells (Supplemental Figure 3D), consistent with the established observation that regulatory T cells do not emerge until after P4 (13). Instead, the majority of T cells from the P1 group were CD25\textsuperscript{+} and likely represent an immature state (Supplemental Figure 3D).

Localization of inflammatory cells. To confirm differences in the P1 and P14 monocyte/macrophage response, we sought to enumerate immune cell types in heart sections using immunostaining. To
assess possible heterogeneity in cellular localization, we analyzed regions both in the ischemic zone (IZ) and the remote zone (RZ) of transverse heart sections (Figure 2A). Serial heart sections, beginning at the ligature and progressing through the apex, were stained for markers specific for macrophages (F4/80) (Figure 2A) and neutrophils (NIMP-R14) (Supplemental Figure 1B). Sections from the area of the heart in which left anterior descending ligation was performed were also taken from sham-operated mice and stained for F4/80. Representative images (Figure 2A) and quantification of the total number of F4/80+ macrophages (Figure 2B) showed significantly more cells in hearts at P1 compared with P14 in the absence of ischemic injury. Seven days following MI at P1, macrophages appeared more abundant in the heart overall and were uniformly distributed throughout the myocardium (Figure 2A). Quantification of the number of macrophages localized to the IZ or RZ confirmed this observation (Figure 2B). In contrast, hearts from mice 7 days following MI at P14 had fewer macrophages overall that were almost exclusively localized to the IZ (Figure 2, A and B). In both the P1 and P14 groups, neutrophils were present in small numbers in both the IZs and RZs of the myocardia (Supplemental Figure 1, B and C). Together with the FACS data, the immunostaining shows that, in regenerating hearts (P1 group), there is an increased abundance of macrophages and a striking difference in macrophage localization compared with nonregenerating hearts (P14 group). Therefore, macrophages may function differently in the regenerating neonatal myocardium following MI.

Depleting macrophages in the neonatal MI model. Significant regeneration takes place by 7 days following MI at P1 (3, 34). Within the first week following MI at P1, monocytes/macrophages had the highest, consistent representation in the heart of the cell types assessed and have been shown to have regenerative effects in other systems (32, 33). We hypothesized that, because of their distinc-
tive abundance and localization, monocytes/macrophages from mice undergoing MI at P1 may have a role in heart regeneration that is different from the role of monocytes/macrophages in non-regenerative MI. To test the role of monocytes/macrophages, we applied a method for clodronate liposome-mediated depletion of monocytes/macrophages to the neonatal MI model (24). Following i.v. injection of liposomes containing clodronate, phagocytosis by monocytes/macrophages results in apoptosis and subsequent depletion of the monocyte/macrophage population. We adapted this method to neonatal MI (Figure 3A). By day 3 after MI, spleens from clodronate liposome-treated (CL2MDP-L-treated) mice were visibly smaller than those of control mice (Figure 3B), and FACS analysis of hearts showed a specific depletion of the mononuclear phagocyte (CD11b+Ly-6G−) population (Figure 3C). Compared with control-treated neonates, P1 mice given CL2MDP-L had more than a 50% reduction in cardiac mononuclear phagocytes but no depletion of neutrophils (Figure 3C). Additional staining of the remaining mononuclear phagocyte population showed that the relative ratio of macrophages/DCs and Ly-6C lo or Ly-6C hi monocytes was unchanged (Figure 3D). By day 7 after MI, CL2MDP-L-treated spleens were significantly smaller, as a ratio to body weight (Figure 3E), and lacked the morphologically distinct red pulp and white pulp compartments that were visible in controls by H&E staining (Figure 3F). Immunohistochemical detection of the macrophage markers F4/80 in the spleen (Figure 3F) and Mac-3 in heart (Figure 3G) showed a marked reduction in the macrophage population.

**Figure 4** Depletion of monocytes/macrophages blocks heart regeneration in neonatal mice. (A) At 7 days after MI, H&E-stained serial heart sections, starting below the ligature and progressing toward the apex (3 sections per heart), show a diminishing infarct area that has been displaced to the periphery in control mice. CL2MDP-L-treated mice maintain an interstitial scar. The injured area is circled. Scale bar: 1 mm. (B) Masson’s trichrome staining of serial sections, starting below the ligature and progressing toward the apex, from control or CL2MDP-L-treated mice 21 days following MI at P1 to visualize fibrosis. The injured area is circled. Scale bar: 1 mm. (C and D) Picrosirius red staining on heart sections from control or CL2MDP-L-treated mice 21 days following MI at P1. (C) Representative images and (D) quantification as a percentage of total section area (≥7 sections per heart) show significantly more fibrosis in the CL2MDP-L-treated group (n = 7–8 mice per group). Scale bar: 1 mm. (E) Cardiac function following MI or sham surgeries was assessed at 28 days after MI by echocardiography. Data are expressed as the percentage of FS (n = 3–4 mice per group). Data are mean ± SEM. *P < 0.05, **P < 0.01.

