Targeting Interleukin-1β Reduces Leukocyte Production After Acute Myocardial Infarction

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Background—Myocardial infarction (MI) is an ischemic wound that recruits millions of leukocytes. MI-associated blood leukocytosis correlates inversely with patient survival, yet the signals driving heightened leukocyte production after MI remain incompletely understood.

Methods and Results—With the use of parabiosis surgery, this study shows that soluble danger signals, among them interleukin-1β, increase bone marrow hematopoietic stem cell proliferation after MI. Data obtained in bone marrow reconstitution experiments reveal that interleukin-1β enhances hematopoietic stem cell proliferation by both direct actions on hematopoietic cells and through modulation of the bone marrow’s hematopoietic microenvironment. An antibody that neutralizes interleukin-1β suppresses these effects. Anti-interleukin-1β treatment dampens the post-MI increase in hematopoietic stem cell proliferation. Consequently, decreased leukocyte numbers in the blood and infarct reduce inflammation and diminish post-MI heart failure in ApoE−/− mice with atherosclerosis.

Conclusions—The presented insight into post-MI bone marrow activation identifies a mechanistic target for muting inflammation in the ischemically damaged heart. (Circulation. 2015;132:1880-1890. DOI: 10.1161/CIRCULATIONAHA.115.016160.)

Key Words: hematopoiesis ■ hematopoietic stem cells ■ interleukin-1β ■ myocardial infarction

Myocardial infarction (MI) inflicts a sterile cardiac wound that, within minutes, recruits leukocytes from circulation at a rate of several hundred thousand cells per day. This demand depletes blood pool leukocytes quickly and requires continuous resupply over the next several days. In mice, the spleen initially serves as a leukocyte reservoir contributing ≈50% of myeloid cells to the infarct in the early hours after coronary ligation. Thereafter, emergency hematopoiesis fuels the increased demand for myeloid cells.5,6 Sympathetic nervous system activity triggers hematopoietic progenitor migration to the spleen, initiating extramedullary myelopoiesis.3

Clinical Perspective on p 1890

The mechanisms of increased hematopoietic system activity after ischemic injury are incompletely understood.5 The sympathetic nervous system activates the bone marrow after MI1 and in mice exposed to chronic psychosocial stress.5 This activation increases hematopoietic stem and progenitor cell (HSPC) proliferation and migration via chemokine C-X-C motif ligand 12 (CXCL12)/C-X-C chemokine receptor type 4 (CXCR4) signaling.7 Soluble factors released from ischemic myocardium into the blood may also signal to the bone marrow to drive hematopoietic stem cell (HSC) proliferation remotely. These post-MI stimuli could act on either HSCs directly or niche cells that regulate the bone marrow microenvironment. Data obtained from mice with infection or injected systemic stimuli suggest that circulating danger signals may activate hematopoiesis.8,9

The proinflammatory cytokine interleukin-1β (IL-1β) may provide 1 such hematopoiesis activation signal. Increased bone marrow progenitor proliferation after injection of the chemical compound alum, which is used as a vaccination adjuvant, was attenuated in IL-1 receptor–deficient mice.10 IL-1β also stimulates myelopoiesis in obesity.11 IL-1β is first synthesized as its cytosolic precursor pro-IL-1β and then gives rise to its active form via caspase-1, an enzyme in turn regulated by the NLRP3 inflammasome.12,13 IL-1β and IL-1α both signal using the receptor IL-1R1.14,15 IL-1R2, the
other IL-1 receptor, functions as a decoy for IL-1β.16,17 Further, IL-1β instigates inflammation in atherosclerotic plaque18 and ischemic myocardium.13 IL-1β rises in patient serum after acute MI,19 and both preclinical and clinical pilot data suggest that anti-IL-1 therapy can provide benefit after MI20–22 and in atherosclerosis.23–25

This study shows that, after MI, soluble factors that reach the bone marrow via the circulation significantly accelerate hematopoiesis. Parabiosis experiments revealed that IL-1β stimulates systemic leukocyte production by (a) directly acting on hematopoietic stem cells and (b) modulating the stem cell microenvironment in the bone marrow. Administration of an anti-mouse IL-1β reduced post-MI emergency hematopoiesis and attenuated leukocytosis. In ApoE−/− mice with atherosclerosis, anti-IL-1β therapy moderated leukocyte overproduction, supported resolution of infarct inflammation, and ameliorated post-MI heart failure.

Methods

A detailed methods description is provided in the online-only Data Supplement.

Experimental Animals

We used female C57BL/6j (wild-type [WT], n=162), B6.129S7-I1r1tm1Imx/J (IL1R1−/−, n=28) and apolipoprotein E−/− mice (ApoE−/−; B6.129P2-Apoetm1Unc/J, n=24) mice aged 8 to 12 weeks (The Jackson Laboratories, Bar Harbor, ME) for our studies. We also used transgenic mice expressing green fluorescent protein under the Nestin-promoter (Nestin-green fluorescent protein, n=10).26,27 Nestin-green fluorescent protein mice were a gift from Dr Gregori Enikolopov (Cold Spring Harbor Laboratory, NY). Age-matched mice were randomly allocated either to control or treatment groups. The study was approved by the Subcommittee on Animal Research Care at Massachusetts General Hospital (Boston, MA).

