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doi:10.1038/nature11260

Myocardial infarction accelerates atherosclerosis

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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.

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*$^{-/-}$ 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 cysteinyl 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*$^{-/-}$ 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 (Fig. 1a, b). In parallel,

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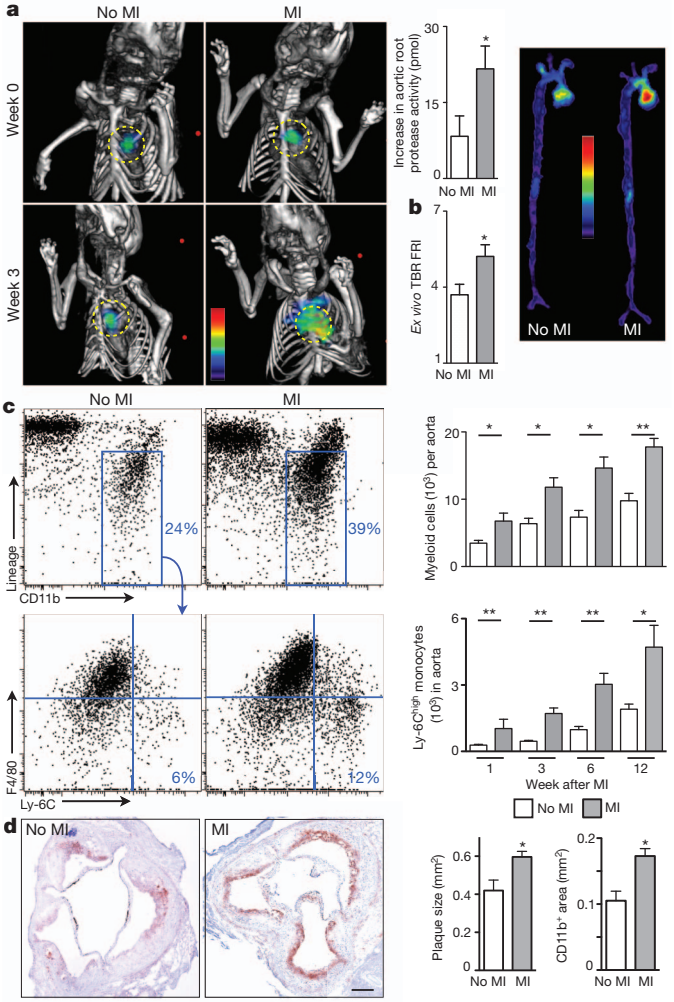

Figure 1 | Increased inflammation in atherosclerotic plaques after MI. **a**, Protease activity was determined by FMT–CT before and 3 weeks after MI. Circles indicate aortic root ($n = 10$ per group). **b**, Protease activity in excised aortae determined by fluorescence reflectance imaging (FRI), expressed as target to background ratio (TBR; $n = 10$ per group). **c**, Flow cytometric quantification of myeloid cells and Ly-6C^{high} 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. $^{*}P < 0.05$, $^{**}P < 0.01$.

expression of the inflammatory cytokine interleukin-6 (*Il6*), *Mmp9*, myeloperoxidase and *Ly-6C* (also known as *Ly6c1*) increased in atherosclerotic plaques (Supplementary Fig. 1). The number of monocytes and macrophages per aorta increased, particularly the inflammatory Ly-6C^{high} monocyte subset (Fig. 1c). Plaque monocyte content also increased in *Apoe*$^{-/-}$ mice without MI, reflecting the natural course of disease in these animals[17,18]. Yet innate immune cell accumulation accelerated distinctively after MI, as indicated by the significantly greater slope obtained when fitting the number of Ly-6C^{high} monocytes in the aorta over time (Supplementary Fig. 2). Neutrophil presence in atheromata also increased (Supplementary Fig. 3) whereas mast cells did not (Supplementary Fig. 4). Histological analysis affirmed increased accumulation of CD11b^{+} myeloid cells and larger lesion size after MI (Fig. 1d). The thickness of the fibrous cap decreased, covering larger necrotic cores (Supplementary Fig. 5). Ly-6C^{high} 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 Fig. 6). Monocyte numbers in the blood and spleen increased consistently for up to 3 months after coronary ligation (Supplementary Fig. 7) but were unaltered in the bone marrow (Supplementary Fig. 8).

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 (Fig. 2 and Supplementary Fig. 9) but not in the bone marrow (Supplementary Fig. 10). Proliferation of progenitors doubled in the spleen (Supplementary Fig. 11). 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 Fig. 12).

