Proliferation and Recruitment Contribute to Myocardial Macrophage Expansion in Chronic Heart Failure

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Rationale: Macrophages reside in the healthy myocardium, participate in ischemic heart disease, and modulate myocardial infarction (MI) healing. Their origin and role in post-MI remodeling of nonischemic remote myocardium, however, remain unclear.

Objective: This study investigated the number, origin, phenotype, and function of remote cardiac macrophages residing in the nonischemic myocardium in mice with chronic heart failure after coronary ligation.

Methods and Results: Eight weeks post MI, fate mapping and flow cytometry revealed that a 2.9-fold increase in remote macrophages results from both increased local macrophage proliferation and monocyte recruitment. Heart failure produced by extensive MI, through activation of the sympathetic nervous system, expanded medullary and extramedullary hematopoiesis. Circulating Ly6C<sup>hi</sup> monocytes rose from 64±5 to 108±9 per microliter of blood (P<0.05). Cardiac monocyte recruitment declined in Ccr2<sup>−/−</sup> mice, reducing macrophage numbers in the failing myocardium. Mechanical strain of primary murine and human macrophage cultures promoted cell cycle entry, suggesting that the increased wall tension in post-MI heart failure stimulates local macrophage proliferation. Strained cells activated the mitogen-activated protein kinase pathway, whereas specific inhibitors of this pathway reduced macrophage proliferation in strained cell cultures and in the failing myocardium (P<0.05). Steady-state cardiac macrophages, monocyte-derived macrophages, and locally sourced macrophages isolated from failing myocardium expressed different genes in a pattern distinct from the M1/M2 macrophage polarization paradigm. In vivo silencing of endothelial cell adhesion molecules curbed post-MI monocyte recruitment to the remote myocardium and preserved ejection fraction (27.4±2.4 versus 19.1±2%; P<0.05).

Conclusions: Myocardial failure is influenced by an altered myeloid cell repertoire. (Circ Res. 2016;119:853-864. DOI: 10.1161/CIRCRESAHA.116.309001.)

Key Words: heart failure ■ hypertrophy ■ macrophage ■ monocyte ■ myocardial infarction

Heart failure commonly complicates ischemic heart disease. Incomplete reperfusion, large infarcts, or multiple infarcts cause left ventricular remodeling, increased cardiac volumes, hypertrophy of remote myocardium, and reduced ejection fraction. Loss of contractile units due to ischemic injury initiates chronic remodeling of the remote nonischemic myocardium, promoting progressive pump failure. These patients have unacceptable mortality rates<sup>1–4</sup> and ultimately can require left ventricular assist devices and heart transplantation to sustain life. The remodeling process of the remote myocardium involves myocyte hypertrophy, apoptosis, and fibrosis and leads to dilatation of the ventricle. In addition to the changes observed in myocytes, remodeling also alters nonmyocyte parenchymal cells. Post–myocardial infarction (MI) remodeling and heart failure associate with inflammation, documented by increased tumor necrosis factor α and circulating innate immune cells. 7–11

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Resident macrophages represent 6% to 8% of the noncardiomyocyte population in the healthy mouse myocardium, a number comparable to the frequency of resident macrophages in other tissues. Cardiac macrophage frequency is higher in newborn mice and increases orders of magnitude in ischemic myocardium. The innate immune response after MI consists of an early inflammatory phase dominated by neutrophils and inflammatory monocytes. Around day 3, monocytes and macrophages with resolution phenotypes implement a transition to a reparative phase. These 2 phases are essential for healing of the infarct in mice and also occur in humans. The inflammatory response receives reinforcement beyond resident cardiac macrophages; cells recruited to the infarct derive from the bone marrow and spleen, and increased sympathetic signaling to the bone marrow enhances hematopoiesis in the days after acute ischemia. The activation of monocyte production in the bone marrow and spleen begins with increased cell cycle entry and migration of Ccr2+ (chemokine (C-C motif) receptor 2) hematopoietic progenitor cells, expanding the systemic pool of innate immune cells.

Macrophages have salutary functions in immune defense, wound healing, and tissue homeostasis. For instance, macrophages may regulate angiogenesis, a process involved in adaptation to myocyte hypertrophy. Yet, macrophages also promote tissue destruction, for instance, in atherosclerotic plaques and, if inflammation resolution is delayed, in the acute infarct. The roles of cardiac-resident macrophages during chronic post-MI remodeling are incompletely understood. Preclinical data indicate that macrophages increase in the remote zone after MI but have not revealed their sources and functions. It was unclear whether changes in cardiac macrophages during left ventricular remodeling result from local proliferation (as in the steady state), monocyte recruitment (as in acute ischemia), or both. Here, we test hypotheses on the origins of remote myocardial and systemic monocyte/macrophages and their progenitors during post-MI remodeling. We detail supply routes and mechanisms that trigger cell proliferation in the heart and hematopoietic organs. Fate mapping supports the hypothesis that both increased monocyte recruitment and local macrophage proliferation contribute to the expanded cell population in failing myocardium. Reinforcements to resident monocytes derive from increased production in the bone marrow and spleen. We report that mechanical strain, which rises in the left ventricular wall after MI, elicits macrophage proliferation. Expression profiling of steady-state, recruited, and locally sourced macrophages reveals that their phenotypes differ reflecting their complex phenotypic plasticity. Finally, inhibition of monocyte recruitment with nanoparticle-enabled RNAi of endothelial cell adhesion molecules reduces myocardial macrophage numbers and post-MI ventricular dilation.

Methods

Mice
Female C57BL/6J mice, female C57BL/6-Tg (UbcGFP) 30Scha/J mice, male and female B6.129P2(Cg)-Cx3cr1tm2.1(cre/ERT2)Litt/WganJ mice expressing a Cre-ERT2 fusion protein and enhanced YFP (yellow fluorescent protein), male and female B6.Cg-Gt(ROSA)26Sor tm9(CAG-ttdTomato)Hze/J mice, and female B6.129S4-Ccr2 tm1Ifc/J mice were purchased from Jackson. Male and female FVB/N-Adrb3 tm1Lowl/J mice were donated by P. Frenette (Albert Einstein College of Medicine New York, NY) and B. Lowell (Beth Israel Deaconess Medical Center, Boston, MA.). All procedures were approved by the Institutional Animal Care and Use Committee Subcommittee on Research Animal Care, Massachusetts General Hospital.