Myocardial Infarction Surgery

Myocardial infarction was induced by permanent ligation of the left anterior descending coronary artery as described previously.3 Ischemia reperfusion injury was induced and assessed as described previously.29 Please also see the online-only Data Supplement.

Neutralizing IL-1β

The IL-1β-neutralizing antibody was a donation from Novartis (Basel, Switzerland). The antibody selectively binds IL-1β, thus blocking the interaction of the cytokine with its receptors. We used a monoclonal, mouse anti-mouse IL-1β IgG2a/k antibody derived from an IgG2a/k antibody as described by Geiger et al.29 The in vitro potency IC50 is ~25 pmol/L, and its affinity to murine IL-1β is ~300 pmol/L. The t1/2 in mice is 14 days.29 We initiated treatment 2 hours after the induction of MI with subcutaneously injecting 10 mg/kg bodyweight of the IL-1β antibody (or a mouse monoclonal IgG2a antibody raised against cyclosporine A as isotype control). We repeated injections once weekly over the study period.

Bromodeoxyuridine Experiments

To assess proliferation, we used FITC/APC bromodeoxyuridine (BrdU) flow kits (BD Bioscience). For these BrdU pulse experiments, 1 mg BrdU was injected intraperitoneally 24 hours before euthanization and subsequent organ harvest. After surface staining, intracellular BrdU staining was performed according to the manufacturer’s protocol. Flow cytometry and cell sorting procedures are described in the online-only Data Supplement.

Bone Marrow Reconstitution

We lethally irradiated WT or IL1R1−/− mice with 950 cGy. We then lethally irradiated mice with 2×106 whole bone marrow cells from either WT or IL1R1−/− mice. Two to 4 months later mice received coronary ligation. Please also see the online-only Data Supplement.

Administration of Recombinant Mouse IL-1β

We injected 2.5 µg of recombinant mouse IL-1β (R&D Systems, Minneapolis, MN) intraperitoneally daily over 2 days as described elsewhere31 and then harvested the bone marrow 48 hours after the first administration.

Statistics

Statistical analyses were performed using GraphPad Prism software version 6 (GraphPad Software, Inc). Results are displayed as mean±standard deviation (SD). First, values were tested for Gaussian distribution (D’Agostino-Pearson omnibus normality test). For 2-group comparisons, an unpaired t test was applied to normally distributed variables, and a Mann-Whitney test was applied to nonnormally distributed variables. For comparing >2 groups, a 1-way analysis of variance test, followed by a Sidak test for multiple comparisons, was applied. P values of <0.05 indicated statistical significance.

Results

IL-1β Released After MI Activates Bone Marrow Hematopoiesis

It is unclear how the hematopoietic system, which responds to acute myocardial ischemia with increased proliferation, receives signals to increase leukocyte production. We asked whether HSC activation relies on a circulating factor, released after MI, that reaches the bone marrow via the blood. To test this hypothesis, we joined the circulation of 2 WT mice in parabiosis. After 2 weeks, a period necessary to establish shared circulation, 1 parabiont underwent coronary ligation but the other did not. Two days later, we assessed HSC proliferation in the bone marrow of the noninfarcted parabiont by BrdU incorporation (Figure 1A). Lin− Sca-1+ c-Kit+ CD48− CD150+ HSC proliferation in noninfarcted parabionts increased significantly (Figure 1A), indicating that factor(s) circulating in blood contribute to hematopoietic activation after MI. In humans, IL-1β levels rise in the blood after MI.9 Accordingly, we found IL-1β mRNA and protein levels increased in the infarcted heart (Figure 1B), in circulation and in the bone marrow (Figure 1C). These data indicate this cytokine transfers signals from the site of ischemic injury to the hematopoietic system. Injecting recombinant mouse IL-1β into naive mice triggered HSC proliferation similar to that observed after MI (Figure 1D). We next joined WT and interleukin-1 receptor 1 knockout mice (IL1R1−/−) in parabiosis. Two days after coronary artery ligation in 1 parabiont, increased HSC proliferation observed in the nonischemic WT parabionts significantly diminished in IL1R1−/− mice (Figure 1A). Taken together, these data indicate that IL-1β crossed from the circulation of the infarcted parabiont to that of the noninfarcted parabiont and instigated increased HSC proliferation in the bone marrow.

IL-1β Effector Cells

Bone marrow stromal cells form a microenvironment that harbors HSPCs.3 These niche cells maintain hematopoiesis
by producing factors that signal to HSPCs and regulate their dormancy, retention, and lineage bias. Therefore, our next question addressed how IL-1β stimulates HSC proliferation: does IL-1β affect hematopoietic cells directly or act on niche cells regulating HSC activity? To address this issue, we investigated which bone marrow cells can sense IL-1β.

