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

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

Hendrik B. Sager[1,*], **Maarten Hulsmans**[1,*], **Kory J. Lavine**[2], **Marina B. Moreira**[3], **Timo Heidt**[1], **Gabriel Courties**[1], **Yuan Sun**[1], **Yoshiko Iwamoto**[1], **Benoit Tricot**[1], **Omar F. Khan**[4], **James E. Dahlman**[4,5], **Anna Borodovsky**[6], **Kevin Fitzgerald**[6], **Daniel G. Anderson**[4,5,7], **Ralph Weissleder**[1,8], **Peter Libby**[3], **Filip K. Swirski**[1], and **Matthias Nahrendorf**[1,9]

[1]Center for Systems Biology and Department of Imaging, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

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

[3]Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

[4]Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA, USA

[5]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA

[6]Alnylam Pharmaceuticals, Cambridge, MA, USA

[7]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

[8]Department of Systems Biology, Harvard Medical School, Boston, MA, USA

[9]Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

Abstract

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

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

Corresponding author: Matthias Nahrendorf, Center for Systems Biology, 185 Cambridge Street, Boston, MA 02114, Tel: (617) 643-0500, Fax: (617) 643-6133, mnahrendorf@mgh.harvard.edu, Hendrik B. Sager, German Heart Center Munich, Department of Cardiology, Lazarettstrasse 36, 80636 Munich (Muenchen), Germany, hendrik.sager@tum.de.
*These authors contributed equally.

Disclosures

J.E.D., and D.G.A. have filed intellectual property protection related to 7C1 nanoparticles. The authors declare that they have no further competing interests.

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^{high} monocytes rose from 64±5 to 108±9 /μl blood ($p<0.05$). Cardiac monocyte recruitment declined in *Ccr2*$^{-/-}$ 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 MAPK pathway, while 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 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 vs.19.1±2%, $p<0.05$).

Conclusions—Myocardial failure is influenced by an altered myeloid cell repertoire.

Keywords

macrophage; monocyte; heart failure

Subject codes

Inflammation; heart failure

Introduction

Heart failure commonly complicates ischemic heart disease. Incomplete reperfusion, large 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 non-ischemic myocardium, promoting progressive pump failure. These patients have unacceptable mortality rates[1–4], 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[5, 6]. In addition to the changes observed in myocytes, remodeling also alters non-myocyte parenchymal cells. Post-MI remodeling and heart failure associate with inflammation, documented by increased TNFα and circulating innate immune cells[7–11].

Resident macrophages represent about 6–8% of the non-cardiomyocyte population in the healthy mouse myocardium[12, 13], a number comparable to the frequency of resident macrophages in other tissues. Cardiac macrophage frequency is higher in newborn mice[14] and increases orders of magnitude in ischemic myocardium[15]. 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[15]. These two phases are essential for

healing of the infarct in mice[16, 17] and also occur in humans[18]. 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[19, 20]. The activation of monocyte production in the bone marrow and spleen begins with increased cell cycle entry and migration of $Ccr2^{+}$ hematopoietic progenitor cells[21], 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[22, 23]. Yet, macrophages also promote tissue destruction, for instance in atherosclerotic plaques[24, 25] and, if inflammation resolution is delayed, in the acute infarct[26]. The roles of cardiac resident macrophages during chronic post-MI remodeling are incompletely understood. Preclinical data[27, 28] 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[12, 29]), monocyte recruitment (as in acute ischemia[12, 29]), or both.

Here we test hypotheses regarding 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 (*Ubc*^{GFP}) 30Scha/J) mice, male and female B6.129P2(Cg)-Cx3cr1$^{tm2.1(cre/ERT2)Litt}$/WganJ mice expressing a Cre-ERT2 fusion protein and enhanced YFP, male and female B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/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, USA) and B. Lowell (Beth Israel Deaconess Medical Center, Boston, MA, USA). All procedures were approved by the Institutional Animal Care and Use Committee Subcommittee on Research Animal Care, Massachusetts General Hospital.

Myocardial infarction was induced by permanent ligation of the proximal left anterior descending coronary artery as described previously[30]. Mice were anesthetized with

isoflurane, and received buprenophine (0.1 mg/kg i.p.) twice daily for three days, starting on the day of the surgery.

Parabiosis

Mice were joined in parabiosis as described perviously[30]. Mice were anesthetized with isoflurane and received buprenophine (0.1 mg/kg i.p.) twice daily for three 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 0.67Hz for 24h) 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 ± standard error of mean unless stated otherwise. For two-group comparisons, an unpaired t-test was applied to normally distributed variables, and a Mann-Whitney test to non-normally distributed variables. For comparing more than two groups, an ANOVA test, followed by a Sidak's test for multiple comparisons, was applied. P values of < 0.05 indicated statistical significance.

siRNA formulation into 7C1 nanoparticles was done as previously described[34, 35].

Please see the supplemental material for siRNA dosing, flow cytometry, MRI, 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 non-ischemic, 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 four ($p<0.0001$, n=15–20 per group) and 7.23 mg/g at eight 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–C). Macrophage numbers in the mature infarct scar were low in comparison to the non-ischemic myocardium and declined over time (Figure I in the Online Data Supplement). We detected blood monocytosis in HFrEF mice (Figure 1D and 1E), a finding that resembles findings in heart failure patients[9, 10].