Figure 1. Expansion of cardiac macrophages in heart failure with reduced ejection fraction. A–C, Gating and quantification of myeloid cells in steady state vs 4 and 8 wk after myocardial infarction (MI) in the remote area (ie, the myocardium that was never ischemic), n=8 to 23 wild-type (WT) mice per group, mean±SEM, *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. D and E, Blood monocytes in steady state vs 4 and 8 wk after MI, n=8 to 23 WT mice per group, mean±SEM, *P<0.05 and **P<0.01.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>HFrEF</td>
<td>heart failure with reduced ejection fraction</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MI</td>
<td>myocardial infarction</td>
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MI was induced by permanent ligation of the proximal left anterior descending coronary artery as described previously.30 Mice were anesthetized with isoflurane and received buprenorphine (0.1 mg/kg IP) twice daily for 3 days, starting on the day of the surgery.

Parabiosis
Mice were joined in parabiosis as described previously.30 Mice were anesthetized with isoflurane and received buprenorphine (0.1 mg/kg IP) twice daily for 3 days, starting on the day of the surgery. Experiments began 14 days after parabiosis surgery, as required to establish a shared circulation.

Mechanical Cell Strain
Mechanical deformation (distortion of 4% on surface area at 0.67 Hz for 24 hours) was applied to cultured cells with a device that produces biaxial strain.31–33 For the preparation of cells subjected to mechanical strain, autoclaved membranes were coated with 2 mg/mL of human serum fibronectin (Sigma-Aldrich) at 4 °C.

Statistics
Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc). Results are mean±SEM unless stated otherwise. For 2-group comparisons, an unpaired t test was applied to normally distributed variables and a Mann–Whitney test to non-normally distributed variables. For comparing >2 groups, an ANOVA test, followed by a Sidak test for multiple comparisons, was applied. P values of <0.05 indicated statistical significance. Small interfering RNA (siRNA) formulation into 7C1 nanoparticles was done as previously described.34,35

Please see the Methods section in the Online Data Supplement for siRNA dosing, flow cytometry, magnetic resonance imaging, histology, ELISA, and cell culture.

Results
Cardiac Macrophages Expand During the Development of Chronic Heart Failure Post MI
Macrophages populate the heart in the steady state,36,37 die rapidly in acutely ischemic myocardium,12 and are replaced by monocyte-derived macrophages.12,29 The behavior of macrophages that reside in the nonischemic, remote myocardium after MI is less well understood, and it is unclear if and how they contribute to the development and progression of chronic heart failure. To examine this cell population, we ligated the left coronary artery proximally to induce large infarcts in mice. The heart to body weight ratio increased from 4.48 mg/g at steady state to 6.65 mg/g at 4 weeks (P<0.0001; n=15–20

Figure 2. Contribution of recruitment to cardiac macrophage expansion in heart failure with reduced ejection fraction.
A, Experimental design. B and C, Gating and quantification of resident vs bone marrow–derived cardiac macrophages in steady state vs 4 wk after myocardial infarction (MI), n=4 to 8 per group, mean±SEM. ****P<0.0001. D, Experimental design. E and F, Gating and quantification of chimerism for blood monocytes and cardiac monocytes and macrophages in steady state vs 4 wk after MI, n=4 to 10 pairs per group, mean±SEM, **P<0.01. G, Relative contribution of monocyte-derived vs locally sourced macrophages to total remote monocyte/macrophage population 4 wk after MI, n=4 to 10 pairs per group, mean±SEM. H, Phenotyping of resident vs bone marrow–derived cardiac macrophages using fate mapping outlined in (A; 4 wk after MI, n=4–8 per group, mean±SEM, *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001). FACS indicates fluorescence-activated cell sorting; GFP, green fluorescent protein; SSC-A, side scatter-area; and YFP, yellow fluorescent protein.
per group) and 7.23 mg/g at 8 weeks (P<0.0001; n=9–20 per group) after MI. In remote myocardium, which was isolated by microdissection and did not contain tissue from the infarct or the border zone, inflammatory monocyte and macrophage numbers per mg tissue increased progressively at 4 and 8 weeks after MI (Figure 1A through 1C). Macrophage numbers in the mature infarct scar were low in comparison to the nonischemic myocardium and declined over time (Online Figure I). We detected blood monocytes in heart failure with reduced ejection fraction (HFrEF) mice (Figure 1D and 1E), a finding that resembles data in heart failure patients.9,10

**Origins of Macrophages in Post-MI Heart Failure**

To determine whether accumulation of remote myocardial macrophages relies on recruitment of monocytes from the blood or alternatively from local proliferation, we pursued fate-mapping experiments in Cx3cr1CreER/ R26tdTomato/ mice. In these mice, all fractalkine receptor (Cx3cr1)–expressing cells, including circulating monocytes and cardiac-resident macrophages, express YFP. After injection of tamoxifen, all Cx3cr1pos cells also express the red fluorescent protein tdTomato. Thus, shortly after tamoxifen challenge, blood monocytes and resident macrophages exhibit red and yellow fluorescence (Online Figure II). Three weeks later, circulating monocytes are replaced by newly made cells, which derive from hematopoietic progenitors that do not express Cx3cr1. At this time point, blood monocytes and their progeny no longer express tdTomato (Online Figure II), whereas cells arising from local proliferation of Cx3cr1pos resident cardiac macrophages continue to express tdTomato. We infarcted mice 3 weeks after the last tamoxifen injection (Figure 2A) and assessed the myocardial frequencies of blood monocyte–derived YFPpos tdTomatoneg cells and locally sourced YFPpos tdTomatopos macrophages. A minor monocyte contribution to the cardiac macrophage pool in the steady state (9%) rose significantly in the remote myocardium of mice with HFrEF (21%; P<0.0001; Figure 2B and 2C).

