Monocytes in ischemic heart disease
van der Laan, A.M.

Link to publication

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Chapter 9

Myocardial infarction accelerates atherosclerosis


Nature 2012;487:325-329

*Authors contributed equally
ABSTRACT

During progression of atherosclerosis, myeloid cells destabilize lipid-rich plaques in the arterial wall and cause their rupture, thus triggering myocardial infarction and stroke. Survivors of acute coronary syndromes have a high risk of recurrent events for unknown reasons. Here we show that the systemic response to ischaemic injury aggravates chronic atherosclerosis. After myocardial infarction or stroke, Apoe\(^{-/-}\) mice developed larger atherosclerotic lesions with a more advanced morphology. This disease acceleration persisted over many weeks and was associated with markedly increased monocyte recruitment. Seeking the source of surplus monocytes in plaques, we found that myocardial infarction liberated haematopoietic stem and progenitor cells from bone marrow niches via sympathetic nervous system signalling. The progenitors then seeded the spleen, yielding a sustained boost in monocyte production. These observations provide new mechanistic insight into atherogenesis and provide a novel therapeutic opportunity to mitigate disease progression.
INTRODUCTION

Today, survival after a first myocardial infarction (MI) approaches 90%. However, re-infarction occurs commonly and has a high mortality. In a representative trial, new myocardial ischaemia occurred in 54% of patients within the first year after MI.1 The largest population study so far showed a 17.4% 1-year risk of re-infarction.2 Conventional wisdom infers that these very high rates of secondary events reflect later stages of linear disease progression. This study tested the alternative hypothesis that a first infarct—triggering a burst of acute systemic inflammation aimed at repair of the injured heart—could accelerate atherosclerosis.

Monocytes infiltrate lesions and, together with their lineage-descendant macrophages, instigate inflammation and deliver proteolytic enzymes that digest extracellular matrix and render atherosclerotic plaques unstable.3-7 Elevated levels of circulating monocytes provide an expanded pool of inflammatory cells available for recruitment to growing arterial lesions, potentially promoting plaque rupture. Leukocytosis after MI predicts an increased risk of re-infarction and death.8,9 During acute MI, blood monocyte levels spike, and these cells accumulate in the evolving myocardial wound.10,11 Thus, the organism experiences an acute inflammatory event (for example, MI) superimposed on a pre-existing chronic inflammatory disease (atherosclerosis), both of which involve the same myeloid cell type. Given the frequency of re-infarction, we investigated whether acute myocardial injury accelerates pre-existing chronic atherosclerosis.

We found that in Apoe<sup>−/−</sup> mice with atherosclerosis, MI increased plaque size and induced a ‘vulnerable’ lesion morphology with higher inflammatory cell content and protease activity, fuelled by persistently increased myeloid cell flux to atherosclerotic sites. Earlier clinical studies described an increase of haematopoietic stem and progenitor cells (HSPCs) in the circulation of patients shortly after MI.12 We thus proposed that release of these progenitors may increase the availability of monocytes. We found that in response to heightened sympathetic nervous system (SNS) activity—provoked by pain, anxiety and heart failure in patients with MI—HSPCs departed bone marrow niches and produced prolonged amplified extramedullary monocytopoiesis in mice after coronary ligation.

MI ACCELERATES ATHEROSCLEROSIS

Proteases, including metalloproteinases and cysteiny1 cathepsins, can catabolize the extracellular matrix of the plaque’s fibrous cap and render it prone to rupture.13,14 Therefore, protease activity may serve as a marker in mice of processes associated with lesion vulnerability in humans.15 To test the hypothesis that MI changes the course of atherosclerotic disease, we serially imaged protease activity in aortic plaques of Apoe<sup>−/−</sup> mice, before and 3 weeks after coronary ligation, using hybrid fluorescence molecular tomography–X-ray computed tomography (FMT–CT).16 Imaging showed a sharp increase of plaque protease activity within 3 weeks after MI (Figure 1a and 1b). In parallel, expression of the inflammatory cytokine interleukin-6 (Il6), Mmp9, myeloperoxidase and Ly-6C (also known as Ly6c1) increased in
Figure 1 Increased inflammation in atherosclerotic plaques after MI. (a) Protease activity was determined by FMT–CT before and 3 weeks after MI. Circles indicate aortic root (n = 10 per group). (b) Protease activity in excised aortae determined by fluorescence reflectance imaging (FRI), expressed as target to background ratio (TBR; n = 10 per group). (c) Flow cytometric quantification of myeloid cells and Ly-6Chigh monocytes in aorta (n = 5–9 per group). Dot plots 3 weeks after MI are shown. (d) CD11b staining and lesion size (n = 9–10 per group). Scale bar represents 150 μm. Data are shown as mean ± s.e.m.. *P<0.05, **P<0.01.
atherosclerotic plaques (Supplementary Figure S1). The number of monocytes and macrophages per aorta increased, particularly the inflammatory Ly-6C<sup>high</sup> monocyte subset (Figure 1c). Plaque monocyte content also increased in Apoe<sup>−/−</sup> mice without MI, reflecting the natural course of disease in these animals.<sup>17,18</sup> Yet innate immune cell accumulation accelerated distinctively after MI, as indicated by the significantly greater slope obtained when fitting the number of Ly-6C<sup>high</sup> monocytes in the aorta over time (Supplementary Figure S2). Neutrophil presence in atheromata also increased (Supplementary Figure S3) whereas mast cells did not (Supplementary Figure S4). Histological analysis affirmed increased accumulation of CD11b<sup>+</sup> myeloid cells and larger lesion size after MI (Figure 1d). The thickness of the fibrous cap decreased, covering larger necrotic cores (Supplementary Figure S5). Ly-6C<sup>high</sup> monocytes isolated from atherosclerotic lesions exhibited higher levels of messenger RNAs encoding inflammatory genes. Il1b and cathepsin B were expressed at higher levels 3 weeks after MI, whereas arginase (Arg1) and TGF-β, markers associated with alternatively activated macrophages, were expressed at lower levels (Supplementary Figure S6). Monocyte numbers in the blood and spleen increased consistently for up to 3 months after coronary ligation (Supplementary Figure S7) but were unaltered in the bone marrow (Supplementary Figure S8).

Figure 2 Elevated levels of progenitor cells in the spleen of Apoe<sup>−/−</sup> mice after MI. (a) Quantification of HSPCs, MDPs and GMPs at different time points after MI (n = 3–15 per group). The gating strategy is shown in Supplementary Figure S10. (b) Number of colony-forming units (c.f.u.). Data are shown as mean ± s.e.m.. *P<0.05, **P<0.01.
Chapter 9

EXTRAMEDULLARY MONOCYTOPOIESIS AFTER MI

Because the spleen has the ability to host extramedullary haematopoiesis,19-21 we measured splenic monocyte progenitor content in mice after MI. Haematopoietic progenitor cell numbers in the spleen increased after MI (Figure 2 and Supplementary Figure S9) but not in the bone marrow (Supplementary Figure S10). Proliferation of progenitors doubled in the spleen (Supplementary Figure S11). In patients who died after an acute MI, we found increased numbers of c-kit+ cells in the spleen, some of which co-localized with the proliferation marker Ki-67 (Supplementary Figure S12).