Using quantitative real-time polymerase chain reaction in fluorescence-activated cell sorted bone marrow (for gating; please see Figure 2A), we found that HSC, Lin- Sca-1+ c-Kit+ (LSK), and all stromal cells regulating HSPC activity, including mesenchymal stromal cells, endothelial cells, and osteoblasts, express the IL1R1 (Figure 2A), with higher expression in the nonhematopoietic niche cells. We then reconstituted lethally irradiated WT mice with whole bone marrow from either WT (recipients' bone marrow HSC and bone marrow stromal cells are IL1R1+/+) or from IL1R1–/– mice (recipients' bone marrow HSC lack IL1R1, whereas the bone marrow stromal cell are IL1R1 competent). Four months later, mice underwent coronary ligation. Subsequent analysis showed significantly decreased HSC proliferation in mice reconstituted with IL1R1–/– bone marrow (Figure 2B). In these mice, in which hematopoietic but not niche cells were unresponsive to IL-1β, BrdU incorporation in HSC was still markedly above chimeras without MI (Figure 2B). The data indicate that the hematopoietic effect of IL-1β involves direct and indirect action on hematopoietic cells. To compare IL-1β effects on HSC with indirect effects on hematopoiesis via niche cells, we transplanted WT bone marrow into lethally...
irradiated IL1R1−/− recipients (Figure IA in the online-only Data Supplement). In this scenario, bone marrow hematopoietic cells are responsive to IL-1β but the marrow’s stromal cells are not. In a second group, lethally irradiated WT mice received IL1R1−/− bone marrow, resulting in the opposite situation. We then investigated the relevance of these 2 scenarios for post-MI recovery (Figure IB and IC in the online-only Data Supplement). Cardiac function by MRI was similar in both groups 21 days after coronary ligation, and blood leukocyte numbers were also comparable (Figure ID through IF in the online-only Data Supplement) indicating that the direct action of IL-1β on HSPCs and indirect action via stromal cells are equally important for the post-MI increase in leukocyte production.

Neutralizing IL-1β Reduces Post-MI Bone Marrow HSPC Proliferation and Leukocytosis

Thus far, these data show IL-1β involvement in triggering post-MI emergency hematopoiesis. This activated heart–bone marrow axis may represent a therapeutic target for reducing leukocyte overproduction after MI, a process that may lead to heart failure and secondary ischemic events. To test how IL-1β neutralization changes leukocyte production, we chose the clinically relevant murine version of canakinumab. Treatment began 2 hours after coronary ligation, and we assessed bone marrow HSPC proliferation by measuring BrdU incorporation 48 hours later. HSC and LSK proliferation were markedly lower in treated mice than in a control IgG-treated cohort that also underwent coronary artery ligation (Figure 3A). The treatment effects were somewhat lower than the decrease in HSC proliferation observed in IL-1R−/− mice, possibly because IL-1R1−/− mice lack both IL-1α and IL-1β. Nevertheless, antibody treatment significantly reduced frequencies of HSC and LSK in the active S+G2/M phase of the cell cycle, as determined by Ki67 and DAPI staining for cell cycle analysis (Figure 3B). A third method of measuring hematopoietic progenitor proliferation detected a lower number of colony forming units derived from the bone marrow of treated mice (Figure 3C). Reduced hematopoietic progenitor activity led to fewer circulating neutrophils on day 3 and decreased neutrophil and Ly6Chigh monocyte levels on day 7 after MI (Figure IIA in the online-only Data Supplement and Figure 3D).

Neutralizing IL-1β Reduces Extramedullary Hematopoiesis

The bone marrow chimera data indicate that IL-1β may act on bone marrow niche cells. Indeed, anti-IL-1β treatment preserved mRNA levels of vascular cell adhesion molecule 1, angiopoietin-1, and osteopontin after coronary ligation (Figure 4A). Cell-specific treatment effects are shown in Figure III in the online-only Data Supplement. These effects likely contributed to the treatment-induced decrease in myeloid cell production, because angiopoietin-1, osteopontin,
and CXCL12 regulate hematopoietic progenitor quiescence. Vascular cell adhesion molecule 1 retains HSPCs in the hematopoietic niche. When vascular cell adhesion molecule 1 expression falls after MI, HSPCs release into circulation and seed the spleen. Indeed, HSPC release from the bone marrow into the blood decreased in mice treated with the anti-IL-1β antibody (Figure 4B). Consequently, spleens of mice treated with anti-IL-1β antibody contained fewer LSKs and granulocyte-macrophage progenitors than controls (Figure 4C).

Neutralizing IL-1β Reduces Inflammation in Myocardial Infarcts

Leukocytes mobilize rapidly to the infarct after onset of ischemia, and, because of their fast turnover, the extent of their accumulation depends on their supply from the circulation. We consequently tested whether lowering blood leukocyte numbers in the anti-IL-1β treatment group would reduce leukocyte recruitment to the ischemic heart. Indeed, we detected fewer recruited myeloid cells on day 3 and 7 after coronary ligation by flow cytometry and immunohistochemistry (Figure 5 and Figure IIB in the online-only Data Supplement). These data corroborate previous reports by the Frangogiannis group.