In addition, we used parabiosis to follow HFrEF-induced changes in monocyte recruitment to failing myocardium. We surgically joined a UbcGFP mouse, in which all leukocytes express GFP (green fluorescent protein), with a wild-type mouse (Figure 2D). Two weeks later, when the parabionts established a shared circulation, we induced a large infarct in the wild-type parabiont (Figure 2D) and compared the chimerism of GFPpos monocytes and macrophages in the blood and heart to steady-state parabionts without MI. The contribution of recruited monocytes to the macrophage population in the remote myocardium rose 2.3±0.3-fold in infarcted parabionts (P<0.01; Figure 2E and 2F). On the basis of these data, we estimate that recruited monocytes contribute about one third to the expanded macrophage population in failing myocardium at 4 weeks after MI (Figure 2G; see the Methods section in the Online Data Supplement for calculation).

To address the question whether macrophages in failing myocardium and those of different origins display distinct phenotypes, we isolated respective cell populations from the myocardium of Cx3cr1CreER/ R26tdTomato/ mice and compared their gene expression to steady state by quantitative polymerase chain reaction. Macrophages isolated from healthy and failing myocardium differed significantly in gene expression (Figure 2H). Monocyte-derived macrophages isolated from failing myocardium expressed more Il1β, Ym-1, and Vegfa, whereas locally sourced macrophages had higher mRNA for Tnfa, Tgfb3, and Mrc-1. These differences diverge from the canonical M1/M2 macrophage polarization pattern. For instance, locally sourced macrophages in failing hearts expressed not only more Tnfa (a prototypical M1 gene) but also more Mrc-1 and Fizz-1 (both M2 genes) than monocyte-derived macrophages.

We next tested the role of the Ccl2/Ccr2 interaction in recruiting monocytes to the failing remote myocardium.
Examination of the cellular source of Ccl2 in the remote myocardium revealed that capillary and arteriolar endothelial cells and, to a lesser degree, also macrophages produce Ccl2 (Online Figure III). Hence, we induced MIs in Ccr2−/− mice, which lack the Ccr2 chemokine receptor–binding Ccl2. Monocyte release from the bone marrow into the blood and the recruitment of monocytes to inflammatory sites requires Ccl2/Ccr2 interaction. Although neutrophil numbers did not change, monocyte counts fell in the blood of Ccr2−/− mice 4 weeks after MI (Figure 3A). Infarcted Ccr2−/− mice recruited significantly fewer Ly6Chigh monocytes to the remote myocardium. Thus, numbers of remote myocardial macrophages decreased (Figure 3B), which indicates that Ccr2-dependent monocyte bone marrow release and Ccr2-dependent monocyte recruitment to the heart contribute to the expanded macrophage pool in HFrEF.

Mechanical Strain Stimulates Local Macrophage Proliferation

Steady-state cardiac macrophages self-renew through local proliferation. Hence, we investigated the contribution of local proliferation to the increase in the remote myocardial macrophage population in mice with HFrEF. Remote macrophage proliferation rose significantly in HFrEF when compared with the steady state (Figure 4A).

Among many other adaptations during left ventricular remodeling, mechanical forces, which influence macrophage behavior, change in the left ventricular wall. During chronic post-MI remodeling and acutely after a large infarct, ventricular filling pressures can increase substantially from a normal left ventricular end-diastolic pressure of ≈5 mm Hg to values that can exceed 30 mm Hg. This alteration profoundly increases wall tension and stress, leading to myocyte slippage and chamber dilation. Increased mechanical strain also acts on macrophages, which can sense tissue forces. We therefore hypothesized that increased strain accelerates macrophage proliferation. We isolated murine peritoneal macrophages, plated them on a membrane covered with a fibronectin layer, and exposed them to biaxial mechanical strain (4% membrane deformation, 0.67 Hz) for 24 hours. Mechanical strain increased proliferation and consequently the numbers of macrophages (Figure 4B and 4C). In addition to flow cytometry, confocal microscopy of strained...
dishes documented increased proliferation and numbers of primary peritoneal murine macrophages (Figure 4D). To probe the pathways involved, we assayed mitogen-activated protein kinase (MAPK) signaling, which relies on the protein kinases Fak and Src that both associate with cytoskeletal structures altered by deformation.52–55 Further downstream, activated MAPK (Erk1/2, p38 MAPK) leads to expression of genes that regulate cell cycle entry.56 Indeed, strain activated the MAPK pathway in cultured peritoneal macrophages, indicated by increased Erk phosphorylation (Figure 4E). Mitogen-activated protein kinase kinase (Mek)-1/2 inhibition diminished strain-induced proliferation in vitro (Figure 4F).

In vivo, Mek1/2 inhibition for 3 weeks, starting 1 week after coronary artery ligation, reduced cardiac macrophage proliferation (Figure 4G) and numbers (Figure 4H). Mek1/2 inhibition did not affect blood monocytes (Figure 4I). Using primary human macrophages, we likewise observed higher cell numbers after exposure to mechanical strain (Figure 4I). Human samples obtained from patients with ischemic cardiomyopathy undergoing left ventricular assist device implantation had more Ki67+ macrophages in regions remote to chronic infarcts when compared with control samples from unused donor hearts (Figure 5B).

HFrEF Stimulates Hematopoiesis

Because mice with HFrEF develop blood monocytosis (Figure 1), we investigated the contribution of the bone marrow and spleen to increased monocyte supply. In the bone marrow, hematopoietic stem and progenitor cells proliferated more vigorously during HFrEF than in steady state (Figure 6A). In line with these data, the number of colony-forming units increased in the bone marrow of mice with HFrEF (Figure 6A). To explore which mechanism increased bone marrow myelopoiesis, we investigated the sympathetic nervous system, which undergoes activation in patients with heart failure.57 Mice with HFrEF had higher bone marrow noradrenaline levels (Figure 6C), a neurotransmitter that signals to hematopoietic niche cells through the β1 adrenergic receptor. As a result, mesenchymal stromal cells alter their supply of hematopoietic niche factors.58 Indeed, Cxcl12 (chemokine (C-X-C motif) ligand 12) and Angiopoietin-1, both promoters of hematopoietic stem cell (HSC) quiescence,59,60 fell in the bone marrow of mice with HFrEF (Figure 6D). In addition, HFrEF reduced stem cell factor and vascular cell adhesion molecule 1 (Figure 6D), which retain HSC in the bone marrow niche.61 When we neutralized bone marrow sympathetic nervous system signaling by generating HFrEF in Adrb3−/− mice, Cxcl12 expression was preserved (Figure 6E), resulting in unchanged HSC proliferation (Figure 6F). Blood neutrophil and monocyte levels did not increase in Adrb3−/− mice 4 weeks after MI (Figure 6G).