Origins of macrophages in post-MI heart failure

To determine if accumulation of remote myocardial macrophages relies on recruitment of monocytes from the blood, or alternatively from local proliferation, we pursued fate mapping experiments in *$Cx_3cr1^{CreER/+}$ $R26^{tdTomato/+}$* mice. In these mice, all fractalkine receptor (Cx_3cr1) expressing cells, including circulating monocytes and cardiac resident macrophages, express yellow fluorescent protein (YFP). After injection of tamoxifen, all Cx_3cr1^{pos} cells also express the red fluorescent protein tdTomato. Thus, shortly after tamoxifen challenge, blood monocytes and resident macrophages exhibit red and yellow fluorescence (Figure II in the Online Data Supplement). Three weeks later, circulating monocytes are replaced by newly-made cells which derive from hematopoietic progenitors that do not express Cx_3cr1. At this time point, blood monocytes and their progeny no longer express tdTomato (Figure II in the Online Data Supplement) while cells arising from local proliferation of Cx_3cr1^{pos} resident cardiac macrophages continue to express tdTomato. We infarcted mice three weeks after the last tamoxifen injection (Figure 2A) and assessed the myocardial frequencies of blood monocyte-derived YFP^{pos} $tdTomato^{neg}$ cells and locally sourced YFP^{pos} $tdTomato^{pos}$ 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 *Ubc^{GFP}* mouse, in which all leukocytes express green fluorescent protein (GFP), 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 GFP^{pos} 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). Based on 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 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 *$Cx_3cr1^{CreER/+}$ $R26^{tdTomato/+}$* mice and compared their gene expression to steady-state by qPCR. 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*, while locally sourced macrophages had higher mRNA for *Tnfα*, *Tgfβ1* and *Mrc-1*. These differences diverge from the canonical M1/M2 macrophage polarization pattern. For instance, locally sourced macrophages in failing hearts expressed more *Tnfα* (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 (Figure III in the Online Data Supplement). 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 for the recruitment of monocytes to inflammatory sites requires Ccl2/Ccr2 interaction[38–40]. While neutrophil numbers did not change, monocyte counts fell in the blood of *Ccr2*$^{-/-}$ mice four weeks after MI (Figure 3A). Infarcted *Ccr2*$^{-/-}$ mice recruited significantly fewer Ly6C^{high} 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[12, 17, 29]. 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 to the steady-state (Figure 4A).

Among many other adaptations during left ventricular remodeling, mechanical forces, which influence macrophage behavior[32, 41–45], 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 ~ 5mmHg to values that can exceed 30mmHg. This alteration profoundly increases wall tension and stress[46], leading to myocyte slippage[47, 48] and chamber dilation[5, 6, 49, 50]. Increased mechanical strain also acts on macrophages, which can sense tissue forces[32, 51]. 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, 4C). In addition to flow cytometry, confocal microcopy 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). Mek1/2 inhibition diminished strain-induced proliferation in vitro (Figure 4F). In vivo, Mek1/2 inhibition for three weeks, starting one 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 5A). 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 to 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 (HSPC) 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 6B). To explore which mechanism increased bone marrow myelopoiesis, we investigated the sympathetic nervous system (SNS), 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 β_3 adrenergic receptor. As a result, mesenchymal stromal cells alter their supply of hematopoietic niche factors[58]. Indeed, *Cxcl12* and *Angiopoietin1*, both promoters of HSC quiescence[59, 60], fell in the bone marrow of mice with HFrEF (Figure 6D). In addition, HFrEF reduced *Scf* and *Vcam-1* (Figure 6D), which retain HSC in the bone marrow niche[61]. When we neutralized bone marrow SNS 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), HSPC release into blood increased (Figure 7A). These cells seeded the spleen and induced extramedullary hematopoiesis: eight weeks after MI, spleen weight (Figure 7B), splenic HSPC proliferation (Figure 7C) and splenic numbers of innate immune cells rose (Figure 7D–F). 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 Icam-1, Vcam-1, E- and P-selectin increased significantly 4 weeks post MI (Figure 8A). Employing a recently established in vivo RNAi approach[34, 35], we silenced all 5 major adhesion molecules in cardiac endothelial cells (Figure 8A). This method relies on nanoparticle delivery of siRNA to endothelial cells and curbs leukocyte recruitment, as previously shown for atherosclerotic plaque and acute ischemia[35]. 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 five cell adhesion molecules or an irrelevant control siRNA. The treatment began one week after coronary artery ligation to reduce interference with the acute inflammatory phase after MI. After three weeks of RNAi, neutrophil, monocyte and macrophage numbers decreased significantly in the remote myocardium while blood leukocyte levels did not change (Figure 8B). We then examined the consequences of reduced myeloid cell recruitment by cardiac MRI. RNAi treatment led to smaller end-diastolic volumes and less impaired left ventricular ejection fraction (Figure 8C), while it did not influence infarct volume (Figure IV in the Online Data Supplement). 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[63]. 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 β_3 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 one week after coronary ligation, attenuates post-MI remodeling.

Data on the abundance, origin and function of macrophages 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 cardiovascular disease, macrophages have received considerable attention in atherosclerotic plaque and in the acutely ischemic heart. Macrophage functions likely help maintain cardiovascular health; however, activation of their inflammatory actions can unleash functions that promote disease[15, 37]. 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.