When we splenectomized mice at the time of MI, atherosclerosis did not accelerate (Supplementary Figure S13). The number of progenitor cells in liver tissue after MI was much lower than in the spleen; however, splenectomy increased progenitor cell presence in the liver 4 days after MI (Supplementary Figure S14). We concluded that the infarct-induced monocytosis resulted primarily from augmented production in the spleen, but that other extramedullary sites may contribute.22 This observation raised the question whether monocytes of splenic and bone marrow origin differ qualitatively. Surprisingly, Ly-6Chigh monocytes isolated from the spleen or bone marrow on day 4 after MI had significantly different mRNA levels in 11 of the 32 genes assessed (Supplementary Figure S15). For instance, Il1b and cathepsin B mRNA levels were 60- and 6-fold higher in inflammatory monocytes isolated from the spleen, matching the increased expression of these genes in Ly-6Chigh monocytes isolated from atherosclerotic plaques after MI (Supplementary Figure S6). Therefore, post-MI extramedullary myelopoiesis may not only increase the availability of inflammatory cells but also change their functional program. To test whether another form of acute tissue injury prevalent in atherosclerotic patients would accelerate splenic myelopoiesis, we analysed Apoe−/− mice 6 weeks after ischaemic stroke. The number of myeloid cells and Ly-6Chigh monocytes in atherosclerotic plaques increased after stroke, in parallel with expanded splenic monocytopoiesis (Supplementary Figure S16).

BONE MARROW HSPC RELEASE AFTER MI

As granulocyte macrophage progenitors (GMPs) and macrophage dendritic cell progenitors (MDPs) have a limited self-renewal capacity,23,24 we tested whether upstream progenitors released from their bone marrow niches sustain the splenic proliferative activity after MI. Indeed, blood levels of HSPCs increased 2-, 7- and 24-fold at 6, 48 and 96h after MI, respectively (Figure 3a). The number of splenic Flk2− HSPCs increased markedly after MI (Supplementary Figure S17). This mobilization of upstream HSPCs with high capacity for self-renewal probably explains the long-term boost in splenic monocyte production in Apoe−/− mice after MI.

Anxiety, pain and impaired left ventricular function during MI can all activate the SNS. Accordingly, levels of tyrosine hydroxylase, the rate-limiting enzyme for production of noradrenaline in sympathetic fibres,25 increased in the bone marrow of mice after MI and hence indicated a higher sympathetic tone (Figure 3b). SNS activity may liberate haematopoietic stem cells from their niches by signalling through
AMI and atherosclerosis

Nestin+ mesenchymal stem cells express this receptor, which regulates the production of stem cell retention factors. Because acute MI raises blood progenitor levels in patients, we investigated whether SNS activity causes the release of HSPCs from the bone marrow after MI. Blood HSPCs decreased by 100, 75 and 50% at 6, 48 and 96h after MI in mice treated with a β3-adrenoceptor antagonist (Figure 3a). The stem cell retention factor Cxcl12, angiopoietin and stem cell factor (Scf, also known as Kitl) underwent similar regulation (Figure 3c). Levels of the adhesion molecule Vcam1, which also retains HSPCs in the bone marrow, decreased after MI but did not change after β3-adrenoceptor blocker administration (Figure 3c). These data indicate that increased sympathetic tone after MI causes withdrawal of stem cell retention factors by β3-adrenoceptor-expressing niche cells.

Figure 3 β3-Adrenoceptor-mediated progenitor release after MI. (a) Flow cytometric analyses of HSPCs in blood of C57BL/6 mice (n = 6–11 per group). (b) Immunostaining for tyrosine hydroxylase (TH). Scale bar represents 10μm. Insets depict low-magnification overview. Bar graph shows quantification of TH+ area (n = 5 per group). (c) Expression of HSPC retention factors (relative to GAPDH) in the bone marrow of C57BL/6 mice on day 4 after MI (n = 8 per group). Data are shown as mean ± s.e.m.. *P<0.05, **P<0.01.
Treatment with a $\beta_3$-adrenoceptor blocker reduced splenic accumulation of progenitors in wild-type mice shortly after MI (Supplementary Figure S18) and consequently diminished their output of myeloid cells (Supplementary Figure S19). In Apoe$^{-/-}$ mice 3 weeks after MI, $\beta_3$-blocker treatment reduced the number of GMPs and their progeny in the spleen and blood (Supplementary Figure S20). Retrospective analysis of a clinical trial revealed that prior $\beta$-blocker therapy was associated with a reduction in monocytes after an acute coronary syndrome (Supplementary Table S1). The mechanism that led to this decrease is unclear, also because some clinically used $\beta$-blockers have a lower affinity for the $\beta_3$-adrenoceptor subtype; however, these associative data show an interesting parallel to our findings in mice.

In Apoe$^{-/-}$ mice after MI, $\beta_3$-blocker treatment lowered protease activity, myeloid cell content, and mRNA levels of inflammatory cytokines in the plaque (Supplementary Figure S21). When we adoptively transferred GFP$^+$ GMPs to wild-type mice with MI, $\beta_3$-blocker treatment did not alter their splenic differentiation (Supplementary Figure S22). Sympathetic denervation with 6-hydroxydopamine (6-OHDA) increased bone marrow mRNA levels of the stem cell retention factor Cxcl12, reduced levels of HSPCs in blood, decreased circulating monocyte levels, and attenuated the accumulation of myeloid cells in atherosclerotic lesions (Supplementary Figure S23). Combination of $\beta_3$ blockade and splenectomy showed no additive effects (Supplementary Figure S24). Neither MI nor $\beta_3$ blockade changed blood cholesterol and high-density lipoprotein levels (Supplementary Figure S25).

**INTRAVITAL MICROSCOPY OF HSPC DEPARTURE**

We adoptively transferred lineage$^-\text{c-kit}^+\text{Sca-1}^+\text{Flk2}^-$ (Sca1 also known as Ly6a) HSPCs labelled with a fluorescent membrane dye (DiD) to examine their release with serial intravital microscopy (IVM). DiD$^+$ cells were quantified after they had settled into the bone marrow, and then again 4 days after MI. Concomitant with the post-MI increase of progenitors in circulation, 52% of cells that were present during the first imaging session departed from the bone marrow, which was inhibited by the $\beta_3$-adrenoceptor antagonist (Figure 4). Post-imaging flow cytometry corroborated the trafficking of DiD$^+$ cells (Supplementary Figure S26). We next investigated the relocation of bone marrow cells to the spleen directly. Lineage$^-\text{c-kit}^+\text{Sca-1}^+\text{Flk2}^-$ HSPCs were harvested from CD45.2$^+$ donors and labelled with a photoconvertible dye before transfer into CD45.1$^+$ recipients. These cells engrafted into the skull bone marrow, where we photoconverted them with laser illumination. Only if mice underwent coronary ligation, photoconverted CD45.2$^+$ DAPI$^+$ cells were detected in splenic cell suspensions 4 days later (Supplementary Figure S27).