In agreement with reduced bone marrow HSC retention factors in HFrEF (Figure 6D), hematopoietic stem and progenitor cell release into blood increased (Figure 7A). These cells seeded the spleen and induced extramedullary hematopoiesis: 8 weeks after MI, spleen weight (Figure 7B), splenic hematopoietic stem and progenitor cell proliferation (Figure 7C), and splenic numbers of innate immune cells rose (Figure 7D through 7F). Taken together, these data indicate that HFrEF leads to increased extramedullary myelopoiesis, which contributes to the expanded systemic pool of innate immune cells.

Monocyte-Derived Macrophages Contribute to Adverse Remodeling After MI

Adhesion molecules are necessary for extravasation of leukocytes and their recruitment into sites of inflammation.62 Myocardial expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E- and P-selectin increased significantly 4 weeks post MI (Figure 8A). Using a recently established in vivo RNAi approach,34,35 we silenced all 5 major adhesion molecules in cardiac endothelial cells.
This method relies on nanoparticle delivery of siRNA to endothelial cells and curbs leukocyte recruitment, as previously shown for atherosclerotic plaque and acute ischemia. To examine whether monocyte-derived macrophages contribute causally to the development of HFrEF, we treated mice with endothelial-avid nanoparticles that contained either siRNA silencing all 5 cell adhesion molecules or an irrelevant control siRNA. The treatment began 1 week after coronary artery ligation to reduce interference with the acute inflammatory phase after MI. After 3 weeks of RNAi, neutrophil, monocyte, and macrophage numbers decreased significantly in the remote myocardium, whereas blood leukocyte levels did not change (Figure 8B). We then examined the consequences of reduced myeloid cell recruitment by cardiac magnetic resonance imaging. RNAi treatment led to smaller end-diastolic volumes and less impaired left ventricular ejection fraction (Figure 8C), whereas it did not influence infarct volume (Online Figure IV). The treatment reduced myofibroblast content, collagen deposition, and capillary frequency in the myocardium (Figure 8D). Myocardial mRNA levels of IL1β, TNFα, and VEGFA declined as well (Figure 8E).

Discussion

A large MI usually leads to left ventricular dilation, infarct expansion, hypertrophy of the remote myocardium, and reduced cardiac output. Current standard-of-care measures do not completely interrupt this pathogenic sequence, hence the need for orthogonal strategies, based on newly identified pathways. Here, we describe that the expansion of remote myocardial macrophages in mice with MI depends on both local macrophage proliferation and recruitment of monocytes that derived from hematopoietic progenitors in the bone marrow and spleen. We identify increased sympathetic input through the β3 adrenergic receptor as a signal that fuels cell cycle entry of bone marrow hematopoietic progenitor cells, which leads to higher systemic numbers of monocytes. These monocytes enter the remote myocardium through canonical CCL2/CCR2 chemokine/chemokine receptor interaction, and through increased expression of endothelial cell adhesion molecules. We further identify mechanical strain as a putative cue leading to increased local proliferation of cardiac macrophages. Finally, we show that dampening monocyte recruitment, by means of adhesion molecule silencing started 1 week after coronary ligation, attenuates post-MI remodeling.
associated remodeling occurs in the remote myocardium that subside in the ischemic zone, whereas active, inflammation-
after coronary ligation indicate that inflammatory processes in cardiovascular disease, macrophages have received considerable at-
immunologic organ that harbors panoply of immune cell types. Some of these splenocytes may have autoimmunity after MI, these data highlight the complexity that our current fate-mapping studies confirm. Comparable to the diseased vessel wall, monocyte recruit-
place to ischemic vascular complications, organ damage, and maladaptive infarct repair. Thus, depending on location, number, and phenotype, macrophages can protect or harm, rendering indiscriminate targeting of the immune system unlikely to succeed as a therapy. Hence, there is a need for a precise understanding of macrophage physiology and both their salutary and detrimental actions.

Previous work in humans and in mice indicate that, at least at chronic time points investigated by us, the infarct scar is relatively stable. In clinical delayed-enhancement magnetic resonance imaging, the tissue volume of bright infarct signal decreases over time.66,67 Fundamental studies in rodents after coronary ligation indicate that inflammatory processes subside in the ischemic zone, whereas active, inflammation-
ated remodeling occurs in the remote myocardium that was initially not ischemic.68–70 Hence, our study focused on macrophages residing in the myocardium remote from the ischemic zone.

The question of macrophage origin had previously been addressed for cardiovascular tissues in the steady state,29,71 acute myocardial injury,12 and atherosclerotic plaque.72 In early atherogenesis, plaque macrophages derive from blood monocytes, but in established lesions, the population expands due to local proliferation of these monocyte-derived cells.72 In the healthy adult myocardium, blood monocytes do not contribute to the cardiac-resident population to a major extent,12,29 a finding that our current fate-mapping studies confirm. Decreasing blood monocyte recruitment by in vivo RNAi improved remodeling and preserved left ventricular ejection fraction. These data support that blood monocyte levels could serve a prognostic marker.

The relevance of macrophage origins rests on whether or not recruited and locally sourced macrophages differ in function, which the difference in gene expression detected in this study implies. Monocyte-derived macrophages arise in the bone marrow and spleen. In mice with acute ischemia19 or exposure to chronic stress,73 sympathetic nervous signaling induces higher myeloid cell output. A similar mechanism results in chronically elevated HSC activity in post-MI heart failure, as mice with a genetic deficiency of the β3 adrenergic receptor were protected from bone marrow microenvi-
vironmental signals that push hematopoietic progenitors into active cell cycle phases. Sympathetic nervous signaling and the resulting reduction of Cxcl12 also induces bone marrow

Data on the abundance, origin, and function of macro-
phages are expanding rapidly.64,65 Within the last decade, we have learned that most organs harbor resident macrophages, including the healthy heart and arteries. These cells form a network interspersed between parenchymal cells. In cardio-
vascular disease, macrophages have received considerable at-
tention in atherosclerotic plaque and in the acutely ischemic heart. Macrophage functions likely help maintain cardiovas-
cular health; however, activation of their inflammatory actions can unleash functions that promote disease.15,17 Their oversupply leads to ischemic vascular complications, organ damage, and maladaptive infarct repair. Thus, depending on location, number, and phenotype, macrophages can protect or harm, rendering indiscriminate targeting of the immune system unlikely to succeed as a therapy. Hence, there is a need for a precise understanding of macrophage physiology and both their salutary and detrimental actions.