Prior work in humans and in mice indicates that, at least at chronic time points investigated by us, the infarct scar is relatively stable. In clinical delayed enhancement MRI, 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, while active, inflammation-associated remodeling occurs in the remote myocardium that was initially not ischemic[68, 69, 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. Comparable to the diseased vessel wall, monocyte recruitment increases in the chronically failing myocardium. Yet, local proliferation contributes the majority of cells at 4 weeks after

MI. Whether the ratio of locally sourced versus blood monocyte derived macrophages changes dynamically while heart failure evolves remains to be investigated. 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 ischemia[19] 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 β_3 adrenergic receptor were protected from bone marrow microenvironmental signals that push hematopoietic progenitors into active cell cycle phases. Sympathetic nervous signaling and the resulting reduction of Cxcl12 also induces bone marrow release of myeloid cells and their progenitors[19, 58]. These progenitors take up residence in the spleen to establish extramedullary hematopoiesis in HFrEF. Prior splenectomy experiments revealed that about half of all infarct macrophages derive from the organ[20, 74]. Previous work has also implicated the spleen in heart failure, and splenectomy reduced chronic heart failure in mice[28]. Astonishingly, injection of splenocytes from a mouse with MI led to heart failure in an otherwise healthy recipient[28], perhaps suggestive of autoimmunity. While it remains unclear which antigen induces autoimmunity after MI, these data highlight the complexity of the spleen as an immunological organ that harbors a panoply of immune cell types. Some of these splenocytes may have roles in heart failure, perhaps regulating myocyte health or macrophage activity. Eight weeks after a large MI, the spleen produces Ly6C^{high} monocytes, a function that the organ does not exhibit in the steady-state. Clinical data on the importance of the spleen for cardiovascular health are sparse, with one study reporting increased cardiovascular mortality in patients that lost their spleen in World War II[75]. Confounding due to the predisposition to infections with encapsulated microorganisms in splenectomized humans complicates the interpretation of this observation. Imaging of spleen size, metabolism and proliferation might circumvent the paucity of human spleen tissue available for analysis. ^{18}F-FDG PET data indicate that splenic glucose uptake increases in patients with acute coronary syndromes[76], a finding that may relate to increased myelopoiesis[78].

Wall stress increases in the failing heart, exposing all cells, including macrophages, to increased biomechanical strain. Macrophages respond to strain by inflammatory activation[51] and increased expression of scavenger receptors[32]. These phenomena could be of importance in hypertension, which exposes arterial macrophages to increased mechanical forces[32]. Our in vitro data imply that strain increases macrophage proliferation. While the precise nature of mechanosensing remains uncertain, 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[78], although this effect may also relate to growth factor signaling and oxidative stress[79]. 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 due to 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[34, 35], 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[80–82]. The used nanomaterial directs uptake of siRNA to endothelial cells and leads to sufficient silencing with low toxicity[34]. The therapy began one 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[35], lesions found in patients with ischemic cardiomyopathy. Thus, this treatment approach may not only attenuate left ventricular remodeling but also decrease the risk of re-infarction. 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[23]. Reparative monocytes and macrophages can elaborate Vegfa after MI, and thus support regeneration and healing[14, 16, 83]. While 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. Further, we found a non-significant trend towards higher blood leukocyte numbers in the siCAM5 treatment group (Figure 8B). While 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 CAMs. 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 a number of 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. Further, 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[19], and angiotensin II regulates the release of splenic monocytes[75], suggesting that beta blockers and ACE 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations and acronyms

ADRB3	β_3 adrenergic receptor
Angpt-1	angiopoetin-1
BrdU	5-bromo-2′-deoxyuridine
Ccr2	Chemokine (C-C Motif) Receptor 2
Cxcl12	chemokine (C-X-C motif) ligand 12
DAPI	4,6-diamidino-2-phenylindole
Erk	extracellular signal-regulated kinase
GFP	green fluorescent protein
HFrEF	heart failure with reduced ejection fraction
HSC	hematopoietic stem cells
HSPC	hematopoietic stem and progenitor cells
LVAD	left ventricular assist device
Mapk	mitogen-activated protein kinase
Mek	mitogen-activated protein kinase kinase
MI	myocardial infarction
MRI	magnetic resonance imaging
NA	noradrenaline
Scf	stem cell factor

SNS	sympathetic nervous system
Vcam-1	vascular cell adhesion molecule 1
WT	wild type

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Novelty and Significance

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, the white cell blood count correlates with mortality.
- Macrophages participate in acute and chronic tissue remodeling via phagocytosis and cross talk with stromal cells.

What new information does this article contribute?

- Higher sympathetic tone in mice with heart failure fuels systemic myeloid cell production in hematopoietic tissues.
- Myocardial macrophages proliferate locally, potentially due to higher mechanical strain in the failing heart.
- Inhibition of monocyte recruitment to the post-MI heart reduces heart failure, indicating that inflammatory bone marrow derived cardiac macrophages enhance left ventricular remodeling.

Macrophages reside in the healthy heart, and are recruited in large numbers into acutely ischemic tissues. While it is known that the cells regulate tissue health, their kinetics, sources and functions are not well understood in the setting of chronic heart failure. Here we describe that in the months after cardiac ischemia, macrophage numbers increase in the remote myocardium due to i) local macrophage proliferation and ii) due to monocyte recruitment. Increased local proliferation results in part from mechanic stress in failing hearts. Recruited monocytes, which are made in the bone marrow and spleen, contribute about one third to the increase in cardiac macrophages. If monocyte recruitment is dampened, left ventricular remodeling is attenuated. These data provide causal evidence that monocyte recruitment contributes to post-MI heart failure.