Therapeutically Targeting IL-1β Signaling Improves Infarct Healing and Reduces Post-MI Remodeling

We next evaluated the impact of antibody-mediated IL-1β signaling blockade on infarct healing and post-MI heart failure in ApoE−/− mice on a high-fat diet. We chose to study...
dyslipidemic mice because they mimic 2 aspects of clinical myocardial infarction: an atherogenic milieu and chronically activated innate immunity. Even without MI ApoE−/− mice have augmented myelopoiesis caused by increased progenitor cycling in the bone marrow and the spleen. In a serial imaging trial, delayed enhancement MRI determined
comparable infarct size in both cohorts on day 1 after permanent occlusion of the coronary artery (Figure 6A). In a separate cohort, anti-IL-1β treatment did not change ischemia reperfusion injury (Figure IV in the online-only Data Supplement). On day 7 after MI, mice underwent fluorescence molecular tomography-computed tomography imaging to determine infarct protease activity with a molecular imaging agent (pan-cathepsin reporter). At this time point, this molecular imaging signal reports on the resolution of cardiac inflammation. Fluorescence molecular tomography-computed tomography revealed that anti-IL-1β treatment significantly reduced protease activity in the infarct (Figure 6B) which we located by using the hybrid CT modality. Mice were then reimaged by MRI 3 weeks after MI. In comparison with controls, anti-IL-1β-treated ApoE–/– mice exhibited smaller end-diastolic volumes and a better preservation of the left ventricular ejection fraction (Figure 6C). Histological analysis of wound-healing biomarkers in a cohort of WT mice euthanized on day 7 after coronary ligation showed that anti-IL-1β treatment reduced staining for the myofibroblast marker α smooth muscle actin and collagen-1, whereas the density of sprouting capillaries in the granulation tissue did not change (Figure 7A). In accord with the histological data, TGF-β1 and MMP-3 mRNA fell and TIMP-1 and TIMP-2 mRNA increased in the infarct and border zone of the treatment group 7 days after coronary ligation (Figure 7B). In addition, mRNA levels for the proinflammatory genes tumor necrosis factor-α and interleukin-6 fell, concordant with the observed reduction of inflammatory myeloid cells at this time point.

**Anti–IL-1β Treatment Reduces Adhesion Molecule Expression and Shifts Macrophage Phenotype**

To explore how local mechanisms may contribute to the observed salient effects of anti-IL-1β treatment, we examined the mRNA levels of adhesion molecules in infarct tissue. IL-1β neutralization reduced ICAM 2 and P-selectin expression, whereas ICAM 1, VCAM 1, and E-selectin expression were unchanged on day 7 after coronary artery ligation (Figure 8A), indicating that reduced IL-1β signaling dampens leukocyte recruitment. We also investigated how IL-1β influences the infarct macrophage phenotype. We isolated CD45<sup>hi</sup> CD11b<sup>hi</sup> F4/80<sup>hi</sup> macrophages by flow sorting from 4-day-old infarcts and measured mRNA levels of several M1/M2 macrophage markers (Figure 8B). Anti-IL-1β treatment shifted macrophage polarization away from the inflammatory M1 phenotype, indicating that anti-IL-1β therapy augments resolution of inflammation in acute infarcts.

**Discussion**

Recent experiments link atherosclerosis and MI, its dreaded clinical complication, with bone marrow activation, yet...
whether soluble systemic factors mediate this cross talk between organs remained unknown. This study provides evidence that circulating, blood-borne factors contribute to bone marrow activation during MI. After joining the circulation of 2 mice in parabiosis, 1 mouse underwent coronary ligation. The observed increase in HSC proliferation in the bone marrow of the noninfarcted parabiont must result from signaling by soluble mediators that crossed from the circulation of the infarcted mouse to the noninfarcted mouse. Here, we report that IL-1β participates in the process and contributes to bone marrow activation after MI via direct effects on hematopoietic cells and indirect effects that modulate the hematopoietic bone marrow microenvironment. Finally, we found that neutralizing IL-1β with the murine version of an antibody currently under investigation in a large-scale human trial can limit bone marrow activation. Anti-IL-1β treatment lowered the post-MI increase in HSC proliferation, reducing leukocytes in the blood and ischemic heart. Inhibiting this driver of inflammation favored infarct healing and attenuated left ventricular remodeling in mice with atherosclerosis, a harbinger of the development of heart failure post-MI, as in previous reports on nonatherosclerosis-prone mice.

Although the bone marrow generates billions of cells every day, leukocyte numbers in the steady state follow circadian rhythms within tightly regulated boundaries. Only a minor fraction of HSCs actively cycle, whereas the niche cells forming the hematopoietic microenvironment use signals such as CXCL12 to keep stem cells quiescent. In response to infection and other stressors, a variety of signals, including interferons and Toll-like receptor ligands, increase leukocyte output to meet the heightened demand for these innate immune cells during host defense against invading pathogens. These danger signals may act directly on hematopoietic stem cells that express receptors to sense danger-associated molecular patterns. Alternatively, alarmins may modulate the activity of niche cells, including endothelial cells and mesenchymal...
In the setting of cardiovascular disease, blood leukocytosis correlates with mortality; however, the signals that increase leukocyte production are incompletely understood. In mice with atherosclerosis, insufficient cholesterol efflux from cells may induce increased HSPC cycling in the bone marrow and spleen, thereby leading to hyperlipidemia-associated monocytosis, heightening macrophage recruitment into the vessel wall and progressing inflammation in the plaque. In this regard, a high macrophage burden characterizes plaques that have given rise to MI in humans.