**SPLENIC HSPC ENGRAFTMENT AFTER MI**

Finally, we investigated the mechanisms of splenic progenitor seeding. The mRNA levels of Scf increased in splenic tissue after MI in parallel with the number of
SCF\(^+\) cells in splenic sections (Figure 5a and 5b). Antibody neutralization of SCF decreased retention of adoptively transferred HSPCs in the spleen and proliferation of host HSPCs (Figure 5c and 5d). Co-localization studies identified CD31\(^+\) and occasionally nestin\(^+\) cells (Supplementary Figure S28a and S28b) as a source of SCF, in agreement with a recent report on the role of SCF in the splenic niche during the steady state.\(^{33}\) We found adoptively transferred DiD\(^+\) HSPCs cells in close vicinity to CD31\(^+\) cells (Supplementary Figure S28c). Neutralization of VLA-4 (also known as Itga4), an integrin involved in stem cell retention,\(^{34,35}\) reduced the number of adoptively transferred HSPCs in the spleen after MI, but not in the steady state (Supplementary Figure S29).

DISCUSSION

We have shown that acute MI or stroke increases inflammation in atherosclerotic plaques at a distance. After an ischaemic event, atherosclerotic plaques grew faster and displayed higher protease activity. We identified an increased supply of innate immune cells as a driving force for this phenomenon. On a systems level, pre-existing chronic inflammation flared when mice experienced an additional acute inflammatory stimulus. Increased SNS activity after MI released upstream progenitors from bone marrow niches. On the receiving end, the spleen hosted these cells by increasing SCF production, leading to amplified extramedullary myelopoiesis (Figure 5e). The pro-inflammatory changes in atherosclerotic plaques persisted for several months.

The evolutionary benefit of outsourcing myelopoiesis from the bone marrow may involve the protection of steady state ‘housekeeping’ in this confined...
compartment. Unlike the bone marrow, the spleen is an organ that can rapidly expand in size. In the event of increased leukocyte need after acute injury, the myelopoietic system may proliferate in extramedullary sites to protect quiescent stem cells and to ensure unimpeded production of red cells, platelets and lymphocytes in the bone marrow.

Despite growing understanding of the chronic inflammatory nature of atherosclerosis, specific anti-inflammatory therapy has yet to materialize. Given the central role of myeloid cells in disease promotion and their rapid turnover in inflamed tissue, interrupting the monocyte supply chain may attenuate atherosclerosis. In this case, SNS inhibition abrogated stem cell release from the bone marrow. Because the regulation of progenitor cell migration is multifactorial, there are other targets along this pathway that await exploration, including chemokine receptors and cytokines involved in stem cell activation. In addition, the innate immune response unleashed by acute ischaemic injury may also change the ‘fluid phase’ of blood by augmenting

![Figure 5](Image)

**Figure 5** Splenic progenitor engraftment after MI. (a) Quantitative polymerase chain reaction of SCF in spleen \( (n = 5–6 \text{ per group}) \). (b) Number of SCF\(^+\) cells in spleen of C57BL/6 mice 4 days after MI as determined by immunofluorescence. (c) Enumeration of adoptively transferred GFP\(^+\) HSPCs on day 4 after MI \( (n = 8 \text{ per group}) \). (d) Proliferation of endogenous HSPCs determined by BrdU incorporation \( (n = 8 \text{ per group}) \). (e) Paradigm. BM, bone marrow. Data are shown as mean ± s.e.m., *\( P<0.05 \), **\( P<0.01 \).
circulating acute phase reactants such as fibrinogen and plasminogen activator inhibitor 1, factors that promote thrombosis and counter endogenous fibrinolysis. Our study suggests that patients with an ischaemic complication of atherosclerosis experience a particularly vulnerable disease phase, and that interventions aimed at progenitors of innate immune cells could affect long-term outcomes.

METHODS SUMMARY

Wild-type C57BL/6J, C57BL/6.SJL, C57BL/6-Tg(UBC-GFP)30Scha/J and B6.129P2-Apoetm1Unc/J mice were used in these studies, which were approved by the Subcommittee on Animal Research Care at Massachusetts General Hospital. The patient studies were conducted in accordance with the Declaration of Helsinki. The studies were approved by the Research Committee of the Department of Pathology of the VUmc and by the Ethikkommission Heidelberg University. Detailed procedures are available in Supplementary Information.

Acknowledgements

We thank the CSB Mouse Imaging Program (J. Truelove, D. Jeon, J. Donahoe, B. Marinelli) and K. Naxerova for helpful discussions. This work was funded by grants from the National Institute of Health R01-HL096576, R01-HL095629 (M.N.); R01-EB006432, T32-CA79443, P50-CA086355 (R.W.). F.L. was funded in part by Deutsche Forschungsgemeinschaft SFB 938/Z2. Fig. 5e was produced using Servier Medical Art (http://www.servier.com).
REFERENCES

27. Méndez-Ferrer, S. et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow
SUPPLEMENTARY INFORMATION

Supplementary Methods

Animal models
C57BL/6J, B6.SJL-Ptprca Pepcb/BoyJ, C57BL/6-Tg(UBC-GFP)30Scha/J and B6.129P2-Apoetm1Unc/J mice were purchased from Jackson Laboratory. ApoE/- mice were on C57BL/6 background, but lacked apolipoprotein E that is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. ApoE/- mice were fed a high-cholesterol diet (Harlan Teklad, 0.2% total cholesterol). Myocardial infarction (MI) was performed on apoE/- mice when they were on the high-cholesterol diet for 10 weeks. The group assignment (MI versus no MI) was random. Tissues collected from the mice were analyzed at either 1, 3, 6, or 12 weeks after MI. Tissues from C57BL/6 mice were collected and analyzed at either 6, 48, or 96 hours after MI. To induce MI, mice were intubated and ventilated with 2% isoflurane supplemented with oxygen. Thoracotomy was performed in the fourth left intercostal space. The left coronary artery was identified and permanently ligated with a monofilament nylon 8-0 suture. The thorax was closed with glue. Intraluminal middle cerebral artery occlusion (MCAO) was performed to induce transient cerebral ischemia causing stroke. A middle neck incision was made. Then the left common carotid artery was carefully dissected without harming the vagal nerve and ligated with a 6-0 suture. The left external carotid artery was also ligated. The left internal carotid artery and the left pterygopalatine artery were clipped using microvascular clips. A monofilament (Doccol Corporation) made of 8.0 nylon was introduced through the left carotid artery before its bifurcation. The clipped arteries were opened and the monofilament blocked the middle cerebral artery. After 45 minutes, the monofilament was withdrawn and the skin was closed with suture. Regional cerebral blood flow was measured with Laser Doppler (Instruments Inc.) to confirm occlusion and reperfusion. Rectal temperature was maintained at 37.5 ± 0.5°C during surgery.

Treatment with 6-hydroxydopamine hydrobromide (6-OHDA)
6-OHDA (Sigma-Aldrich) was injected i.p at a dose of 250 mg kg⁻¹ body weight in a final volume of 200 μl one day before MI in C57/BL6 mice. ApoE/- mice were treated with the drug once a week for three weeks starting one day before inducing MI.