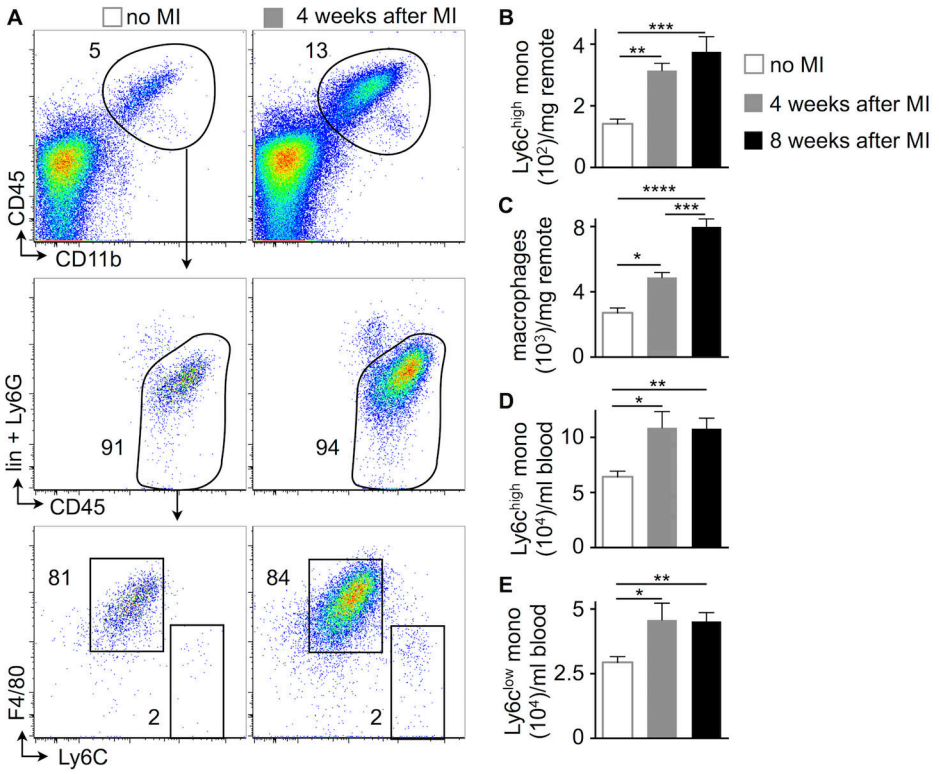

Figure 1. Expansion of cardiac macrophages in HFrEF

A–C, Gating and quantification of myeloid cells in steady-state versus 4 and 8 weeks after MI in the remote area (i.e. the myocardium that was never ischemic), n=8–23 WT mice per group, mean±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

D and E, Blood monocytes in steady-state versus 4 and 8 weeks after MI, n=8–23 WT mice per group, mean±SEM, *p<0.05, **p<0.01.

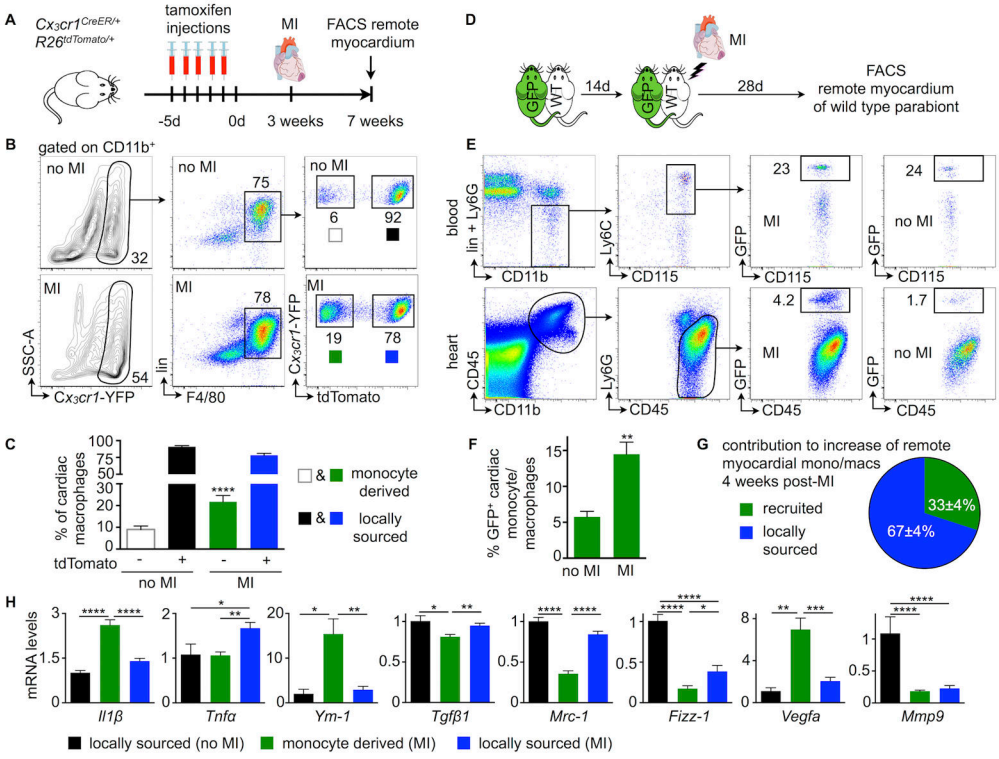

Figure 2. Contribution of recruitment to cardiac macrophage expansion in HFrEF

A, Experimental design.

B and C, Gating and quantification of resident versus bone marrow-derived cardiac macrophages in steady-state versus 4 weeks after MI, n=4–8 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 versus 4 weeks after MI, n=4–10 pairs per group, mean±SEM, **p<0.01.

G, Relative contribution of monocyte-derived versus locally sourced macrophages to total remote monocyte/macrophage population 4 weeks after MI, n=4–10 pairs per group, mean ±SEM.