When we splenectomized mice at the time of MI, atherosclerosis did not accelerate (Supplementary Fig. 13). 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 Fig. 14). 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-6C^{high} 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 Fig. 15). 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-6C^{high} monocytes isolated from atherosclerotic plaques after MI (Supplementary Fig. 6). 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-6C^{high} monocytes in atherosclerotic plaques increased after stroke, in parallel with expanded splenic monocytopoiesis (Supplementary Fig. 16).

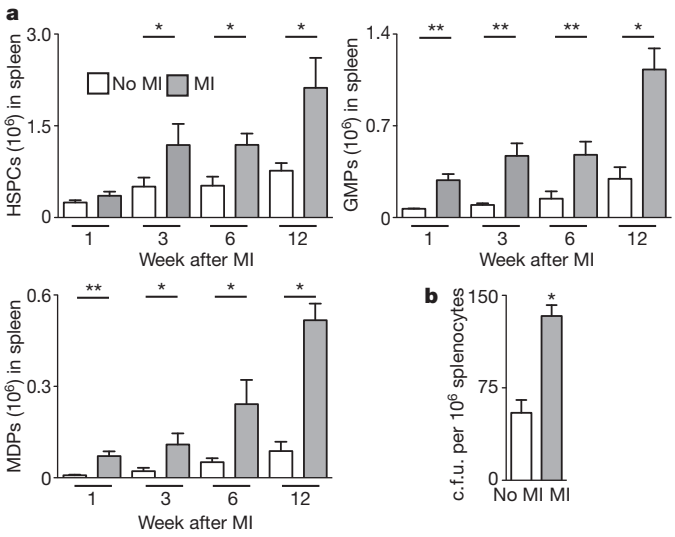

Figure 2 | Elevated levels of progenitor cells in the spleen of *Apoe*$^{-/-}$ mice after MI. **a**, Quantification of HSPCs, MDPs and GMPs at different time points after MI ($n = 3$–15 per group). The gating strategy is shown in Supplementary Fig. 10. **b**, Number of colony-forming units (c.f.u.). Data are shown as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

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 96 h after MI, respectively (Fig. 3a). The number of splenic Flk2$^-$ HSPCs increased markedly after MI (Supplementary Fig. 17). 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 (Fig. 3b). SNS activity may liberate haematopoietic stem cells from their niches by signalling through the β_3-adrenoceptor[26]. Nestin$^+$ mesenchymal stem cells express this receptor, which regulates the production of stem cell retention factors[27]. Because acute MI raises blood progenitor levels in patients[12], 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 96 h after MI in mice treated with a β_3-adrenoceptor antagonist (Fig. 3a). The stem cell retention factor *Cxcl12*, angiopoietin and stem cell factor (*Scf*; also known as *Kitl*)[28] underwent similar regulation (Fig. 3c). Levels of the adhesion molecule *Vcam1*, which also retains HSPCs in the bone marrow, decreased after MI but did not change after β_3-adrenoceptor blocker administration (Fig. 3c). These data indicate that increased sympathetic tone after MI causes withdrawal of stem cell retention factors by β_3-adrenoceptor-expressing niche cells.

Treatment with a β_3-adrenoceptor blocker reduced splenic accumulation of progenitors in wild-type mice shortly after MI (Supplementary Fig. 18) and consequently diminished their output of myeloid cells (Supplementary Fig. 19). In *Apoe*$^{-/-}$ mice 3 weeks after MI, β_3-blocker treatment reduced the number of GMPs and their progeny in the spleen and blood (Supplementary Fig. 20). Retrospective analysis of a clinical trial[29] revealed that prior β-blocker therapy was associated with a reduction in monocytes after an acute coronary syndrome (Supplementary Table 1). The mechanism that led to this decrease is unclear, also because some clinically used β-blockers have a lower affinity for the β_3-adrenoceptor subtype[30]; however, these associative data show an interesting parallel to our findings in mice.

In *Apoe*$^{-/-}$ mice after MI, β_3-blocker treatment lowered protease activity, myeloid cell content, and mRNA levels of inflammatory cytokines in the plaque (Supplementary Fig. 21). When we adoptively transferred GFP$^+$ GMPs to wild-type mice with MI, β_3-blocker treatment did not alter their splenic differentiation (Supplementary Fig. 22). Sympathetic denervation with 6-hydroxydopamine (6-OHDA)[26,31] 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 Fig. 23). Combination of β_3 blockade and splenectomy showed no additive effects (Supplementary Fig. 24). Neither MI nor β_3 blockade changed blood cholesterol and high-density lipoprotein levels (Supplementary Fig. 25).