Treatment with adrenergic β3 receptor antagonist
SR 59230A, a selective antagonist for adrenergic β3 receptor was obtained from Sigma-Aldrich. Mice were injected with the drug intraperitoneally twice daily at a dose of 5 mg kg⁻¹ body weight in a final volume of 100 μl in PBS. ApoE/- mice were treated with the drug for three weeks.

In vivo neutralization assays
C57BL/6 mice were injected i.v with 300 μg of either anti-mouse SCF antibody (R&D systems) or control goat IgG (R&D systems) diluted in 300 μl of PBS on the day before and 2 days after MI. For VLA-4 neutralization, mice were injected i.v. with 200 μg of either anti-mouse VLA-4 (BioXCell) or control rat IgG2b (BioXCell) 2 days
after MI. About 400,000 lineage-c-kit+ cells sorted from GFP+ mice were adoptively transferred i.v. on the day of MI.

Organ and tissue processing
Blood was drawn via cardiac puncture in 50mM EDTA (Sigma-Aldrich), which was followed by red blood cell lysis with 1x RBC lysis buffer (BioLegend). Spleen and bone marrow were processed in PBS with 0.5% bovine serum albumin and 1% fetal bovine serum (FACS buffer). Aorta was excised under a microscope and minced in digestion mixture containing 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I, and 60 U/ml hyaluronidase (Sigma-Aldrich) and incubated at 37o C at 750 rpm for 1 hour. After this the digestion reaction was stopped with 10 ml FACS buffer.

Flow cytometry
The processed single cell suspensions (300 μl) were taken in 5 ml falcon tubes (BD Bioscience) for staining with fluorochrome-labelled antibodies against mouse hematopoietic lineage markers. For monocyte staining, a Phycoerythrin (PE) anti-mouse lineage antibody cocktail containing antibodies directed against CD90 (clone 53-2.1), B220 (clone RA3-6B2), CD49b (clone DX5), NK1.1 (clone PK136), Ly-6G (clone 1A8) and Ter-119 (clone TER-119) was used. Monocytes were then stained with anti-mouse CD11b (clone M1/70), CD11c (clone HL3), F4/80 (clone BM8) and Ly6C (clone AL-21). Monocytes were identified as (CD90/B220/CD49b/NK1.1/Ly-6G/Ter119)low, CD11bhigh, F4/80low, CD11clow/neg, Ly-6Chigh/low. For progenitor cell staining, in addition to the antibodies mentioned above, we also used PE-conjugated antibodies directed against CD11b (clone M1/70), CD11c (clone N418), and IL7Rα (clone A7R34) in the lineage cocktail. The cells were then stained with antibodies against c-kit (clone 2B8), Sca-1 (clone D7), CD16/32 (clone 2.4G2), CD34 (clone RAM34), and CD115 (clone AFS98). Hematopoietic stem and progenitors cells (HSPCs) were identified as lineage (CD90/B220/CD49b/NK1.1/Ly-6G/Ter119/CD11b/CD11c/IL7Rα)low c-kit high Sca-1high. Granulocyte and macrophage progenitors (GMPs) were identified as lineagelow c-kithigh Sca-1low/mid CD11bhigh CD16/32high CD34high. Macrophage dendritic cell progenitors (MDPs) were identified as lineagelow c-kithigh Sca-1low/mid CD115high CD16/32high CD34high. For in vivo BrdU proliferation assays, the mice were injected intraperitoneally with 1 mg of BrdU (BD Pharmingen) in 100 μl PBS. Tissues were collected the next day and intracellular BrdU staining was performed using BrdU Flow Kits (BD Pharmingen).

Cell sorting
In adoptive transfer experiments, donor cells were obtained by crushing all long bones (femur, tibia, fibula, humerus) and spines using a pestle and a mortar in FACS buffer. The cells were passed through 40 μm filters. Red blood cells were lysed and cells were stained with phycoerythrin (PE)-conjugated lineage antibodies as mentioned above, followed by incubation with anti-PE microbeads (Miltenyi). The unconjugated cells, which were enriched for progenitor cells, were separated using magnetic columns according to the manufacturer’s instruction. For progenitor cell isolation, the enriched cells were stained with antibodies discussed above and
sorted using a FACSAria Ilu cell sorter (BD Biosciences) prior to their intravenous adoptive transfer. Depending on the specific experiment, each recipient mouse was injected with 400,000 GFP+ donor lineage- c-kit+ progenitor cells or 100,000 GMPs 6 hours after induction of myocardial infarction. For photoconversion experiments, each recipient was injected with 25,000 to 40,000 Flk-2- HSPPCs 4 days before MI. Tissues from recipient mice were collected and analyzed by flow cytometry 4 days after MI. For isolation of Ly-6Chigh monocytes from the aorta 3 weeks after MI, single cell suspensions were prepared from the aorta as discussed above. Monocyte staining was performed using the antibodies mentioned above. Ly-6C^{high} monocytes were sorted using a FACSAria Ilu cell sorter (BD Biosciences).

Quantitative RT-PCR
Messenger RNA (mRNA) was extracted from aortic roots and bone marrow using a RNeasy Micro Kit (Qiagen) using manufacturer's protocol. One microgram of mRNA was used to generate complimentary DNA (cDNA) using a high capacity RNA to cDNA kit (Applied Biosystems). Taqman gene expression assays (Applied Biosystems) were used to quantify target genes. Variability in loading different amount of cDNA was normalized using Gapdh, a house keeping gene.

PCR
RNA was extracted from Ly-6Chigh monocytes sorted from the aortas of 3 mice per group using a RNeasy Micro Kit (Qiagen). Exactly 3,000 monocytes were isolated from atherosclerotic plaque using a tissue harvesting and antibody incubation protocols as described under Cell sorting using a FACSAria III cell sorter. QuantiTect Whole Transcriptome kit (Qiagen) was used to prepare cDNA from total RNA and make enough cDNA for the array by linear amplification according to manufacturer’s protocol. In short, reverse transcription reaction was performed at 37°C for 30 minutes to generate cDNA from mRNA. The reaction was stopped by an incubation at 95°C for 5 minutes. The synthesized cDNA was ligated using a ligation mix at 22°C for 2 hours. Finally, whole transcriptome amplification was performed at 30°C for 8 hours to generate cDNA. Three sets of primers were designed using online tools of Integrated DNA Technologies (http://www.idtdna.com/Scitools/Applications/RealTimePCR/). The specificity of the primers was validated using Sybr Green dissociation curves. Sybr Green-based quantitative PCR was performed using a 7300 real-time PCR system (Applied Biosystems). All samples were run in triplicates. Raw Ct values were normalized according to expression of Ly-6C and a heat map of relative normalized expression (Δct) data was generated using Cluster3 and Java Treeview applying an uncentered correlation metric for hierarchical clustering. For comparison of Ly-6C^{high} monocyte phenotype in bone marrow versus spleen, cells were isolated as described under cell sorting using a FACSAria Ilu cell sorter from 6 mice per group, on day 4 after coronary ligation. Monocyte staining was performed as discussed in the ‘Flow Cytometry’ section after depleting the lineage+ cells. Exactly 100,000 Ly-6Chigh monocytes were sorted from each organ per mouse and analyzed by PCR. cDNA was prepared from the total RNA extracted from the cells. Linear amplification of cDNA and Sybr Green-based quantitative PCR were performed for 32 genes. Raw Ct values were normalized to expression of Ly-6C, which was similar
AMI and atherosclerosis in both populations. A heat map of ΔCt values after mean centering for each gene was generated using the R environment for statistical computing. P-values were calculated using a two-sided t-test and Bonferroni corrected for multiple testing.