H, Phenotyping of resident versus bone marrow-derived cardiac macrophages using fate mapping outlined in 2A (4 weeks after MI, n=4–8 per group, mean±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

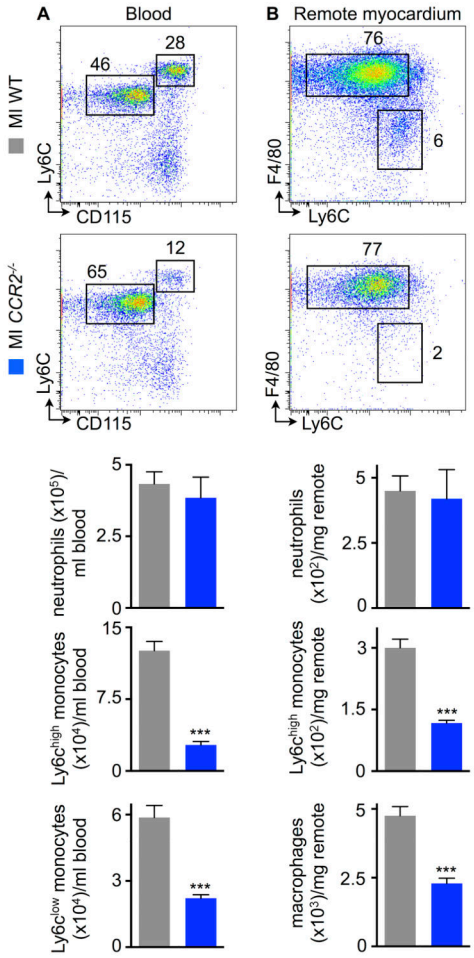

Figure 3. Ccr2 dependent monocyte recruitment contributes to cardiac macrophage expansion

A, Gating and quantification of blood myeloid cells in WT vs. *Ccr2*$^{-/-}$ mice, 4 weeks after MI, n=6–8 per group, mean±SEM, ***p<0.001.

B, Gating and quantification of cardiac myeloid cells in WT vs. *Ccr2*$^{-/-}$ mice, 4 weeks after MI, n=6–8 per group, mean±SEM, ***p<0.001.

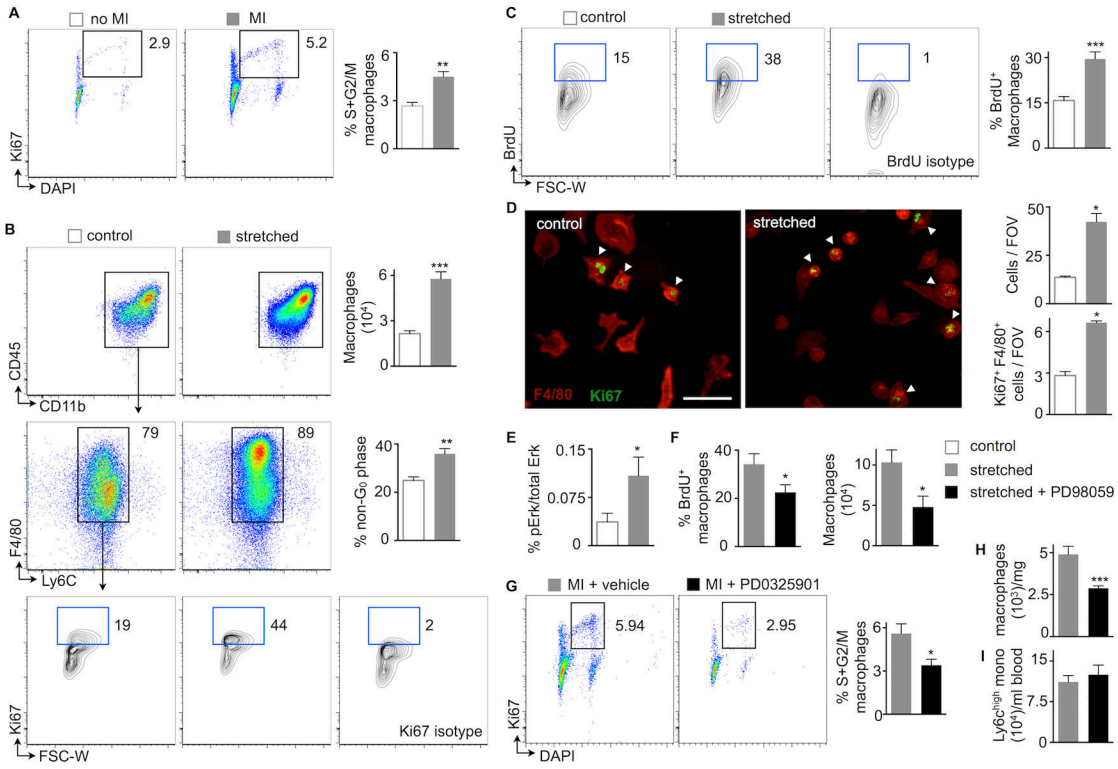

Figure 4. In situ macrophage proliferation and the role of biomechanical strain

A, Gating and quantification of cardiac macrophage proliferation in steady-state versus 4 weeks after MI, n=6–9 per group, mean±SEM, **p<0.01.

B and C, Gating, quantification of cell numbers and proliferation with Ki67 (B) and BrdU (C) in stretched vs. non-stretched cultured murine peritoneal macrophages (n=6–8 dishes per group, mean±SEM, **p<0.01, ***p<0.001).

D, In-dish confocal microscopy, macrophage numbers and macrophage proliferation in stretched versus non-stretched murine cultured peritoneal macrophages (n=5 per group, mean±SEM, *p<0.01).

E, PhosphoErk1/2 (pT202/Y204) to total Erk1/2 ratio in stretched versus non-stretched cultured peritoneal murine macrophages by ELISA (n=6 per group, mean±SEM, *p<0.05).

F, Cell numbers and BrdU incorporation in stretched cultured peritoneal murine macrophages that were treated with Mek inhibitor (n=6 per group, mean±SEM, *p<0.05).

G and H, Gating and quantification of cardiac macrophage proliferation and numbers in mice with HFrEF, treated with a Mek inhibitor (4 weeks after MI, n=7–8 per group, mean ±SEM, *p<0.05, ***p<0.001).

I, Blood monocytes in mice with HFrEF, treated with a Mek inhibitor (n=7–8 per group, mean±SEM).