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Figure 7. Heart failure with reduced ejection fraction activates splenic myelopoiesis. A, Blood colony-forming unit (CFU) assay in steady state vs 4 wk after myocardial infarction (MI), n=5 to 12 per group, means±SEM, **P<0.01. B, Spleen weight in steady state vs 4 and 8 wk after MI, n=9 to 20 per group, mean±SEM, *P<0.05. C, Splenic hematopoietic stem and progenitor cell proliferation in steady state vs 4 and 8 wk after MI, n=7 to 16 per group, means±SEM, *P<0.05. D–F, Splenic myeloid cells in steady state vs 4 and 8 wk after MI, n=7 to 16 per group, means±SEM, *P<0.05 and **P<0.01. LSK indicates hematopoietic lineage-, sca-1+, c-kit+ cells.
metabolism, and proliferation might circumvent the paucity of human spleen tissue available for analysis. $^{18}$F-FDG PET (18F-fluorodeoxyglucose positron emission tomography) data indicate that splenic glucose uptake increases in patients with acute coronary syndromes, a finding that may relate to increased myelopoiesis.

Wall stress increases in the failing heart, exposing all cells, including macrophages, to increased biomechanical strain. Macrophages respond to strain by inflammatory activation and increased expression of scavenger receptors. These phenomena could be of importance in hypertension, which exposes arterial macrophages to increased mechanical forces. Our in vitro data imply that strain increases macrophage proliferation. Although the precise nature of mechanosensing remains unclear, straining of cells in culture activated MAPK, and Mek1/2 inhibition suppressed macrophage proliferation both in vitro and in vivo. Given that macrophage functions may differ substantially when cultured, the interpretation of these data requires caution. The MAPK pathway is activated in human heart failure, although this effect may also relate to growth factor signaling and oxidative stress. In addition to direct mechanical strain effects on macrophage proliferation, para- and autocrine pathways may contribute to the observed phenomenon. Our data motivate further study of the link between macrophage proliferation and strain, especially in the context of evidence for strain-induced proliferation in other cell types.

The origin of cells that promote disease may instruct the design of therapeutic interventions: if harmful cells accumulate because of recruitment, then inhibition of recruitment or dampening hematopoietic supply may prove beneficial. The result of the RNAi experiment (Figure 8), which used recently
established technology to silence endothelial adhesion molecules, suggests that dampening myeloid cell recruitment might mitigate myocardial ischemic injury. These data also provide evidence for causal involvement of monocyte-derived macrophages in the evolution of heart failure. Several clinical trials indicate that in vivo RNAi is a clinically viable strategy. The used nanomaterial directs uptake of siRNA to endothelial cells and leads to sufficient silencing with low toxicity. The therapy began 1 week after coronary artery ligation to avoid interference with recruitment during the acute inflammatory phase after MI. Late effects of treatment on the infarct-healing process are probably minor but cannot be excluded entirely. A similar siRNA delivery strategy reduced the recruitment of monocytes to atherosclerotic plaque, lesions found in patients with ischemic cardiomyopathy. Thus, this treatment approach may not only attenuate left ventricular remodeling but also decrease the risk of reinfarction. Safety studies will have to reveal whether such treatment compromises host defense against infection. In addition, we detected a small but significant reduction of Vegfa mRNA and reduced capillary density, in line with the high Vegfa mRNA levels in monocyte-derived cardiac macrophages. This observation sounds a cautionary note, as a mismatch of capillaries to hypertrophying myocytes might aggravate the balance between left ventricular oxygen supply and demand. Reparative monocytes and macrophages can elaborate Vegfa after MI and thus support regeneration and healing. Although the RNAi treatment that targeted monocyte recruitment proved overall beneficial, these considerations should prompt careful monitoring and dose selection to avoid undue decreases in myocardial Vegfa concentrations. Furthermore, we found a nonsignificant trend toward higher blood leukocyte numbers in the siCAM5 treatment group (Figure 8B). Although retention of leukocytes in circulation might partially explain this observation, we do not know how the treatment interferes with leukocyte homing to recycling organs such as bone marrow and spleen. These organs remove aging cells based on their elasticity, potentially independent of cell adhesion molecules. Additionally, siCAM5 treatment also reduced remote myocardial neutrophil numbers. Thus, lower neutrophil counts might also contribute to the beneficial effects of RNAi on remodeling.

Our data raise many interesting questions. For instance, they suggest prioritization of genome-wide expression profiling of cardiac macrophage subsets isolated from failing myocardium. Such an undertaking could reveal unexpected functions and therapeutic options, if cell-specific deletion of identified genes improves post-MI remodeling. Furthermore, our observations suggest that monitoring of macrophage numbers and functions should inform novel therapeutic approaches to the treatment of heart failure and may furnish mechanistic insights into their mode of action. The production of monocytes depends partially on β-adrenergic receptors, and angiotensin II regulates the release of splenic monocytes, suggesting that β blockers and angiotensin-converting enzyme inhibitors may act in part by altering leukocyte dynamics. Moreover, these data highlight the need to explore further the direct communication of macrophages with myocytes, fibroblasts, and endothelial cells in cardiac pathophysiology.

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Disclosures
J.E. Dahlman and D.G. Anderson have filed intellectual property protection related to 7C1 nanoparticles. The other authors report no conflicts.

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What Is Known?

- After large myocardial infarction, the myocardium undergoes a remodeling process that leads to hypertrophy, chamber dilation, heart failure, and death.
- In patients with heart failure, white blood cell count correlates with mortality.
- Macrophages participate in acute and chronic tissue remodeling via phagocytosis and crosstalk with stromal cells.

What New Information Does This Article Contribute?

- Higher sympathetic tone in mice with heart failure increases systemic myeloid cell production in hematopoietic tissues.
- Myocardial macrophages proliferate locally, possibly because of higher mechanical strain in the failing heart.
- Inhibition of monocyte recruitment to the myocardium after myocardial infarction reduces heart failure, indicating that inflammatory bone marrow–derived cardiac macrophages enhance adverse left ventricular remodeling.