Regeneration depends on macrophages. Next, we determined the ability of P1 mice depleted of monocytes/macrophages to regenerate their hearts in response to MI. By day 7 after MI, control mice already exhibited substantial regeneration (Figure 4A). H&E staining of sections at and below the ligature revealed replacement of dead myocardia and subsequent marginalization of the remaining scar to the tissue periphery in control mice (Figure 4A, left to right). In contrast, sections from mice treated with CL2MDP-L had visible transmural IZs that extended toward the apex at day 7 after MI, suggesting that regeneration was interrupted within the first week following MI (Figure 4A).

By 21 days following MI at P1, control mice had completely regenerated their myocardia from the ligature to the apex. Masson’s trichrome and Picrosirius red staining for collagens showed that only a small, localized fibrotic response around the ligation site was visible in some control samples (Figure 4, B and C). Strikingly, the CL2MDP-L-treated group showed visible collagen staining throughout the infarct area to the apex. Furthermore, the area of fibrotic tissue quantified by Picrosirius red staining was significantly higher in CL2MDP-L-treated mice, suggesting that regeneration was monocyte/macrophage dependent (Figure 4D). Consistent with increased fibrotic scar formation, cardiac function was significantly depressed in CL2MDP-L-treated mice following MI compared with that in saline controls or sham-operated mice treated with CL2MDP-L, further supporting a role for monocytes/macrophages in functional regeneration of the neonatal heart after MI (Figure 4E).

Monocyte/macrophage populations differ between P1 and P14 mice. It has been well documented that adult macrophages mediate scar formation (24, 35, 36), not regeneration, following ischemic heart injury. Therefore, we reasoned that monocytes/macrophages from P1 mice must differ functionally from P14 monocytes/macro-
To understand how monocytes/macrophages participate in cardiac regeneration, we sought to identify phenotypic differences between populations from P1 and P14 mice. Macrophages can be subdivided into M1 or M2 populations based on their expression of multiple markers. We used real-time PCR analysis to compare expression of M1 genes (Cdh86, Fcgr1, and Nos2) and M2 genes (Chi3l3 and Arg1) in the heart at 3 days after MI (Figure 5A). Following MI of P14 mice, the M2 marker arginase 1 (Arg1) was the most dramatically upregulated, while 2 of the 3 M1 markers were unchanged or decreased. In contrast, following MI at P1, both types of markers were modestly regulated in the heart, with no clear bias toward M1 or M2.

To gain a more comprehensive understanding of the cardiac monocyte/macrophage genes important for regeneration, we assessed the transcriptional profiles of sorted monocytes/macrophages from hearts of mice 3 days following MI at P1 or P14 by microarray. Using the same staining and gating strategy outlined in Figure 1A, quantitative real-time PCR (qPCR) analysis of RNA isolated from the sorted mononuclear phagocyte population showed enrichment for the expression of monocyte/macrophage-specific genes and depletion in expression of fibroblast genes compared with RNA from the negatively sorted cell population (Supplemental Figure 4A). The analysis of triplicates for each sample allowed us to identify 42 genes that were significantly upregulated in cardiac monocytes/macrophages from mice 3 days following MI at P1 compared with P14 and 41 genes that were downregulated (2-fold or greater cutoff, \( P < 0.01 \)). A majority of the transcripts that were significantly upregulated in P1 samples participate in inflammation or immune function, angiogenesis, or the oxidative stress response (Figure 5B). Furthermore, gene ontology cluster analysis revealed a striking enrichment for genes coding for known or predicted soluble factors (16 of 42). Interestingly, individual M2 genes were significantly higher in both the P1 and P14 groups, suggesting that neither population fits linearly into M1 or M2 polarization. Other genes significantly higher in monocytes/macrophages isolated from hearts following MI at P14 included MHC class II molecules, genes related to immune function, and G protein–coupled receptors. Of note, only one soluble factor (IL10) was increased in the P14 monocytes/macrophages. The most robustly regulated genes are identified in Supplemental Figure 4B. Collectively, the gene expression data suggest that while both P1 and P14 mice mount a monocyte/macrophage response following MI, the magnitude and polarization of the response varies. The differences in M1/M2 marker expression and in the transcriptional profiles of sorted monocyte/macrophage populations likely reflect functional differences. This suggests that monocytes/macrophages from P1 mice may have regenerative functions unique from the profibrotic and scar-forming activities of adult monocytes/macrophages.
Cardiomyocyte proliferation and angiogenesis in macrophage-depleted neonates. Lineage-tracing studies have demonstrated that cardiac regeneration in neonatal mice (2, 3) and zebrafish involves proliferation of preexisting cardiomyocytes (37). In order to understand how monocytes/macrophages regulate regeneration, we examined the ability of neonatal cardiomyocytes to proliferate following MI in the monocyte/macrophage-depleted model. First, heart weights from control and Cl2MDP-L–treated mice were compared relative to body weight after sham operation or MI and showed no significant differences (Supplemental Figure 5A). Serial heart sections from control or Cl2MDP-L–treated mice costained for pH3 and Troponin T showed that the number of pH3+ cardiomyocytes was not different between macrophage-depleted and control mice at day 7 after MI (Supplemental Figure 5B), suggesting that cardiomyocyte proliferation is not directly controlled by monocytes/macrophages.