During MI, increased sympathetic nervous system activity may trigger an additional burst of hematopoiesis by reducing bone marrow levels of CXCL12 and stem cell factor, important HSC retention factors that induce quiescence. We hypothesized that circulating factors also contribute to activating hematopoiesis, as seen after infection or other systemic injury. We specifically tested IL-1β because previous studies have implicated this cytokine in regulating hematopoiesis after the injection of the chemical compound alum and because IL-1β levels increase after myocardial infarction in rats and humans. Our data indicate that post-MI, IL-1β signaling in the bone marrow acts through 2 mechanisms: IL-1β directly binds IL-1R1 expressed by HSCs and indirectly affects leukocyte production through signaling to the niche cell compartment. In this microenvironment, antagonizing IL-1β preserved several HSC retention factors and inhibited the HSC and LSK release after MI, reducing their migration to the spleen and attenuating extramedullary hematopoiesis.

Systemically neutralizing IL-1β lowers blood levels of inflammatory monocytes and neutrophils. These myeloid cells drive plaque inflammation, and myeloid cell supply in blood likely influences the rates of recruitment into the arterial wall and ischemic myocardium. In atherosclerotic plaque, lower leukocyte numbers may lead to smaller lesion size and reduced vulnerability. In the MI, reducing blood leukocytosis supports the resolution of inflammation and mitigates subsequent left ventricular dilation. The ejection fraction was preserved 3 weeks after coronary ligation, likely also because of altered macrophage polarization that enhanced myocardial tissue repair. Our observations of IL-1β’s systemicwide action correlate well with previous reports on its local effects in the infarcted heart.

Our results have direct clinical relevance, because these experiments used a species-appropriate antibody to neutralize IL-1β in mice with MI equivalent to the clinically approved drug for syndromes associated with function gain of the NALP3 inflammasome, which generates active IL-1β. The large-scale trial Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) is testing whether the Food and Drug Administration–approved anti-human IL-1β antibody can limit recurrent events in stable post-MI patients who have residual inflammation, despite full standard-of-care treatments, as gauged by a highly sensitive C-reactive protein concentration in blood above median. The data presented here provide novel mechanistic insight on this drug’s actions. In addition, our results suggest that patients who might have recurrent acute MI while receiving anti-IL-1β antibody would not be at greater risk for enhanced post-MI heart failure. Indeed, these observations support a trial in acute MI, a proposition tested in the MRC trial with a much shorter acting IL-1 receptor antagonist that inhibits both the α- and β-isoforms of this multipotent mediator.

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Disclosures

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atherosclerosis in the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial.

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The acutely ischemic myocardium recruits millions of inflammatory blood leukocytes. To provide these cells, the bone marrow increases neutrophil and monocyte production, cells that are involved in reperfusion injury and infarct healing. Signals that communicate the increased demand of leukocytes after ischemia to the bone marrow are incompletely understood. Here, we provide data that support the role of circulating danger signals in alerting the bone marrow after myocardial infarction. Specifically, interleukin-1β levels increase systemically and result in accelerated hematopoietic stem and progenitor cell proliferation, myeloid cell production, and cell supply to the infarct. These actions occur directly via interleukin-1β signaling to hematopoietic cells and indirectly via bone marrow niche cells. Neutralization of interleukin-1β signaling with an antibody dampened bone marrow output of inflammatory leukocytes and supported inflammation resolution in the injured heart. This treatment also led to reduced post–myocardial infarction heart failure. Of note, a similar drug is clinically approved for cryopyrin-associated periodic syndromes and is currently being investigated for anti-inflammatory treatment in patients with atherosclerosis in the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial.

CLINICAL PERSPECTIVE
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SUPPLEMENTAL MATERIAL

Targeting Interleukin-1β reduces leukocyte production after acute myocardial infarction

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Supplemental Methods

Experimental animals. We used female C57BL/6J (WT, n=162), B6.129S7-I1r1tm1Imx/J (IL1R1−/−, n=28 and apolipoprotein E–deficient (ApoE−/−; B6.129P2-Apoetm1Unc/J, n=24) mice aged 8-12 weeks (The Jackson Laboratories, Bar Harbor, ME, USA) for our studies. We also used transgenic mice expressing green fluorescent protein (GFP) under the Nestin-promoter (Nestin-GFP, n=10). Nestin-GFP mice were a gift from Dr. Grigori Enikolopov (Cold Spring Harbor Laboratory, NY, USA). Nestin-GFP were crossed to C57B6 mice for at least 8 generations. Age-matched mice were randomly allocated either to control or treatment groups. The study was approved by the Subcommittee on Animal Research Care at Massachusetts General Hospital (Boston, MA).

Myocardial infarction surgery. Myocardial infarction was induced by permanent ligation of the left anterior descending coronary artery as described previously. Mice were anesthetized with 2% isoflurane (with O₂ 2 l/min), intubated and ventilated. All mice scheduled for infarct surgeries were injected twice daily with buprenophine (0.1 mg/kg i.p.) for three days, starting on the day of the surgery.

Ischemia Reperfusion Injury was induced and assessed as described previously. In brief, we ligated the the left anterior descending coronary artery and injected 360,000 fluorescent microspheres (10 µm size, excitation/emission wavelength 580/605 nm, Invitrogen) into the left ventricle 5 min thereafter. After 35 min of ischemia we released the suture waited for another 10 min and then injected 10 mg/kg bodyweight of the IL-1β antibody (or a mouse monoclonal IgG2a antibody raised against cyclosporine A as isotype control). Twenty-four hours after the induction of the ischemia, we conducted 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) staining to evaluate myocardial necrosis and fluorescence reflectance imaging (FRI) to quantify the ischemic area at risk (OV110, Olympus).
**Parabiosis.** Mice were joined in parabiosis as described perviously\(^5\). Mice were anesthetized with 2% isoflurane (with O\(_2\) 2 l/min). All mice scheduled for parabiosis surgeries were injected twice daily with buprenophine (0.1 mg/kg i.p.) for three days, starting on the day of the surgery.