Intravital microscopy of HSPC departure

We adoptively transferred lineage$^-$ c-kit$^+$ Sca-1$^+$ Flk2$^-$ (Sca1 also known as Ly6a) HSPCs labelled with a fluorescent membrane dye (DiD) to examine their release with serial intravital microscopy

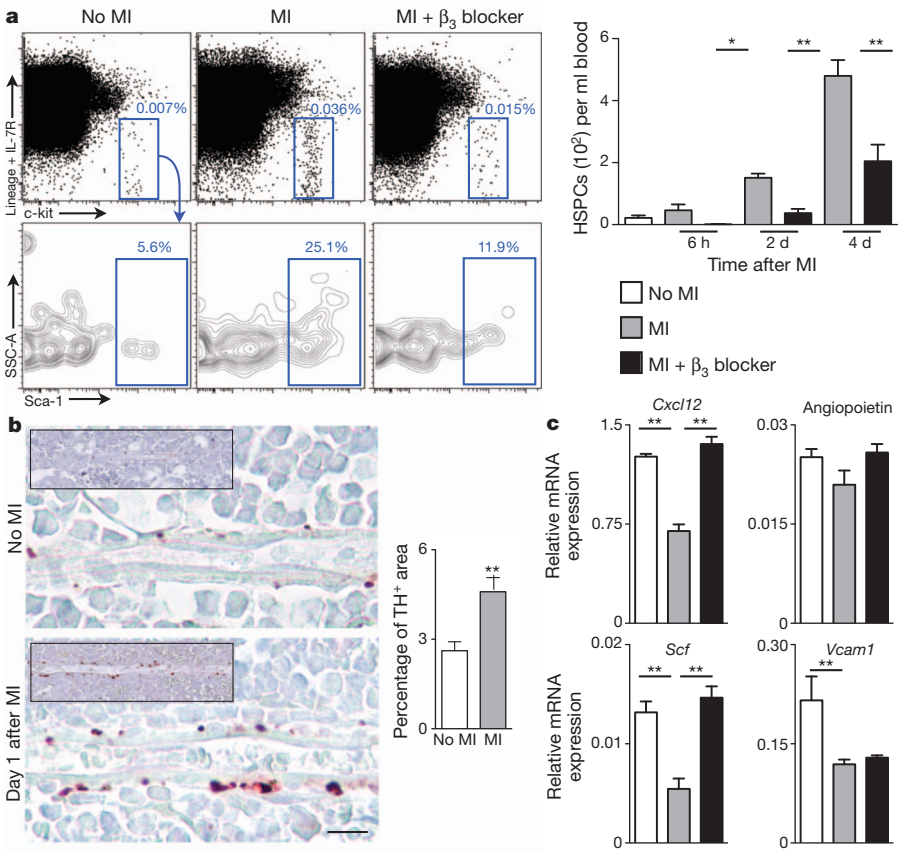

Figure 3 | β_3-Adrenoceptor-mediated progenitor release after MI. **a**, Flow cytometric analyses of HSPCs in blood of C57BL/6 mice ($n = 6$–11 per group). **b**, Immunostaining for tyrosine hydroxylase (TH). Scale bar represents 10 μm. Insets depict low-magnification overview. Bar graph shows quantification 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. $^*P < 0.05$, $^{**}P < 0.01$.

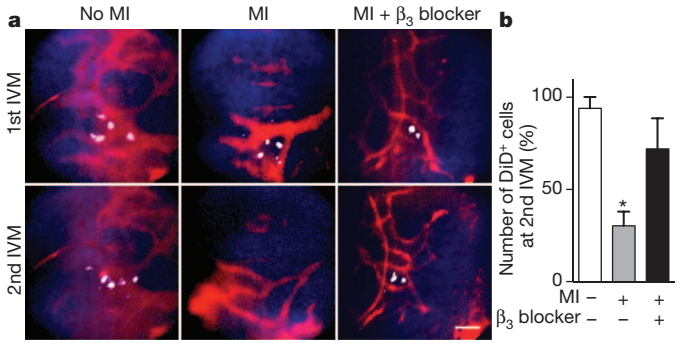

Figure 4 | Serial intravital imaging of progenitor release from the bone marrow. **a**, DiD-labelled-HSPC Flk2$^-$ cells were imaged in the skull bone marrow by IVM before and then again 4 days after MI. DiD–labelled HSPCs are white, blood pool is red, and bone is blue. SSC-A, side scatter. Scale bar represents 50 μm. **b**, Change of HSPC presence between first and second IVM session ($n = 3$ per group). Data are shown as mean ± s.e.m. *$P < 0.05$.

(IVM)[32]. 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 β_3-adrenoceptor antagonist (Fig. 4). Post-imaging flow cytometry corroborated the trafficking of DiD$^+$ cells (Supplementary Fig. 26). We next investigated the relocation of bone marrow cells to the spleen directly. Lineage$^-$ c-kit$^+$ Sca-1$^+$ 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$^+$ 4′,6-diamidino-2-phenylindole (DAPI)$^+$ cells were detected in splenic cell suspensions 4 days later (Supplementary Fig. 27).