**Intravital microscopy**
CD45.1+ wild-type C57BL/6 mice were Flk-2- HSPC donors when recipients were CD45.2+ C57BL/6 mice. After cell sorting, 1–1.5 x 10^6 cells per ml Flk-2- HSPCs were stained with 5 μM DiD in PBS without serum for 10 min at 37°C, washed once in PBS, and injected into the tail vein of recipient mice. Each imaged CD45.2+ mouse received the same number of DiD-labelled CD45.1+ HSPCs (25,000 to 40,000 according to different experiments). Mice were anesthetized and prepared for in vivo imaging as previously described. Immediately before imaging, 25 μl of non-targeted Qdot 800 (Invitrogen) diluted in 100 μl sterile PBS was injected i.v. to allow vasculature visualization. The mouse was held in a heated tube mounted on a precision 3 axis motorized stage (Suter MP385). Cells within the skull bone marrow cavity were imaged with 635 nm laser illumination using a custom-built confocal two-photon hybrid microscope specifically designed for live animal imaging as described previously. At the start of each imaging session, large areas of the skull bone surface were surveyed using video rate second harmonic microscopy generated by collagen in the bone to identify the major anatomical landmarks such as sagittal and coronal sutures. The locations of HSPCs within bone-marrow cavities were identified and their coordinates recorded relative to the intersection of the sagittal and coronal sutures. Data were acquired as Z-stacks at 5 μm steps containing 3 separate channels: bone (assigned to the blue channel), vasculature (assigned to the red channel) and transplanted HSPCs (DiD signal, assigned to the white channel). Image processing was performed using Image J software. After in vivo imaging, the scalp was closed and post-operative care was provided.

**Photoconversion experiments**
Flk-2- HSPCs were harvested from the bone marrow (long bones, spine) of 30 CD45.2+ donor mice per one CD45.1+ recipient. Cells were isolated as described under cell sorting and then labeled with the near IR fluorescent, lipophilic carbocyanine DilC18(7) (DiR, Invitrogen) and injected i.v. into CD45.1 recipients. Four days after adoptive transfer, these cells were located in the skull by intravital microscopy as described above and then photoconverted in vivo by illuminating for 20 seconds with a 750 nm laser (<50 mW on the sample), changing the fluorescence of the dye from 780 nm to 670 nm. Immediately thereafter, converted cells were imaged by IVM in the bone marrow after excitation with the 635 nm laser to ensure that the color of the cells had changed. In one control mouse cells were not photoconverted. Two mice underwent coronary ligation after photoconversion, whereas two control mice did not. Four days after photoconversion, cells were harvested from the spleen. A splenic cell suspension was produced from the entire organ, which was lineage depleted with magnetic beads, stained for DAPI and FITC anti-mouse CD45.2 (Clone 104; BD Pharmingen), mounted on slides and finally imaged ex vivo as described previously.
**Fluorescence Molecular Tomography-Computed Tomography (FMT/CT)**

FMT/CT was performed to investigate how MI changes protease activity of atherosclerotic plaques of apoE-/- mice. Before inducing MI and then again three weeks after the MI, FMT-CT imaging (680/700 nm excitation/ emission) was performed to investigate magnitude of inflammation. ApoE-/- mice without MI were used as control. Five nmol of a pan-cathepsin protease sensor (Prosense-680) were injected i.v. 24 hours before imaging. A quantitative 3D dataset in which fluorescence per voxel was expressed in nM was reconstructed. FMT was followed by CT (Inveon PET-CT, Siemens) to identify anatomic regions. Contrast-enhanced high resolution CT localized the aortic root, a prominent site of plaque formation in apoE-/- mice. This anatomical information guided the placement of the volume of interest in the quantitative protease activity map concomitantly obtained by hybrid FMT. An imaging cartridge containing the anesthetized mouse was placed into a custom machined plexiglas holder that supplies isoflurane during imaging. The CT x-ray source with an exposure time of 370-400 ms was operated at 80 kVp and 500 μA. During CT, isovue-370 was infused continuously at 55 μL/min through a tail vein catheter. The CT reconstruction protocol performed bilinear interpolation, used a Shepp-Logan filter, and scaled pixels to Hounsfield units. Image fusion relied on fiducial markers and used Osirix software (The Osirix Foundation, Geneva).

**Fluorescence reflectance imaging (FRI)**

The mice were sacrificed after the second FMT-CT. Aortas were excised under a microscope and imaged using a planar fluorescent reflectance imaging system (OV-110, Olympus) with an excitation wavelength 680 nm. Light and near infrared fluorescence (NRIF) images were obtained with respective exposure times between 75 msec and 60 seconds.

**Histology**

Aortic roots were collected, embedded in O.C.T. compound (Sakura Finetek), and flash-frozen in a 2-methylbutane bath with dry ice. Sections of 5 μm thickness were stained with anti-CD11b antibody (clone M1/70, BD Biosciences) followed by a biotinylated anti-rat secondary antibody (Vector Laboratories, Inc.) VECTA STAIN ABC kit (Vector Laboratories, Inc.) and AEC substrate (DakoCytomation) were used for color development. Cells with CD11b staining were quantified using IPLab (version 3.9.3; Scanalytics, Inc.). For fibrous cap and necrotic core analysis, aortic roots were stained with Trichrome stain (MASSON) (Sigma-Adlrich). Fibrous cap thickness and necrotic core were quantified using Masson stained aortic root sections scanned with NanoZoomer 2.0-RS (Hamamatsu) in 40x magnification as previously described. Briefly, for fibrous cap thickness, 5 measurements of the thinnest fibrous caps per plaque were averaged. Necrotic cores were analyzed by measuring total acellular areas within each plaque. Toluidine Blue staining was carried out to detect mast cells. After aortic roots sections were fixed with 10% formalin solution, the sections were stained in Toluidine blue working solution (1.0g of Toluidine Blue O (Sigma-Adlrich) in 100mL of 70% alcohol and 1% Sodium chloride in distilled water adjusted pH to 2.3), washed in distilled water, and dehydrated quickly prior to clearing in xylene and cover slipping. Femurs were harvested, fixed in 4% paraformaldehyde for 3 hours,
and immersed in 0.375M EDTA in PBS for 10 days for decalcification prior to paraffin embedding. The paraffin-embedded sections were deparaffinized, rehydrated, and stained with anti-tyrosine hydroxylase antibody (Millipore). The slides were scanned with a NanoZoomer 2.0-RS (Hamamatsu) at 40x magnification and quantified using IPLab (version 3.9.3; Scanalytics, Inc.). For immunofluorescence microscopy, frozen spleen sections were stained with SCF (clone H-189, Santa Cruz Biotechnology, Inc.), nestin (clone 7A3, abcam), CD31 (clone MEC13.3, BD Biosciences), and CD31: Alexa Fluor 488 (clone ER-MP12, AbD Serotec). Alexa Fluor 488 chicken anti-rabbit IgG (Molecular Probes) and Alexa Fluor 594 donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc.) were used as secondary antibodies. The slides were cover slipped using a mounting medium with DAPI (Vector Laboratories, Inc.) to identify nuclei. Images were observed and captured using Nikon Eclipse 80i with a Cascade Model 512 B camera (Roper Scientific) with a Cy5.5 filter cube (HQ650/45x EX, dichroic Q680LP BS, and emission filter HQ710/50m EM), Y-2E/C (D560/40x EX, dichroic 595DCLP BS, and emission filter D630/60m EM), GFP/FITC (HQ480/40x EX, dichroic Q505LP BS, and emission filter HQ535/50m EM), and UV (D365/10x EX, dichroic 380DCLP BS, and emission filter E400LPv2 EM, Chroma Technology Corp.)