Macrophages reside in the healthy heart and are recruited in large numbers into acutely ischemic tissues. Although it is known that macrophages regulate tissue health, their kinetics, sources, and functions are not well understood in the setting of chronic heart failure. We report that in the months after cardiac ischemia, myocardial macrophages proliferate locally, possibly because of higher local proliferation due to increased mechanical strain in the failing heart and inhibition of monocyte recruitment to the myocardium after myocardial infarction.
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SUPPLEMENTAL MATERIAL

Proliferation and recruitment contribute to myocardial macrophage expansion in chronic heart failure

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Detailed Methods

**Flow cytometry.** Blood for flow cytometric analysis was collected by cardiac puncture using a 50 mM EDTA (Ethylene Diamine Tetra Acetic Acid) solution (Sigma Aldrich) as an anticoagulant. Erythrocytes were lysed using a red blood cell lysis buffer (BioLegend). Hearts were extensively flushed with PBS and then excised. Remote myocardium was separated from infarct and border zone using a dissection microscope, minced with scissors and digested in collagenase I (450 U/ml), collagenase XI (125 U/ml), DNase I (60 U/ml) and hyaluronidase (60 U/ml) (Sigma-Aldrich) at 37°C at 750 rpm for 1 hour. Hearts were subsequently homogenized through a 40-μm nylon mesh. Spleens were excised and homogenized through a 40-μm cell strainer. Bone marrow was flushed out from bones and homogenized through 40-μm cell strainers. Total viable cell numbers were obtained using Trypan blue (Cellgro, Mediatech, Inc.). For a myeloid cell staining, cells were first labeled with mouse hematopoietic lineage markers including anti-mouse antibodies directed against B220 (BD Biosciences, clone RA3-6B2), CD90 (BioLegend, clone 53-2.1), CD49b (BD Biosciences, clone DX5), Ly-6G (BD Biosciences, clone 1A8), NK1.1 (BioLegend, clone PK136) and Ter-119 (BD Biosciences, clone TER-119). This was followed by a second staining including CD45.2 (BD Biosciences, clone 104), CD11b (BD Biosciences, clone M1/70), CD115 (BioLegend, AFS98), CD11c (BD Biosciences, clone HL3), F4/80 (BioLegend, clone BM8), MhcII (BioLegend, clone M5/114.15.2), Ccr2 (R&D, clone 475301), and Ly6C (BD Biosciences, clone AL-21). Neutrophils were identified as (CD90/B220/CD49b/NK1.1/Ter-119)\textsuperscript{low}, (CD45.2/CD11b)\textsuperscript{high}, CD115\textsuperscript{low/int}, Ly6G\textsuperscript{high}. Monocytes were identified as (CD90/B220/CD49b/NK1.1/Ter-119)\textsuperscript{low}, CD11b\textsuperscript{high}, (F4/80/CD11c)\textsuperscript{low}, Ly-6C\textsuperscript{high/low} or (CD45.2/CD11b)\textsuperscript{high}, Ly6G\textsuperscript{low}, CD115\textsuperscript{high}, Ly-6C\textsuperscript{high/low}. Macrophages were identified as (CD90/B220/CD49b/NK1.1/Ter-119)\textsuperscript{low}, CD11b\textsuperscript{high}, Ly6C\textsuperscript{low/int}, Ly6G\textsuperscript{low}, F4/80\textsuperscript{high}. For a hematopoietic stem/progenitor cell staining, cell suspensions were labeled with biotin-conjugated anti-mouse antibodies (lineage for hematopoietic stem/progenitor staining) directed against B220 (eBioscience, clone RA3-6B2), CD11b (eBioscience, clone M1/70), CD11c (eBioscience, clone N418), NK1.1 (eBioscience, clone PK136), Ter-119 (eBioscience, clone TER-119), Gr-1 (eBioscience, clone RB6-8C5), CD8a (eBioscience, clone 53-6.7), CD4 (eBioscience, clone GK1.5) and Il7ra (eBioscience, clone A7R34) followed by a labeling with an anti-biotin pacific orange-conjugated streptavidin antibody. Cell suspensions were then stained with antibodies directed against c-kit (BD Bioscience, clone 2B8), Sca-1 (eBioscience, clone D7) and SLAM markers CD48 (eBioscience, clone HM48-1) and CD150 (BioLegend, clone TC15-12F12.2). Hematopoietic Lin⁻ Sca-1⁺ c-kit⁺ (LSK) were identified as (B220/CD11b/CD11c/NK1.1/Ter-119/Gr-1/CD8a/CD4/Ill7ra)\textsuperscript{low} c-kit\textsuperscript{high} Sca-1\textsuperscript{high}, hematopoietic stem cells (HSC) as (B220/CD11b/CD11c/NK1.1/Ter-119/Gr-1/CD8a/CD4/Ill7ra)\textsuperscript{low} c-kit\textsuperscript{high} Sca-1\textsuperscript{high} CD48\textsuperscript{low} CD150\textsuperscript{high}. Data were recorded with an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo version 9 (Tree Star, Inc.). For cell cycle analysis, cells were fixed and permeabilized after surface staining using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Intracellular staining was performed by targeting nuclear antigen Ki67 (eBioscience, clone SolA15) and DNA (4,6-diamidino-2-phenylindole, DAPI, FxCycle Violet Stain, Life Technologies).

**Cell sorting.** Before sorting of cardiac macrophages, we stained samples with biotin conjugated CD11b (BD Biosciences, clone M1/70) and subsequently with streptavidin microbeads (Miltenyi), performed a positive selection using magnetic-activated cell sorting (MACS, QuadroMACS Separator and LS Columns, Miltenyi), followed by sorting on a FACSARia II (BD Biosystems).