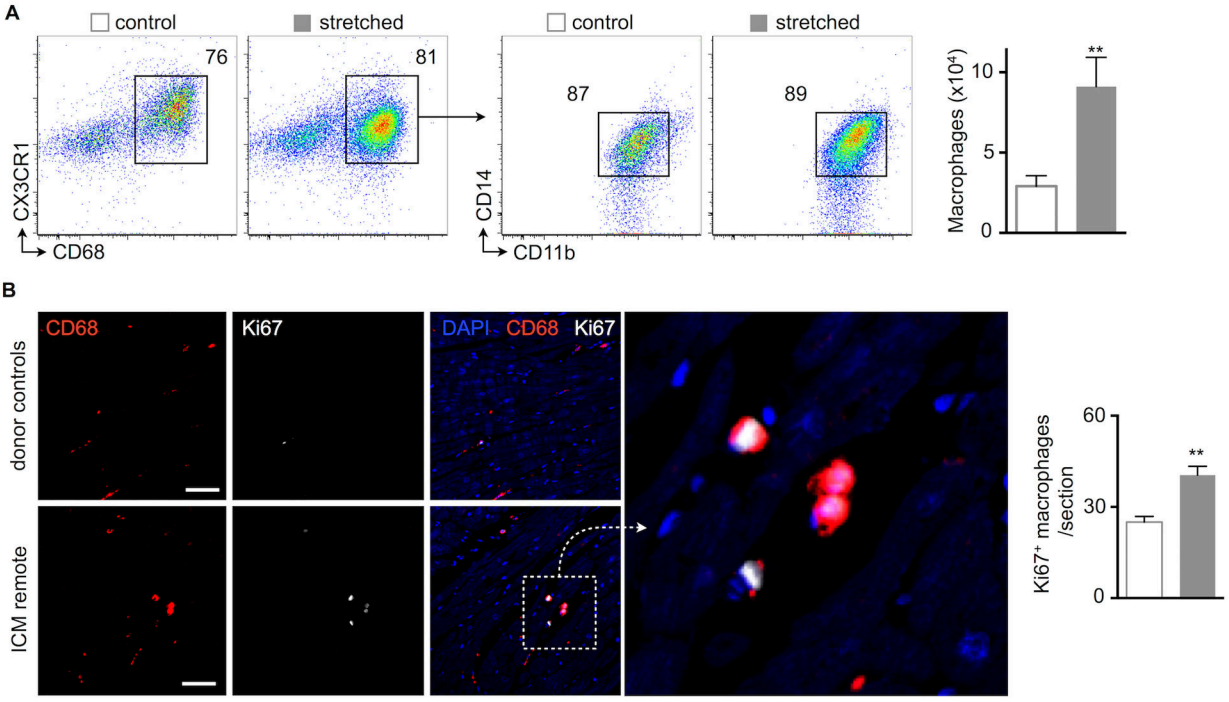

Figure 5. Strain enhances proliferation of human macrophages

A, Strain exposure of human macrophages. Gating and quantification of stretched versus non-stretched human primary macrophages (n=12 per group).

B, Histological evaluation of heart tissue obtained from patients with ischemic cardiomyopathy undergoing left ventricular assist device implantation. Controls are unused donor hearts (n=8–11 per group, mean±SEM, **p<0.01).