**Neutralizing IL-1\(\beta\).** The IL-1\(\beta\) neutralizing antibody was a donation from Novartis (Basel, Switzerland). The antibody selectively binds IL-1\(\beta\), thus blocking the interaction of the cytokine with its receptors. We used a monoclonal, mouse anti-mouse IL-1\(\beta\) IgG2a/k antibody derived from an IgG1/k antibody as described by Geiger et al.\(^6\). The in vitro potency IC50 is about 25 pM, and its affinity to murine IL-1\(\beta\) is about 300 pM. The \(t_{1/2}\) in mice is 14 days\(^7\). We initiated treatment 2 h after induction of MI with subcutaneously injecting 10 mg/kg bodyweight of the IL-1\(\beta\) antibody (or a mouse monoclonal IgG2a antibody raised against cyclosporine A as isotype control). We repeated injections once weekly over the study period.

**Generating cell suspensions.** Blood for flow cytometric analysis was collected by cardiac puncture using a 50 mM EDTA (Ethylene diamine tetra acetic acid) solution (Sigma Aldrich, St. Louis, MO, USA) as an anticoagulant. Erythrocytes were lysed using a red blood cell lysis buffer (Biolegend, San Diego, CA, USA). After organ harvest, single-cell suspensions were obtained as follows. Hearts were extensively flushed with PBS and then removed. Infarct and border zone were separated from remote myocardium 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, St. Louis, MO, USA) at 37\(^\circ\) C at 750 rpm for 1 hour\(^8,9\). Hearts were then homogenized through a 40-\(\mu\)m cell strainer. Bone marrow was flushed out from bones and homogenized through 40-\(\mu\)m cell strainers. To sort stromal cells from the hematopoietic stem cell microenvironment, we harvested bones from nestin-GFP mice as described previously\(^10\). Bone marrow endothelial cells (EC) and mesenchymal stromal cells (MSC) were obtained by flushing out and subsequent digesting bone marrow in 10 mg/ml
collagenase type IV (Worthington) and 20 U/ml DNase I (Sigma). Bone osteoblastic lineage cells were obtained by crushing bones and washing off residual bone marrow cells. We then digested and incubated the bone fragments in collagenase IV and 20 U/ml DNase I as described above. Spleens were removed and then homogenized through a 40-µm cell strainer. Total viable cell numbers were obtained using Trypan blue (Cellgro, Mediatech, Inc., VA, USA).

**Flow cytometry.** For myeloid cell staining, cell suspensions were stained with mouse hematopoietic lineage markers (lineage for myeloid staining) including phycoerythrin (PE) anti-mouse antibodies directed against B220 (BD Bioscience, clone RA3-6B2), CD90 (BD Bioscience, clone 53-2.1), CD49b (BD Bioscience, clone DX5), NK1.1 (BD Bioscience, clone PK136), Ly6G (BD Bioscience, clone 1A8) and Ter-119 (BD Bioscience, clone TER-119). We then applied a second round of staining covering CD45.2 (BD Bioscience, clone 104), CD11b (BD Bioscience, clone M1/70), CD115 (eBioscience, clone M1/70), CD11c (eBioscience, clone HL3), F4/80 (Biolegend, clone BM8) and Ly6C (BD Bioscience, clone AL-21).

Neutrophils were identified as (B220/CD90/CD49b/NK1.1/Ly6G/Ter119)\(^{\text{high}}\) (CD45.2/CD11b)\(^{\text{high}}\) CD115\(^{\text{low}}\), monocytes as (B220/CD90/CD49b/NK1.1/Ly6G/Ter119)\(^{\text{low}}\) (CD45.2/CD11b)\(^{\text{high}}\) (F4/80/CD11c)\(^{\text{low}}\) CD115\(^{\text{high}}\) Ly6C\(^{\text{high/low}}\) and macrophages as (B220/CD90/CD49b/NK1.1/Ly6G/Ter119)\(^{\text{low}}\) (CD45.2/CD11b)\(^{\text{high}}\) Ly6C\(^{\text{low/int}}\) F4/80\(^{\text{high}}\) 8, 9. For hematopoietic stem/progenitor staining, cell suspensions were incubated 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 IL7Rα (eBioscience, clone A7R34) followed by incubation 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/IL7R$\alpha$)$^{\text{low}}$ c-kit$^{\text{high}}$ sca-1$^{\text{high}}$, hematopoietic stem cells (HSC) as (B220/CD11b/CD11c/NK1.1/Ter-119/Gr-1/CD8a/CD4/IL7R$\alpha$)$^{\text{low}}$ c-kit$^{\text{high}}$ sca-1$^{\text{high}}$ CD48$^{\text{low}}$ CD150$^{\text{high}}$. We acquired data on an LSRII flow cytometer (BD Bioscience) with FACSDiva software (BD Bioscience). Experimental data were later analyzed using FlowJo software (Tree Star Inc.).