**Macrophage calculation from parabiosis experiments.** For calculating the contribution of recruitment to expansion of remote macrophage from the non-ischemic zone, we used data obtained in the parabiosis experiment shown in Figure 2D. We first assessed the chimera of GFP⁺ cells in blood and heart in the infarcted wild type parabiont (Figure 2E). We then
calculated recruitment by normalizing heart to blood chimerism for each mouse. For instance, if blood chimerism was 23% and heart chimerism 4.2%, recruitment equaled 18.3% after normalization to blood: 100% / 23% x 4.2% = 18.3% (Figure 2F). Four weeks after MI, we counted 4,884 macrophages/mg myocardium by flow cytometry, increased from 2,715 in steady-state (Figure 1C). Thus, an additional 2,169 macrophages accumulated per mg failing myocardium. The parabiosis experiment, and above calculation returned that on average, 14.5% of the total 4,884 macrophages originated from recruitment (on average 708 GFP+ macrophages/mg remote myocardium in non-GFP parabiont’s infarcted heart, experiment outlined in Figure 2D). We then normalized these 708 macrophages to the mean number of macrophages added by heart failure: 100% / 2,169 x 708 = 32.6%, yielding that approximately one third of the macrophage increase was driven by recruitment (Figure 2G).

**Cell culture of murine macrophages.** We euthanized wild-type mice and flushed the peritoneal cavity with 5 mL PBS containing 3% FBS and 0.4% EDTA and recovered the peritoneal fluid. We then stained cells with anti-mouse PE-conjugated antibodies directed against B220 (BD Biosciences, clone RA3-6B2), CD90 (BioLegend, clone 53-2.1), CD49b (BD Biosciences, clone DX5), NK1.1 (BioLegend, clone PK136) and Ter-119 (BD Biosciences, clone TER-119) and subsequently with anti-PE microbeads (Miltenyi). We next performed a negative selection using magnetic-activated cell sorting (MACS, QuadroMACS Separator and LD Columns, Miltenyi) according to the manufacturer’s instructions. After manually counting cell numbers, primary murine peritoneal macrophages were plated at a density of 1x10⁶ cells/dish in 13 mL of cell culture medium (DMEM containing 14% FBS, 2% Pen/Strep, 2% MEM, and 0.00086% β-ME, Corning and Sigma). 48h after plating, we exchanged the cell culture medium and added 1 mg of BrdU (bromodeoxyuridine, BD Bioscience) to each dish where appropriate, and then initiated stretching as described below. 24h later, cells were harvested by scraping the dishes. We then performed cell surface staining with anti-mouse PE-conjugated antibodies directed against B220 (BD Biosciences, clone RA3-6B2), CD90 (BioLegend, clone 53-2.1), CD49b (BD Biosciences, clone DX5), Ly-6G (BD Biosciences, clone 1A8), NK1.1 (BioLegend, clone PK136) and Ter-119 (BD Biosciences, clone TER-119). This analysis was followed by a second staining covering CD45.2 (BD Biosciences, clone 104), CD11b (BD Biosciences, clone M1/70), F4/80 (BioLegend, clone BM8), and Ly6C (BD Biosciences, clone AL-21). Intracellular staining for the nuclear antigen Ki67 (eBioscience, clone SolA15) or BrdU (BrdU flow kits, BD Bioscience) was carried out as described previously to assess proliferation⁷.

**ERK1/2 ELISA.** For the semi-quantitative measurements of phosphoERK1/2 (pT202/Y204) and total ERK1/2 an ELISA kit was used on lysed stretched and non-stretched cultured murine peritoneal macrophages in accordance to the instruction manual (ERK1/2 (pT202/Y204) + Total ERK1/2 SimpleStep ELISA Kit, Abcam).

**In-dish-imaging.** After stretching, we added F4/80 (BioLegend, clone BM8) directly to the culture medium. After 10 min of staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions and then stained with Ki67 (eBioscience, clone SolA15) and 4,6-diamidino-2-phenylindole (DAPI, FxCycle Violet Stain, Life Technologies). In-dish-imaging used a confocal microscope (Olympus IV100).

**Mek1/2 inhibition.** In cell culture experiments, we used the Mek1/2 inhibitor PD98059 (Calbiochem). We seeded primary peritoneal macrophages and replaced the cell culture medium 48h thereafter with PBS containing either 50 mmol/L PD98059 (dissolved in DMSO (dimethyl sulfoxide, Sigma-Aldrich)) or vehicle alone (DMSO). After 1h of incubation, we again replaced the PBS with cell culture medium and initiated stretching for 24h. For in vivo studies, we used PD0325901 (Selleckchem), a second-generation small-molecule inhibitor selective for Mek1/2. When we prepared PD0325901 for injections, we diluted it with Kolliphor EL (Sigma-
Aldrich) and PBS. We injected 10 mg/kg bodyweight PD0325901 i.p. once daily starting 7d after coronary ligation for three weeks. Control mice received vehicle (DMSO, Kolliphor EL, and PBS).

**Cell culture of human macrophages.** Peripheral blood monocytes were obtained from buffy coats from de-identified healthy donors. Monocytes were isolated by density gradient centrifugation with Lymphocyte Separation Medium 1.077 (Lonza). Adherent monocytes were differentiated into macrophages for 10 days in RPMI 1640 (Gibco) with L-glutamine and 5% human serum. Macrophages were rinsed once with sterile Hanks’ buffered saline solution (HBSS, Gibco) and harvested with a cell scraper, then seeded at a density of 1x10^6 on a silicon elastomer membrane assembled for mechanical strain. Surface staining relied on anti-human CX3CR1 (BioLegend, clone 2A9-1), CD14 (BioLegend, clone HCD14), CD11b (BioLegend, clone ICRF44) and CD68 (BioLegend Y1/82A).

**Magnetic resonance imaging (MRI).** MRI was carried out on 4 weeks after permanent coronary ligation as described perviosly. Using a 7 Tesla horizontal bore Pharmascan (Bruker) and a custom-built mouse cardiac coil (Rapid Biomedical), we obtained cine images of the left ventricular short axis which we analyzed using the software Segment (http://segment.heiberg.se). Four weeks after myocardial infarction, scar tissue was identified as akinetic myocardial segments with relevant thinning of the myocardial wall. Scar and remote volumes in each myocardial slice were quantified using OsiriX© software. 7-8 myocardial slice were analyzed per heart.

**CFU-assay.** A semi-solid cell culture medium (Methocult M3434, Stem Cell Technology) was used for performing colony forming unit (CFU) assays as recommended by the manufacturer. We flushed bones with Iscove’s Modified Dulbecco’s Medium (IMDM, Lonza) supplemented with 2% fetal calf serum. We plated 2x10^4 bone marrow cells in duplicates. After incubating for 12 days, we counted colonies on an inverted microscope.