**Human spleen tissue collection**

Autopsy samples of spleen tissue was collected from 29 patients who died hours to days after acute myocardial infarction (mean age 65 ± 3, 21 men) and 13 control trauma cases (42 ± 6 years, 9 men) at Heidelberg University, Germany. Spleen tissue was also stained in additional autopsy studies at the University of Amsterdam, The Netherlands, where the use of patient material after completion for the diagnostic process is part of the patient contract in the VUmc hospital. The study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the institutional medical ethics committee. Deparaffinized and rehydrated sections were incubated with 10% goat serum, followed by incubation with rabbit anti-c-kit (clone YR145, 1:25, Cellmarque, Rocklin, CA, USA) and mouse anti-Ki-67 (clone MIB-1, 1:150, Dako, Copenhagen, Denmark). Sections were then incubated with goat anti-rabbit IgG Alexa Fluor 555 and goat anti-mouse IgG Alexa Fluor 488 (each 1:200; Molecular Probes, Leiden, The Netherlands), and counterstained with Hoechst 33342. After fluorescence labeling, the slides were immersed for 30 min in 70% ethanol supplemented with 0.1% Sudan Black B (Merck, Darmstadt, Germany). Stained sections were examined under Leica DM6000 microscope (Leica Microsystems, Heidelberg, Germany). Immunoenzyme stainings of c-kit and Ki-67-clones were performed on 2-μm paraffin sections of formalin-fixed tissues using standard avidin-biotin anti–alkaline phosphatase techniques (Vectastain; Vector Laboratories). Antigen retrieval was achieved by steam-cooking the slides in 10 mM citrate buffer (pH 6.1; Dako) for 30 min. A solution of 10% Earle’s balanced salt solution (EBSS, Sigma-Aldrich) supplemented with 1% Hepes, 0.2% BSA, and 0.1% saponin (all from Sigma-Aldrich), pH 7.4, was used as a washing and permeabilization buffer. Primary Ab dilutions also were prepared in this buffer with 4% γ-venin (Behring) added and incubated overnight at 4°C. Biotinylated sheep anti-mouse IgG was used as a secondary reagent for 30 min at room temperature.
Naphthol AS-biphosphate (Sigma-Aldrich) with New Fuchsin (Merck) was used as the substrate for alkaline phosphatase.

**Blood cholesterol and HDL measurement**
Using a cardiac puncture, blood was drawn from apoE-/- mice and collected in eppendorf tubes without anti-coagulant. Blood was kept at room temperature for an hour followed by a spin at 40 °C for 20 minutes. Serum was carefully collected without red blood cell contamination and stored at -200°C. Total cholesterol and HDL were measured using an enzymatic colorimetric assay (Cholesterol E, Wako Diagnostics) according to manufacturer’s instructions.

**Analysis of white blood cell and monocyte counts in patients**
The Pravastatin or Atorvastatin Evaluation and Infection Therapy – Thrombolysis in Myocardial Infarction 22 (PROVE IT-TIMI 22) trial was a multicenter, randomized controlled trial of intensive versus standard lipid lowering therapy in patients hospitalized for an acute coronary syndrome within the previous 7-10 days. The current analysis focused on comparing white blood cell and monocyte counts on day 7-10 after acute coronary syndrome in patients with and without beta-blocker use prior to enrollment, as defined by an interview with the patient and a review of medical records by trained researchers. White blood cell and monocyte counts were assessed in the laboratories at local enrolling institution and recorded on a case report form. Descriptive statistics were expressed as median with interquartile range for continuous variables. Differences in white blood cell and monocyte counts between patients with prior beta-blocker use versus those without were assessed by the Wilcoxon rank-sum test. Univariate linear regression analysis was performed to evaluate the relationship between prior beta-blocker use and white blood cell or monocyte counts (both log transformed). Multivariate linear regression analysis was also performed after adjusting for potential confounders: age, gender, time from symptom onset to randomization, history of congestive heart failure, and creatinine clearance.

**Statistics**
Results are expressed as mean ± standard error of mean. Data was tested for normality using the D’Agostino-Pearson normality test, and for equality of variances using the Bartlett’s test. If normality and equality of variances were not rejected at 0.05 significance level, the group means were compared using a t-test (for 2 groups) and ANOVA, followed by Bonferroni post tests (for > 2 groups). P values of <0.05 indicate statistical significance. For non-normally distributed data and data with unequal variances, we applied nonparametric tests, such as Mann-Whitney U.
Supplementary References


Supplementary Figure S1 MI increases inflammatory gene expression in atherosclerotic plaques. Bar graphs show qPCR of aortic roots excised from apoE-/- mice with and without coronary ligation (n = 5 per group, 3 weeks after MI). mRNA levels were normalized to Gapdh Ct values. Mean ± s.e.m., * P < 0.05.

Supplementary Figure S2 Fitting Ly-6C<sup>high</sup> monocyte content in the plaque as a function of age. Myocardial infarction, induced at 20 weeks of age, significantly accelerated accumulation of inflammatory monocytes in atherosclerotic plaque. The graph shows means for apoE-/- mice that received MI (red) or not (blue). The lines represent the linear fit of data from respective groups (n = 30 per group). The slopes of the lines were significantly different (P = 0.037). The goodness of fit (R²) was 0.78 and 0.75, respectively. The right panel shows the increase of F4/80<sup>low</sup> Ly-6C<sup>low</sup> cells in aortae. Here, the slope did not differ significantly between infarct and control cohorts.
Supplementary Figure S3 Neutrophil levels in atherosclerotic plaque after MI. Quantification of neutrophils (lineage+ CD11b+ cells) in atherosclerotic plaques at different time points after MI. Age matched apoE−/− mice without MI served as controls (n = 5−9 per group). Representative dot plots from the experiment at 3 weeks after MI are shown. Mean ± s.e.m., * P < 0.05, ** P < 0.01.
**Supplementary Figure S4** Mast cell levels in aorta after MI. Aortic root sections were stained with toluidine blue to detect mast cells (n = 7–9 per group). Scale bar indicates 25 μm. Mean ± s.e.m.