Figure 6
Angiogenesis is impaired in monocyte/macrophage-depleted neonates following MI. (A) Sections from control or Cl2MDP-L–treated hearts stained with PECAM (red) and Hoechst (blue) to label endothelial cells and nuclei, respectively, 7 days after MI. Vessels are visible in infarct areas (marked by dashed lines) in controls but not in the Cl2MDP-L group. Original magnification, ×10 (top row); ×20 (bottom row). (B–D) The vasculature was visualized by endomucin immunohistochemistry (brown) on serial heart sections starting below the ligature and progressing toward the apex of control and Cl2MDP-L–treated neonates at (B) 7 and (D) 21 days after MI. (B) Two serial heart sections for each mouse show newly forming vessels invading the IZ in control but not Cl2MDP-L–treated neonates, and lines indicate area used to quantify vessel density. (C) Significantly fewer neovessels are present in Cl2MDP-L–treated mice compared with controls. Data are mean ± SEM. ****P < 0.0001. (D) At 21 days after MI, control mice have new myocardia that contain abundant endomucin-positive vasculature, while heart sections from Cl2MDP-L–treated mice contain areas devoid of new vessels. Lines indicate area lacking vessels. Scale bars: 1 mm (top rows); 200 μm (bottom rows).
The reestablishment of adequate blood flow to the ischemic and newly forming myocardium is a key aspect of heart regeneration following MI (3). To determine whether monocytes/macrophages mediate new blood vessel formation or angiogenesis, we visualized the vasculature in heart sections from control or CL2MDP-L–treated neonates by PECAM staining (Figure 6A). Confocal images at low and high magnification revealed numerous PECAM-positive vessels in both the border zone and infarct area of control mice 7 days following MI at P1. In monocyte/macrophage-depleted hearts, PECAM staining was sparse in the IZ (Figure 6A). Neovascularization was also assessed in P1 control or CL2MDP-L–treated hearts at 7 and 21 days after MI by immunohistochemistry and morphometric analysis of endomucin staining (Figure 6, B–D). In control hearts, newly forming vessels were visible in the regenerating infarct by day 7 after MI; serial sections showed vessels invading areas in which new myocardium were actively replacing the infarct and complete vessel coverage in areas distal to the ligation in which the myocardia had already been completely replaced (Figure 6B). Morphometric analysis of the vascularized area showed a significant increase in control hearts compared with that in CL2MDP-L–treated hearts 7 days following MI (Figure 6C). By day 21, neovascularization was complete throughout all sections in control mice (Figure 6D). Neo-