**Quantitative real-time PCR.** RNA was extracted from either bone marrow or hearts using the RNeasy Mini Kit (Qiagen) or from FACS-isolated macrophages using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) according to the manufacturers’ protocol. mRNA was transcribed to complimentary DNA (cDNA) with the high capacity RNA to cDNA kit (Applied Biosystems). Quantitative PCR used Taqman probes (Applied Biosystems). Results were expressed by delta delta Ct values normalized to Gapdh.

**Noradrenaline high sensitivity ELISA.** For measurements of noradrenaline in the bone marrow, a 2–CAT (A–N) Research ELISA (kit EA633/96, Diagnostika GmbH) was carried out according to the manufacturer’s instructions. One femur was snap-frozen and immediately homogenized in a catecholamine stabilizing solution (pH adjusted to 7.5 using sodium hydroxide (1 N)) containing sodium metabisulfite (4 mM), EDTA (1 mM) and hydrochloric acid (0.01 N).

**Histologic analysis of murine hearts.** Hearts were flushed thoroughly with PBS and then harvested. We embedded hearts in O.C.T. compound (Sakura Finetek) and snap-froze them in a 2-methylbutane bath cooled with dry ice. For immunohistochemistry, 6 μm frozen sections were cut and stained using antibodies targeting α-smooth muscle actin (αSma, Abcam, ab5694), collagen I (Abcam, ab21286), and CD31 (BD Biosciences, clone MEC13.3). We then applied the respective biotinylated secondary antibodies and used a the VECTASTAIN ABC kit (Vector Laboratories, Inc.) and an AEC substrate (Dako) for color development. All sections were counterstained with Harris hematoxylin. We scanned slides and quantified the positive areas (five high-power fields per section and per animal) using iVision (version 4.5, Biovis).
**Histologic analysis of human hearts.** Human myocardial specimens were obtained from patients either at the time of a left ventricular assist device (LVAD) placement (ischemic cardiomyopathy samples) or from donors not suitable for transplantation (donor specimens). Ischemic cardiomyopathy tissue specimens consisted of transmural left ventricular myocardium harvested from the apex in patients with inferior and lateral infarcts. The presence of remote myocardium was confirmed by histological analysis. Donor tissue specimens consisted of transmural apical LV myocardium harvested from donors with normal left ventricular function as documented by echocardiography. Following procurement samples were fixed in 10% neutral buffer formalin, embedded in paraffin, and sectioned. For each specimen, at least three 4 µm sections were stained with antibodies specific for human anti-CD68 (Thermo, 1:2000) and Ki67 (Abcam, 1:1000). Antibody detection was performed using a tryamide-based signal amplification system per manufacturer protocol (Perkin Elmer). Samples were counterstained with DAPI and imaged on a Zeiss confocal microscope. Quantification of CD68 and Ki67 staining was then performed in blinded fashion. Human studies were reviewed, approved, and conducted in accordance with the Human Studies Committee Institutional Review Board at Washington University (IRB protocol 201305086).

**siRNA formulation into 7C1 nanoparticles.** Purified 7C1 nanoparticles were synthesized and formulated as previously described. Polyethyleneimine with a molecular weight of 600 (PEI₆₀₀, Sigma Aldrich) was combined with 200 proof anhydrous ethanol (Koptec) and an epoxide-terminated C₁₅ lipid at a lipid:PEI molar ratio equal to 14:1. The mixture was heated at 90°C for 48 hours before purification was performed with a silica column. To formulate nanoparticles, purified 7C1 was combined with 200 proof ethanol and C₁₄PEG₂₀₀₀ (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], Avanti Polar Lipids) at a 7C1:C₁₄PEG₂₀₀₀ molar ratio equal to 4:1 in a glass syringe. siRNA was dissolved in pH 3 10 mM citrate solution (Teknova) in a separate syringe. The two syringes were connected to a syringe pump and the fluid was pushed through a microfluidic device as previously described. The resulting nanoparticles were dialyzed in 1x PBS and filtered through a 0.22 µm sieve before their size was characterized.

**Nanoparticle and siRNA dosing.** Nanoparticles were injected intravenously via tail vein in a volume of 10 µL/g body weight. Particles contained either 3.0 mg/kg of siRNA targeting luciferase (termed siCtrl, non-targeting control siRNA) or 3.0 mg/kg of a combination of five siRNAs encapsulated in the same particle targeting the leukocyte adhesion molecules Icam1, Icam2, Vcam1, E-selectin, and P-selectin (termed siCAM⁵). The selection of siRNA sequences was described previously. The following sequences were used: Icam1, sense CUUUCCUUUGAAUAUAA, antisense UUAUUGAUUAAGGAAG; Icam2, sense AGGACGUUCACUCUUU, antisense GAAAAGUGAGCCGUCUU; Vcam1, sense ACCUGGUGACUUUCAGG, antisense ACCUGAAAGUCAACCCAGU; Eselectin, sense GCCAAGCGCUUCAUCGU, antisense AACGAUUGAAGGCUCUGGC; Pselectin, sense GCACAAAUGUAUGUCGU, antisense AUAGCAUAACUUUGUGC.
Supplemental Figure I.
Gating and quantification of cardiac macrophages in steady-state versus HFREF (6 and 12 weeks after MI, remote area), n=4-5 WT mice per group, mean±SEM, *p<0.05, ****p<0.0001).
Supplemental Figure II.
Timeline for red fluorescence signal in blood monocytes in fate mapping.
A, Experimental design.
B, Representative gating and assessment of tdTomato red fluorescence signal in blood Ly6C^{high} monocytes after a tamoxifen pulse, n=3 mice per group.
Supplemental Figure III.

Cellular source of Ccl2. Representative immunofluorescence histology of remote myocardium 4 weeks after MI (n=5 mice per group). Co-localization of Ccl2 with endothelial cells (CD31), macrophages (Mac3), fibroblasts (DDR2), and myofibroblasts (SMA). Scale bar, 10 µm.
Supplemental Figure IV.
Evaluation of scar area day 28 after MI by cardiac MRI. Treatment with either siCtrl or siCAM5 was initiated 7d after coronary ligation (n=9-11 per group, mean±SD).
Supplemental References