**BrdU incorporation experiments.** To assess proliferation, we used FITC/APC BrdU (bromodeoxyuridine) flow kits (BD Bioscience). For these BrdU pulse experiments, one mg BrdU was injected intraperitoneally 24h prior to euthanization and subsequent organ harvest. After surface staining, intracellular BrdU staining was carried out according to the manufacturer’s protocol.

**Cell cycle analysis.** Following surface staining, intracellular staining was performed as described previously$^{10}$. To assess cell cycles, we stained for the nuclear antigen KI67 (eBioscience, clone SolA15) and DNA (4,6-diamidino-2-phenylindole, DAPI, FxCycle Violet Stain, Life Technologies).

**Cell sorting.** For sorting hematopoietic stem/progenitor cells, bone marrow was collected from WT mice by crushing bones from femurs, tibias, humeri, pelvic bones and spines with a mortar and pestle. To enrich samples for hematopoietic stem/progenitor cells, we used MACS depletion columns (LD columns, Miltenyi) after incubation with a cocktail of biotin-labeled lineage antibodies (lineage for hematopoietic stem/progenitor staining as described above) followed by incubation with streptavidin-coated microbeads (Miltenyi). We then stained cells with c-kit, sca-1, CD48 and CD150 as described above and FACS-sorted LSKs and HSCs using a FACSaria II cell sorter (BD Biosystems). To sort different hematopoietic stem cell niche populations, we utilized Nestin-GFP mice as described previously$^{10}$. Cell suspensions were obtained from either flushed and digested bone marrow or crushed and digested bones. To
purify stromal niche cells from hematopoietic cells, we used MACS depletion columns (LD columns, Miltenyi) after incubation with biotin-labeled lineage antibodies (lineage for hematopoietic stem/progenitor staining as described above) followed by incubation with streptavidin-coated microbeads (Miltenyi). We subsequently stained with CD45.2, sca-1, CD31 (Biolegend, clone 390) and CD51 (Biolegend, clone RMV-7) and FACS-sorted stromal cells. Bone marrow endothelial cells (EC) were identified as lin<sup>low</sup> CD45<sup>low</sup> sca-1<sup>high</sup> CD31<sup>high</sup>, bone marrow mesenchymal stromal cells (MSC) as lin<sup>low</sup> CD45<sup>low</sup> CD31<sup>low</sup> sca-1<sup>high/intermediate</sup> and GFP<sup>+</sup>, bone osteoblastic lineage cells (OB) as lin<sup>low</sup> CD45<sup>low</sup> sca-1<sup>low</sup> CD31<sup>low</sup> CD51<sup>high</sup>. Many bone marrow stromal cells are also positive for the neurofilament protein Nestin<sup>11</sup> among them endothelial cells (EC), mesenchymal stromal cells (MSC) and osteoblastic cells (OBC). We used Nestin-GFP reporter mice for identifying MSC after we gated on cells that are CD45<sup>low</sup>/CD31<sup>low</sup>/sca-1<sup>high</sup> and consequently excluded EC (CD31<sup>high</sup>) and OBC (sca-1<sup>low</sup>).

To sort heart macrophages, we harvested and digested 4d old infarcts (including their leukocyte-rich border zones) from WT mice. We subsequently stained for myeloid cells and FACS-sorted macrophages as described above.

**Bone marrow reconstitution assays.** We lethally irradiated WT or IL1R1<sup>−/−</sup> mice with 950 cGy. We then reconstituted mice with 2x10<sup>6</sup> whole bone marrow cells from either WT or IL1R1<sup>−/−</sup> mice on the day of the irradiation. Two to four months later mice received coronary ligation, and respective experimental procedures were carried out.

**Fluorescence Molecular Tomography-Computed Tomography (FMT/CT).** We measured protease activity in hearts of ApoE<sup>−/−</sup> mice 7d after MI by using fluorescence molecular tomography (FMT) in combination with computed tomography (CT) as described previously<sup>12</sup>. Pan-cathepsin protease sensor (Prosense-680, PerkinElmer, 5 nmol) was injected to evaluate inflammatory activity in infarcts.
**Histologic procedures.** For histological evaluations, WT mice were euthanized on day 7 after MI, and hearts were perfused thoroughly with PBS and then harvested. The hearts were embedded in O.C.T. compound (Sakura Finetek) and subsequently snap-frozen in a 2-methylbutane bath cooled with dry ice. For immunohistochemistry, 6 µm frozen sections were stained using antibodies targeting CD11b (BD Biosciences, clone M1/70), Ly6G (Biolegend, clone 1A8), alpha-smooth muscle actin (αSMA, Abcam, clone ab5694), collagen I (Abcam, clone ab21286) and CD31 (BD Biosciences, clone MEC13.3). The appropriate biotinylated secondary antibodies followed by VECTASTAIN ABC kit (Vector Laboratories, Inc.) were applied. We used an AEC substrate (Dako) for color development. All sections were counterstained with Harris hematoxylin. We scanned slides using a digital scanner, Nanozoomer 2.0RS (Hamamatsu, Japan), and quantified the positive area using IPLab (version 3.9.3; Scanalytics, Inc.). We analyzing five high-power fields per section and per animal.