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 (Fig. 5a, b). Antibody neutralization of SCF decreased retention of adoptively transferred HSPCs in the spleen and proliferation of host HSPCs (Fig. 5c, d). Co-localization studies identified CD31$^+$ and occasionally nestin$^+$ cells (Supplementary Fig. 28a, b) 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 Fig. 28c). 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 Fig. 29).

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 (Fig. 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[3,6,7], 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[35], 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 circulating acute phase reactants such as fibrinogen and

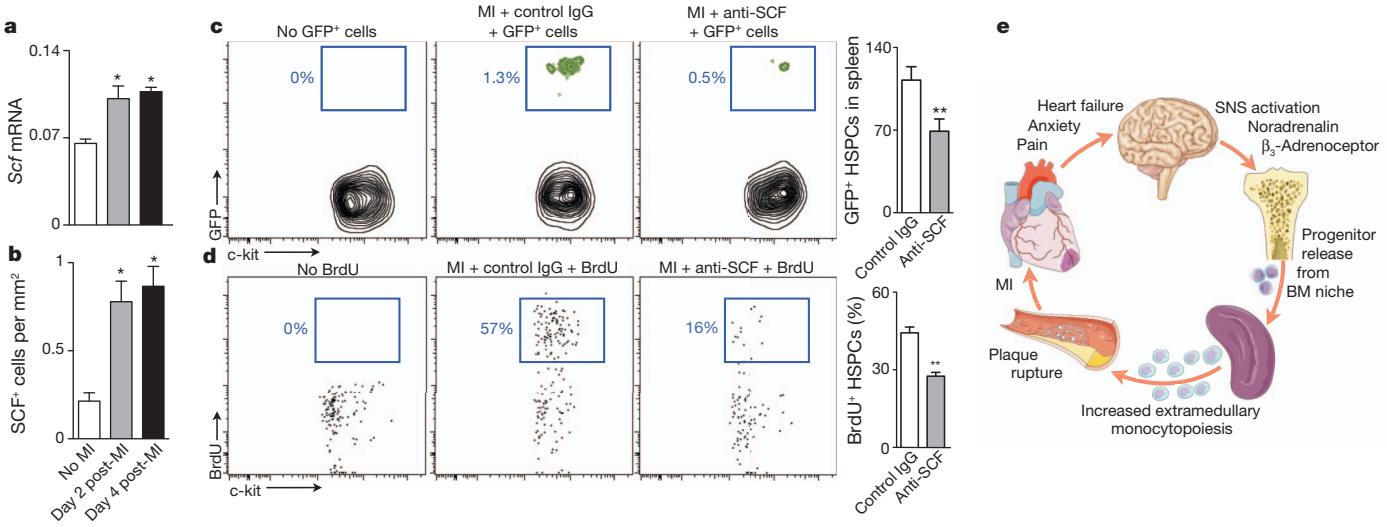

Figure 5 | Splenic progenitor engraftment after MI. **a**, Quantitative polymerase chain reaction of SCF in spleen ($n = 5$–6 per group). **b**, Number of SCF$^+$ cells in spleen of C57BL/6 mice 4 days after MI as determined by immunofluorescence. **c**, Enumeration of adoptively transferred GFP$^+$ HSPCs on day 4 after MI ($n = 8$ per group). **d**, Proliferation of endogenous HSPCs determined by BrdU incorporation ($n = 8$ per group). **e**, Paradigm. BM, bone marrow. Data are shown as mean ± s.e.m. *$P < 0.05$, **$P < 0.01$.

plasminogen activator inhibitor 1, factors that promote thrombosis and counter endogenous fibrinolysis[36]. 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-Apoe^{tm1Unc}/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.

Received 12 December 2011; accepted 25 May 2012.
Published online 27 June 2012.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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).

Author Contributions P.D. and G.C. performed experiments, collected and analysed the data, and contributed to writing the manuscript, R.G. did surgeries and performed experiments, Y.W., F.Le., R.G., C.S.R., Y.I., B.T., A.L.C., T.H., M.D.M., F.La., M.E., P.W., M.T.W., A.T.C., A.M.v.d.L., H.W.M.N., J.J.P., B.B.R., J.B., J.R.S., H.A.K., C.V., S.A.M., D.A.M. and M.S.S. performed experiments, collected, analysed and discussed data, M.A.M., M.J.P., P.L., C.P.L., F.K.S. and R.W. conceived experiments and discussed strategy and results; M.N. designed and managed the study and wrote the manuscript, which was edited and approved by all co-authors.

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