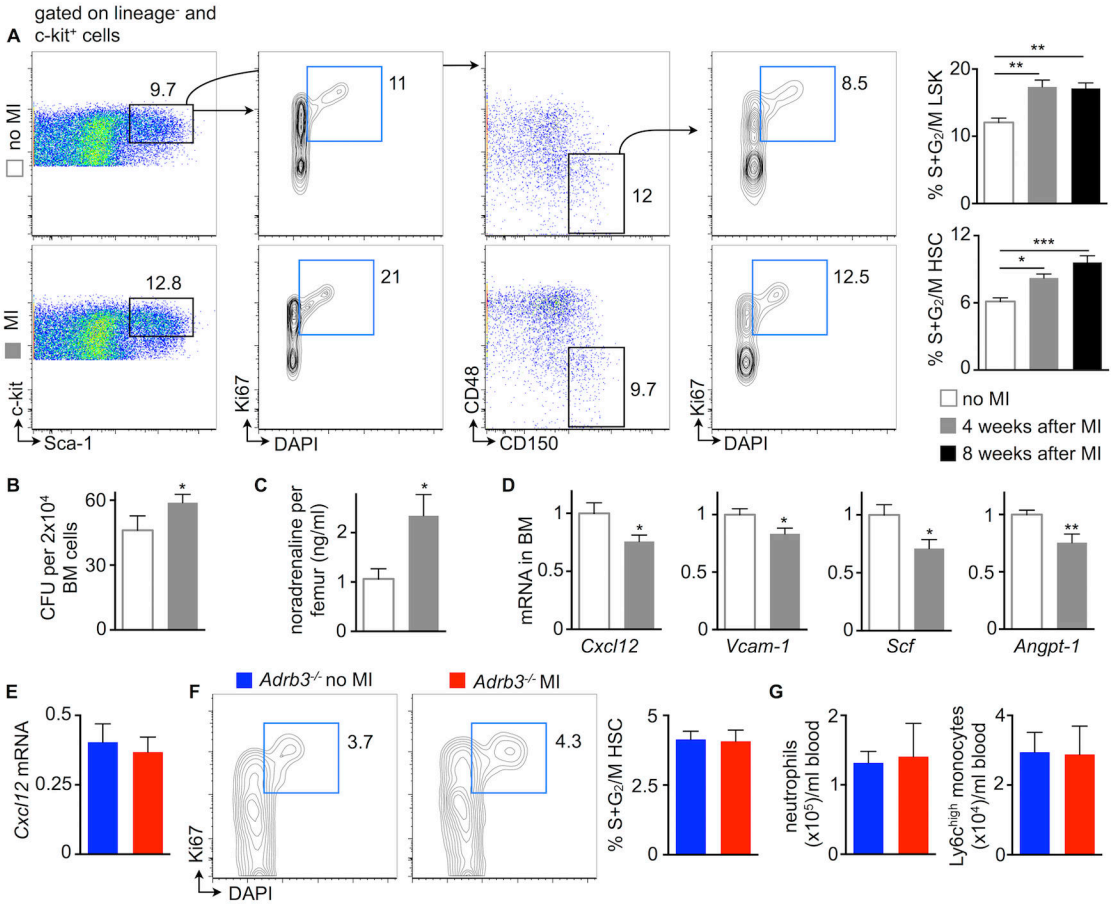

Figure 6. HFrEF activates bone marrow hematopoiesis

A, Gating and quantification of bone marrow hematopoietic stem and progenitor cell proliferation in steady-state versus 4 and 8 weeks after MI, n=9–11 per group, mean±SEM, *p<0.05, **p<0.01, ***p<0.001.

B, Bone marrow colony forming unit (CFU) assay in steady-state versus HFrEF (n=5 per group, mean±SEM, *p<0.05).

C, Bone marrow noradrenaline in steady-state versus 4 weeks after MI, n=5–7 per group, mean±SEM, *p<0.05.

D, mRNA of bone marrow hematopoietic stem cell (HSC) retention factors (*Cxcl12, chemokine (C-X-C motif) ligand 12; Vcam-1, vascular cell adhesion molecule 1; Scf, stem cell factor; Angpt1, angiopoietin-1*) in bone marrow in steady state versus 4 weeks after MI, n=10 per group, mean±SEM, *p<0.05, **p<0.01.

E, mRNA of HSC retention factor *Cxcl12* in steady-state Adrb3$^{-/-}$ versus *Adrb3*$^{-/-}$ mice 4 weeks after MI, n=5–6 per group, mean±SEM.

F, Gating and quantification of bone marrow hematopoietic stem and progenitor cell proliferation in steady-state *Adrb3*$^{-/-}$ versus *Adrb3*$^{-/-}$ mice 4 weeks after MI, n=5–6 per group, mean±SEM.

G, Quantification of blood neutrophils and monocytes in steady-state *Adrb3*$^{-/-}$ versus *Adrb3*$^{-/-}$ mice 4 weeks after MI, n=5–6 per group, mean±SEM.

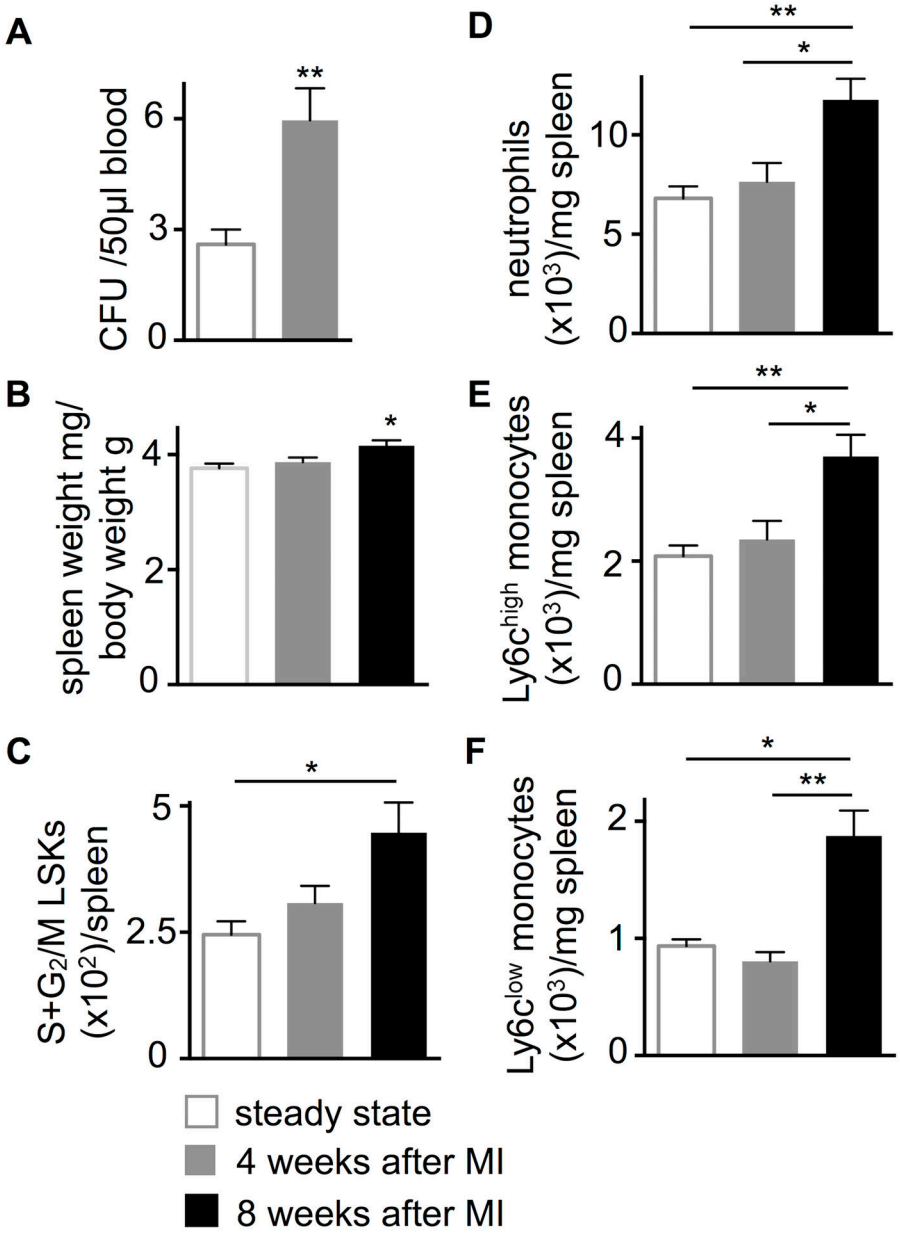

Figure 7. HFrEF activates splenic myelopoiesis

A, Blood colony forming unit (CFU) assay in steady-state versus 4 weeks after MI, n=5–12 per group, mean±SEM, **p<0.01.