**Quantitative real-time PCR.** Messenger RNA (mRNA) was extracted from 7d old infarcts (+border zones) or from flushed bone marrow, using the RNeasy Mini Kit (Qiagen), or from FACS-sorted cells, using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems), according to manufacturers’ protocols. We transcribed one microgram of mRNA to complimentary DNA (cDNA) with the high capacity RNA to cDNA kit (Applied Biosystems). We used Taqman primers (Applied Biosystems) for the quantitative real-time PCR. We expressed the results as Ct values normalized to the housekeeping gene Gapdh (with the control set as 1).

**CFU-assay.** Colony forming unit (CFU) assays were performed, as recomended by the manufacturer, using a semi-solid cell culture medium (Methocult M3434, Stem Cell Technology). We flushed bones with Iscove’s Modified Dulbecco’s Medium (IMDM, Lonza) supplemented with 2% fetal calf serum. We plated 2x10⁴ bone marrow cells on a 35 mm plate in duplicates. After incubating for 12 days, colonies were counted using a low magnification inverted microscope.
**Magnetic resonance imaging (MRI).** MRI was carried out on days 1 and 21 after permanent coronary ligation as described perviously\(^\text{13, 14}\). We obtained cine images of the left ventricular short axis by using a 7 Tesla horizontal bore Pharmascan (Bruker) and a custom-built mouse cardiac coil (Rapid Biomedical). Acquisition was done as described previously\(^\text{13}\). Images were analyzed using the software Segment (http://segment.heiberg.se).

**IL-1β ELISA.** Blood was acquired by cardiac puncture and allowed to clot for 20 min at room temperature. After spinning down for 15 min at 2000 G, 50 µl serum was used for further ELISA applications. Infarct and border zone parts were removed from heart samples and then snap frozen in liquid nitrogen. A mechanical tissue disruption was performed in 250 µl RIPA lysis buffer (Millipore). After spinning down for 20 min at 12000 G, 5 µl of the supernatant was used for further ELISA applications. For measuring protein levels of IL-1β in hearts and serum we used an ELISA kit (R&D Systems, MN, USA) according to the manufacturer’s instructions.

**Administration of recombinant mouse IL-1β.** We injected 2.5 µg of recombinant mouse IL-1β (R&D Systems, MN, USA) i.p. daily over two days as described elsewhere\(^\text{15}\) and harvested the bone marrow 48h after the first administration.

**Statistics.** Statistical analyses were carried out using GraphPad Prism software version 6 (GraphPad Software, Inc.). Results are displayed as mean ± standard deviation (SD). First, values were tested for Gaussian distribution (D'Agostino-Pearson omnibus normality test). For two-group comparisons, an unpaired t-test was applied to normally distributed variables, a Mann-Whitney test to non-normally distributed variables. For comparing more than two groups, a one-way ANOVA test, followed by a Sidak’s test for multiple comparisons, was applied. P values of < 0.05 indicated statistical significance.
Supplemental Figure 1. IL-1β drives HSC proliferation both directly and indirectly.
(a) Experimental set-up of the BM reconstitution experiment. Lethally irradiated wild type (WT) or IL1R1−/− mice were reconstituted with IL1R1−/− or WT bone marrow, respectively. Eight weeks later, mice received MI and were subjected to the described procedures. (b) Quantification of heart to total body weight 21d after coronary ligation (n = 8-10 per group, mean ± SD). (c) Evaluation of post-MI remodeling by cardiac MRI. End-diastolic volumes (EDV) and left-ventricular ejection fraction (LV-EF) were examined on day 21 after coronary ligation (n = 8-10 per group, mean ± SD). (d) Flow cytometric gating for blood leukocyte subsets 7 d after coronary ligation. (e,f) Quantification of blood leukocyte numbers 3 d (e) and 7 d (f) after coronary ligation (n = 10-12 per group, mean ± SD).
Supplemental Figure 2. IL-1β effects on day 3 after MI. 
(a,b) Flow cytometric quantification of (a) blood and (b) heart neutrophils and monocytes/macrophages 3d after coronary ligation (n = 13-14 per group, mean ± SD, *p<0.05).
Supplemental Figure 3. Neutralizing IL-1β changes the bone marrow microenvironment.
(a) Flow cytometric gating for sorting respective bone marrow (BM) stromal cell populations (EC, bone marrow endothelial cells; MSC, bone marrow mesenchymal stromal cells; OB, bone osteoblastic lineage cells). (b) mRNA quantification of HSC retention factors (CXCL12, chemokine (C-X-C motif) ligand 12, VCAM-1, vascular cell adhesion molecule 1; SCF, stem cell factor and OPN, osteopontin) in respective BM stromal cells with qRT-PCR 48h after MI (n = 7-8 per group, mean ± SD, *p<0.05, **p<0.01).
Supplemental Figure 4. Anti-IL-1β treatment does not alter the infarct size in ischemia reperfusion injury.
Quantification of the infarct area (upper panel, dashed line shows TTC negative area) to the area at risk (lower panel, dashed line shows area spared from fluorescent microspheres as assessed with fluorescence reflectance imaging) 24 h after 35 min of ischemia. Percent infarction over area at risk is calculated as TTC-negative area / FRI-negative area * 100% (n = 5-7 per group, mean ± SD).
Supplemental References