**Supplementary Figure S5** Fibrous cap thickness and necrotic core area in plaque after MI. Masson staining on aortic roots harvested from apoE−/− mice 3 weeks after MI and age-matched control apoE−/− mice (n = 7–11 per group). Scale bar indicates 50 μm, arrows point at fibrous cap above necrotic core. Bar graphs show fibrous cap thickness and necrotic core area per section. Mean ± s.e.m., *P < 0.05.
Supplementary Figure S6 Plaque monocytes express higher levels of inflammatory genes after MI. Ly-6Chigh monocytes were isolated from aortae by flow sorting and then analyzed by qPCR (n = 3 apoE-/- mice per group, MI: 3 weeks after coronary ligation).
Supplementary Figure S7 Monocytosis in the spleen and blood after MI. FACS quantification of myeloid cells and Ly-6C<sup>high</sup> monocytes in the spleen (a) and blood (b) of apoE<sup>-/-</sup> mice at different time points after MI (n = 3–9 per group). Blue rectangular gates show myeloid cells and lower right quadrants show Ly-6C<sup>high</sup> monocytes. Age matched apoE<sup>-/-</sup> mice without MI served as controls. Mean ± s.e.m., * P < 0.05, ** P < 0.01.
Supplementary Figure S8 Monocyte levels in the bone marrow. Levels of total monocytes (left) and Ly-6C<sup>high</sup> monocytes in the bone marrow at different time points after MI were measured by flow cytometry (n = 3–9 per group). Age-matched apoE<sup>-/-</sup> mice without MI were used as control (No MI). Mean ± s.e.m.

Supplementary Figure S9 Flow cytometric gating strategy for progenitor cells. Flow cytometric gating for HSPCs, MDPs, and GMPs in the spleen of apoE<sup>-/-</sup> mice with and without MI. Hematopoietic stem and progenitor cells (HSPCs) were identified as lineage<sup>−</sup> (CD90/CD45/B220/CD49b/NK1.1/Ly-6G/Ter119/CD11b/CD11c/IL7Rα)<sup>low</sup> c-kit<sup>high</sup> Sca-1<sup>high</sup>. Granulocyte and macrophage progenitors (GMPs) were identified as lineage<sup>−</sup> c-kit<sup>high</sup> Sca-1<sup>low</sup> CD115<sup>high</sup> CD16/32<sup>high</sup> CD34<sup>high</sup>. Macrophage dendritic cell progenitors (MDPs) were identified as lineage<sup>−</sup> c-kit<sup>high</sup> Sca-1<sup>low</sup> CD115<sup>low</sup> CD16/32<sup>low</sup> CD34<sup>high</sup>. Absolute numbers of progenitors in spleen are shown in Figure 2.
Supplementary Figure S10 Progenitor cell numbers in the bone marrow. Levels of HSPCs, GMPs and MDPs in the bone marrow of apoE-/ mice were unchanged 3 weeks after MI (n = 12–15 per group). Mean ± s.e.m.
Supplementary Figure S11 Splenic progenitor proliferation after MI. Proliferation of progenitor cells in C57BL/6 mice was measured by in vivo BrdU incorporation assay. BrdU was injected intraperitoneally on day 3 after MI and BrdU+ HSPCs, GMPs and MDPs in the bone marrow (a) and spleen (b) were quantified by flow cytometry on day 4 after MI. Naive C57BL/6 mice without MI injected or not injected with BrdU served as controls (n = 4–5 per group). Mean ± s.e.m., * P < 0.05.
Supplementary Figure S12 Splenic progenitors in patients with MI. (a) Immunoreactive staining for c-kit and Ki-67 in a patient with subacute MI. Scale bar indicates 14 μm. (b) Enumeration of c-kit+ mast cell tryptase- cells in the red pulp of the spleen in patients that died hours to days after myocardial infarction (MI, n = 29) or trauma control cases (No MI, n = 13). Mean ± s.e.m., * P = 0.038 in one tailed Mann-Whitney test.
Supplementary Figure S13 Splenic monocytes contribute to increased cell numbers in atherosclerotic plaque after MI. Splenectomy was performed in apoE-/- mice on the day of coronary ligation. (a) Protease activity in aortic roots measured by FMT-CT before and then again 3 weeks after MI and splenectomy. Circles show aortic root area (n = 8–10 per group). (b) Myeloid cells and Ly-6Chigh monocytes in the aorta were quantified and compared with apoE-/- mice with MI but without splenectomy (n = 6–12 per group). (c) Immunohistochemical staining of aortic roots for CD11b (n = 10 per group). The scale bar represents 150 μm. (d) qPCR analyses of aortic roots (n = 5 per group). Mean ± s.e.m., * P < 0.05, ** P < 0.01.
Chapter 9

Supplementary Figure S14 Progenitor cell levels in the liver after MI and splenectomy. The liver can contribute to prenatal hematopoiesis; hence, we also investigated progenitors in this organ. Progenitor cell (Flk-2- HSPCs, HSPCs and GMPs) numbers in the liver and spleen of C57BL/6 mice were quantified using flow cytometry on day 4 after MI (with or without splenectomy) (n = 3–4 per group). Mean ± s.e.m., * P < 0.05.
Supplementary Figure S15 mRNA profile in splenic and bone marrow Ly-6Chigh monocytes after MI. Ly-6Chigh monocytes were sorted from the spleen and bone marrow on day 4 after coronary ligation in six C57BL/6 mice per group, a mRNA heat map is shown. * P < 0.05 ANOVA with Bonferroni post test comparing spleen versus bone marrow mRNA level.
Supplementary Figure S16 Stroke increases monocyte numbers in atherosclerotic plaque. Organs were harvested 6 weeks after stroke in apoE−/− mice and analyzed by flow cytometry. Myeloid cells and Ly-6Chigh monocytes were quantified in the aorta (a), blood (b), and spleen (c). (d) GMPs and MDPs in the spleen. ApoE−/− mice without stroke were used as control (n = 6–7 per group). Mean ± s.e.m., * P < 0.05, ** P < 0.01.
Supplementary Figure S17 Flk-2- HSPCs in the spleen after MI. (a) Gating strategy for Flk-2- HSPCs: CD90/B220/CD49b/NK1.1/Ly-6G/Ter119/CD11b/CD11c/ IL7Rα)low c-kit high Sca-1 high FLK-2low CD34 high/low cells. (b) Quantification of Flk-2- HSPCs in the spleen 4 days after MI by flow cytometry (n = 3–6 per group). Mean ± s.e.m., * P < 0.05.