In adult tissues, inflammation is central to fibrosis and other processes that heal the injury but impede regeneration. However, effective cardiac repair requires an orchestrated response, and activation of monocytes/macrophages following cardiac injury has both beneficial and deleterious effects on scar formation. Following cryoinjury in adult mice depleted of monocytes/macrophages with clodronate liposomes, lack of collagen deposition, failed clearance of necrotic cells, and reduced angiogenesis impair scar formation, leading to rupture and increased mortality (24). Also, optimal healing from MI requires a precise balance of Ly-6Chi and Ly-6Clo monocytes/macrophages to remove debris and form a scar that is compatible with heart function. Conditions that often accompany or precede MI, such as atherosclerosis, obesity, and diabetes, have inflammatory components that disrupt this balance, causing impaired healing (38). Regeneration supersedes wound healing and scar formation in P1 neonatal hearts. Cardiomyocyte cell cycle withdrawal clearly plays a major role in defining the regenerative window in the neonatal mouse heart. However, our data show that neonatal monocytes/macrophages are also critical for vascularization of
regenerated neonatal myocardium, a role that differs from their profibrotic function in adulthood. Myeloid cells exert many proangiogenic roles during development and tissue repair, such as production of growth factors like VEGF, physically assisting endothelial tip fusion, or providing temporary scaffolds for vascular expansion (39). It is likely that P1 monocytes/macrophages are unique in their ability to foster neovascularization without simultaneously perpetuating cell damage through protease production or promoting fibrosis.

**Macrophage heterogeneity.** Tissue macrophages are derived from monocytes, as they enter tissue from the periphery or from local proliferation of resident cells (40, 41). Here, we focus on both monocytes and macrophages, given that many of the macrophages identified in the heart and spleen by our analysis may be monocyte derived. Furthermore, while our sorting strategy is standard for identification of monocytes/macrophages, we cannot rule out the potential presence of a small subset of CD11b+ DCs in these populations.

Monocytes/macrophages exhibit considerable heterogeneity that is not yet fully understood. Our expression profiling on sorted cardiac monocytes/macrophages from P1 and P14 mice highlights this diversity and revealed differences between regenerative and nonregenerative monocytes/macrophages that, in addition to the expected inflammatory-related genes, included expression of several soluble factors, angiogenesis genes, and oxidative stress responders. Given that phenotypic differences in monocyte/macrophage populations often reflect functional variation that influences disease (42), it is likely that P1 cells may perform different functions than their P14 (and adult) counterparts.

The link between regeneration and immunity — therapeutic implications. A link between development of an “advanced” or mammalian-like immune system and the loss of regenerative capacity has been recognized for decades (30). Studies with antiinflammatory and proinflammatory agents demonstrate that inflammation controls regeneration in *Xenopus* hind limb (30) and inflammation is necessary and sufficient to induce zebrafish neurogenesis following injury (43). A recent study in axolotls, salamanders that retain regenerative capacity throughout life, showed a requirement for macrophages in limb regeneration following amputation (31). Examples in mammals suggesting that monocytes/macrophages can have regenerative effects are also beginning to emerge. In skeletal muscle, regeneration following toxic injury depends on monocyte/macrophage stimulation of myogenic proliferation (33) and remyelination in the aging central nervous system is stimulated by recruitment of young monocytes (32).

By using a novel model of mammalian heart regeneration, our studies provide direct evidence that the immune system, and specifically monocytes/macrophages, play a role in heart regeneration. Given that cardiac regeneration can only occur within a narrow developmental window in mammals, the features that define this window immunologically will be of particular therapeutic relevance. However, regeneration clearly involves additional processes specifically monocytes/macrophages, play a role in heart regeneration. Given that cardiac regeneration can only occur within a narrow developmental window in mammals, the features that define this window immunologically will be of particular therapeutic relevance. However, regeneration clearly involves additional processes.

**Methods**

*N=Neonatal MI model and clodronate liposome treatment. MI surgeries were performed on neonatal mice (ICR/CD-1 strain, Charles River Laboratories), as previously described (3). Briefly, after anesthetizing on ice, lateral thoracotomy was performed and a 6-0 prolene suture (Ethicon) was tied through the left anterior descending coronary artery to induce infarction. Sham-operated mice underwent the same procedure without left anterior descending ligation. After surgery, incisions were sutured with a 6-0 nonabsorbable prolene suture, and neonates were allowed to recover under a heat lamp for several minutes. For MI surgeries at P14, mice were anesthetized with isoflurane, followed by endotracheal intubation for ventilation by using a small animal ventilator (Harvard Apparatus). Clodronate liposomes were obtained from the nonprofit consortium Foundation Clodronate Liposomes and stored for 4 to 6 weeks at 4°C during use. Briefly, room temperature CL-MDP-L (10 μg/ml) was administered iv. 4 hours before MI and again at 1, 3, and 6 days after MI. Due to limitations in iv access, the final dose was administered by intraperitoneal injection.