B, Spleen weight in steady-state versus 4 and 8 weeks after MI, n=9–20 per group, mean ±SEM, *p<0.05.

C, Splenic hematopoietic stem and progenitor cell proliferation in steady state versus 4 and 8 weeks after MI, n=7–16 per group, mean±SEM, *p<0.05.

D–F, Splenic myeloid cells in steady-state versus 4 and 8 weeks after MI, n=7–16 per group, mean±SEM, *p<0.05, **p<0.01.

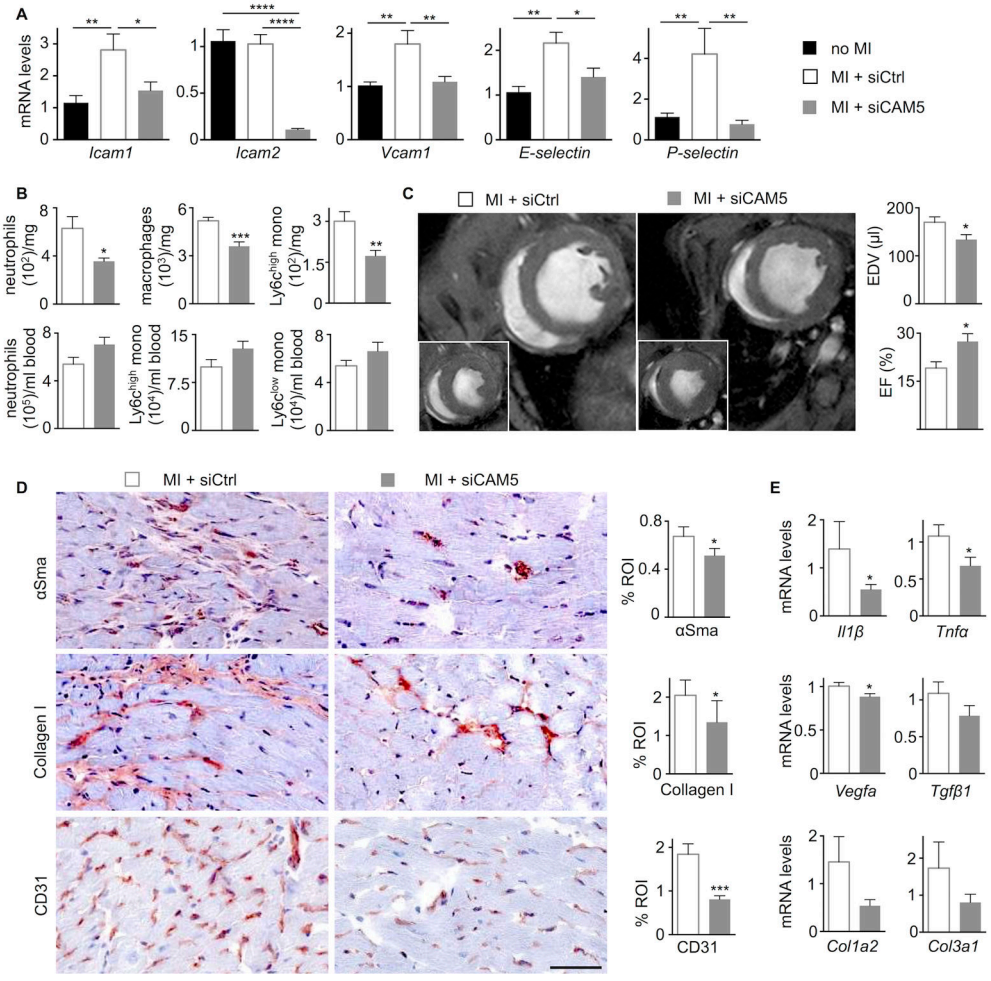

Figure 8. Recruited macrophages contribute to HFrEF development

A, Endothelial cell adhesion molecule mRNA levels in remote myocardium, values normalized to *Gapdh* (treatment with either siCtrl or siCAM5 for three weeks starting one week after MI, n=9–11 per group, mean±SEM, *p<0.05, ***p<0.001, ****p<0.0001).

B, Blood and cardiac myeloid cells in mice with HFrEF that received RNAi treatment with either siCtrl or siCAM5 for three weeks starting one week after MI (n=9–11 per group, mean±SEM, *p<0.05, **p<0.01, ***p<0.001).

C, Evaluation of post-MI remodeling by cardiac MRI. Each panel shows the mid-ventricular short axis view at end-diastole and end-systole (inset). End-diastolic volumes (EDV) and left ventricular ejection fraction (EF) were measured on day 28 after MI (n=9–11 per group, mean±SEM, *p<0.05).

D, Immunohistochemical evaluation of remote myocardium in mice with HFrEF for myofibroblasts (α-smooth muscle actin, αSMA), collagen (collagen-1), and vessels (CD31). Bar graphs show percentage of positive staining per region of interest (ROI) or number of vessels per high-power field (hpf). Scale bar, 50 µm (n=9–11 per group).

E, mRNA levels in remote myocardium, values normalized to *Gapdh* (treatment with either siCtrl or siCAM5 for three weeks starting one week after MI (n=9–11 per group, mean ±SEM, *p<0.05).