Supplementary Figure S18 β3 receptor antagonist treatment after MI reduces progenitor numbers in the spleen of wild type mice. C57BL/6 mice were treated with a β3 receptor antagonist intraperitoneally twice daily after inducing MI. HSPCs and GMPs numbers in the spleen were analyzed by flow cytometry (n = 4–9 C57BL/6 mice per group). Mean ± s.e.m., * P < 0.05, ** P < 0.01.
Supplementary Figure S19 β3 receptor antagonist treatment after MI reduces monocyte levels in the spleen of wild type mice. C57BL/6 mice were treated with a β3 receptor antagonist intraperitoneally twice daily after inducing MI. (a) Flow cytometric plots showing myeloid cells (blue gates in the upper panel) and Ly-6C\textsuperscript{high} monocytes (lower right quadrant in the lower panel) in different treatment groups. (b) Quantification of myeloid cells, monocytes, and Ly-6C\textsuperscript{high} monocytes. Untreated mice with or without MI served as controls (n = 4–7 per group). Mean ± s.e.m., * P < 0.05, ** P < 0.01.
Supplementary Figure S20 β3 receptor antagonist treatment after MI reduces monocyte and GMP levels in apoE-/- mice. ApoE-/- mice with MI were treated with a β3 receptor antagonist twice daily for three weeks (n = 6 per group). (a) Flow cytometric plots showing GMPs (left) and quantification of GMPs (right) in the spleen. Their progeny, myeloid cells and Ly-6Chigh monocytes, were quantified in the spleen (b) and blood (c) and compared with untreated apoE-/- mice with MI. Mean ± s.e.m., * P < 0.05, ** P < 0.01.
Supplementary Figure S21  Treatment with a β3 receptor antagonist reduces atherosclerosis after MI.  

(a) Protease activity in aortic roots measured by FMT-CT before and then again 3 weeks after MI. Circles show aortic root area (n = 8 per group).  

(b) Flow cytometric plots showing myeloid cells (blue gates in the upper panel) and Ly-6Chigh monocytes (lower right quadrant in the lower panel) in apoE-/- mice 3 weeks after MI, treated with a β3 receptor antagonist (n = 6 per group).  

(c) Immunohistochemical staining of aortic roots for CD11b after treatment with a β3 receptor antagonist (n = 10 per group). The scale bar represents 150 μm.  

(d) qPCR analyses of aortic roots (n = 5 per group). Mean ± s.e.m., * P < 0.05, ** P < 0.01.
Supplementary Figure S22 β3 blocker treatment does not affect splenic monocyte differentiation. Flow cytometric analysis of splenocytes 4 days after MI in C57BL/6 mice treated or not treated with a β3 blocker. (a) Proliferation of host GMPs determined by BrdU incorporation (n = 5 per group). (b) Quantification of GFP+ monocytes 4 days after MI and adoptive transfer of GFP+ GMPs (n = 5 per group).
Supplementary Figure S23 Chemical sympathectomy reduces atherosclerosis after MI. (a) Quantification of HSPCs 4 days after MI in blood of C57BL/6 mice treated or not treated with 6-OHDA (n = 5–16). (b) mRNA level of CXCL12, an HSPC retention factor in the bone marrow of C57BL/6 mice with or without 6-OHDA treatment (n = 4–5 per group). Myeloid cells and Ly-6C<sub>high</sub> monocytes were quantified in blood (c) and the aortae (d) of apoE<sup>−/−</sup> mice treated or not treated with 6-OHDA for three weeks after MI (n = 5–7 per group). Mean ± s.e.m., * P < 0.05, ** P < 0.01.

Supplementary Figure S24 The effects of β3 blocker treatment with or without splenectomy on aortic myeloid cell content after MI. ApoE<sup>−/−</sup> mice received splenectomy on the day of MI, and 2 cohorts of apoE<sup>−/−</sup> mice were treated with a β3 blocker for three weeks. Myeloid cells and Ly-6C<sub>high</sub> monocytes were quantified in the aortae by flow cytometry. Age matched apoE<sup>−/−</sup> mice without MI served as control (n = 5–9 per group). Mean ± s.e.m., * P < 0.05 versus no MI, MI + splenectomy, and MI + β3 blocker treatment.
MI or treatment with a β3 receptor antagonist does not change blood cholesterol and HDL levels. Serum was separated from blood drawn from apoE−/− mice that either received MI 3 weeks prior or were treated with a β3 receptor antagonist twice daily for three weeks. Serum of naive apoE−/− mice was used as control. All groups received high cholesterol western type diet. Total cholesterol and HDL were measured in the serum using an enzymatic colorimetric assay (n = 11–12 per group).

Release of DiD+ HSPCs from the bone marrow after MI by flow cytometry. DiD labelled-HSPC cells assessed in the bone marrow by flow cytometry following intravital microscopy. Bone marrow cells were harvested, gated on Lin− IL7R− c-kit+ Sca-1+ cells. Blue gate shows adoptively transferred CD45.1+ DiD+ cells. These data confirm IVM results shown in Figure 4.
Supplementary Figure S27 Bone marrow Flk-2- HSPCs relocate to the spleen after MI. (a) Experimental outline. (b) DiR labelled HSPCs in the bone marrow by intravital microscopy (IVM) before (blue) and after photoconversion (red). The scale bar represents 50 μm, the inset is a higher magnification view of cells on the left (arrows). 18 ± 4 cells were photoconverted per mouse. (c) Photoconverted cells were identified as cDiR+DAPI+CD45.2+ in splenic cell suspensions 4 days after induction of MI by ex vivo imaging. The scale bar represents 20 μm. The bar graph depicts detected cells in the spleen as percentage of cells converted in the skull for individual cases.
Supplementary Figure S28 SCF-expressing cells in the spleen. Co-localization of SCF, CD31 and DAPI (a); and SCF, nestin and DAPI (b) by immunofluorescence microscopy of spleen sections 4 days after MI. (c) Detection of adoptively transferred DiD+ Flk-2- HSPCs in proximity to CD31+ cells. Scale bar indicates 10 μm.
Supplementary Figure S29 VLA-4 neutralization and splenic engraftment of progenitors after MI and in steady state. Flow cytometric enumeration of adoptively transferred GFP+ HSPCs in mice with MI (a) or no MI (b) treated with either anti-mouse VLA-4 antibody or control IgG (n = 4 per group). Mice without treatment and cell transfer served as a negative control. Previous work showed no effect of neutralizing VLA-4 on stem cell retention in the spleen after bone marrow transplantation into irradiated recipients, a contrast most likely caused by a different splenic milieu in non-irradiated mice with myocardial infarction. Mean ± s.e.m. * P < 0.01 versus control IgG.
Supplementary Table S1 β-adrenergic blocker administration associates with reduction in white blood cell and monocyte counts in patients after an acute coronary syndrome. (a) Schematic representation of study design and retrospective analysis. (b) Association between prior beta-blocker use and white blood cell and monocyte counts (expressed as 10^9/l) in patients 7 to 10 days after an acute coronary syndrome. (c) Linear regression analysis of patients with prior beta-blocker use and log white blood cell and log monocyte counts. Multivariate analysis was adjusted for age, gender, time from symptom onset to randomization, history of chronic heart failure, and creatinine clearance.