**RT-PCR and qPCR analysis.** Total RNA was isolated from mouse hearts and spleens using TRIzol (Invitrogen), and RT-PCR was performed using random hexamer primers with the SuperScript III Kit (Invitrogen). qPCR was performed using Sybr probes with the following sequences: Arg1-for 5′-GAAACCCAGAGGAGCATGA-3′; rev 5′-TTTTGCCAGACGACGCTTT-3′; Cdc6-for 5′-CTTACGGAGAACCCATGAT-3′; rev 5′-CCATTGAATAAGCTTGGC-3′; Cd64-for 5′-AGGGTCTCCATGAC-3′; rev 5′-ATCCCTCCTCGTCGAC-3′; iNOS-for 5′-AAAAGGCTCTGGCTGTTG-3′; Neural differentiation of several soluble factors, angiogenesis genes, and oxidative stress responders.

**Flow cytometry.** Single cell suspensions of hearts and spleens from P1 or P14 mice were generated immediately before analysis by flow cytometry, as previously described (44). Briefly, pooled hearts were minced and digested in collagenase/DNase solution in PBS with 20 μM HEPES (125 U/ml collagenase type X1, 60 U/ml hyaluronidase, 60 U/ml DNase I, and 450 U/ml collagenase type I). Single cell suspensions were obtained from pooled hearts at 3 days following MI at P1 or P14 by collagenase digestion and stained with antibodies to distinguish mononuclear phagocytes from other cell types. Clodronate liposomes were administered iv. 4 hours before MI and again at 1, 3, and 6 days after MI. Due to limitations in iv access, the final dose was administered by intraperitoneal injection.
cytes (described above). The mononuclear phagocyte population and remaining cells were separated by FACS using a MoFlo (Beckman Coulter) high-speed sorter. Microarray analysis was performed on RNA samples extracted from the sorted populations by the University of Texas southwestern Microarray Core Facility, which were analyzed for gene expression profiles using the Illumina Mouse-6 Beadchip (Illumina). Briefly, cDNA was column purified following synthesis according to the manufacturer’s protocol and then used for in vitro transcription to generate cRNA. After purification, cRNA quality was checked using an Agilent 2100 Bioanalyzer before proceeding with hybridization. Samples were hybridized overnight at 58°C with rocking, washed, blocked, and then stained with SA-Cy3. Slides were dried and scanned using the Direct Hybridization program on the BeadArray Reader. A fold-change cutoff of 2.0 was considered significant. Data are available from GEO (accession no. GSE54530).

Transmural echocardiography. Cardiac function and heart dimensions were evaluated by 2-dimentional echocardiography using a Visual Sonics Vevo 2100 Ultrasound (Visual Sonics) on conscious mice. M-mode tracings were used to measure anterior and posterior wall thicknesses at end diastole and end systole. Left ventricular internal diameter (LVIDd) was measured as the largest anteroposterior diameter in either diastole (LVIDd) or systole (LVIDs). A single observer blinded to mouse experimental groups performed echocardiography and data analysis. Fractional shortening (FS) was calculated according to the following formula: 

$$FS(\%) = \frac{(LVIDd - LVIDs)}{LVIDd} \times 100$$

Histology and immunostaining. Heart and spleen tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5-μm intervals. H&E, Masson’s trichrome, and Picrosirius red stains were performed using standard procedures. Immunohistochemistry for endomucin to mark vasculature and double immunofluorescence staining for phospho–histone H3 and cardiac troponin T was performed as previously described (2). PECAm staining was performed using standard methods and imaged by confocal microscopy.

For immunohistochemical staining, the following antibodies were used: F4/80 (1:200, Serotec); Mac-3 (1:200, BD Pharmingen); and NIMP-R14 (1:200, Abcam). Cryosections were fixed for 10 minutes in ice-cold acetone. Slides were treated with 0.3% H2O2 for 20 minutes before blocking in 10% rabbit serum for 1 hour at room temperature. Slides were incubated with unconjugated primary antibodies listed above overnight at 4°C. Slides were washed 3 times in PBS and incubated with rabbit biotinylated anti-rat secondary (1:200, Vector) for 30 minutes at room temperature, followed by SA-HRP (1:500, Vector) for 30 minutes at room temperature. Staining was visualized with the DAB Kit (Dako) and counterstained with hematoxylin before mounting.

Statistics. Results are expressed as the mean ± SEM. We used a 2-tailed, unpaired Student’s t test for all pair-wise comparisons (GraphPad Prism version 5). P values of less than 0.05 were considered significant.

Study approval. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

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