Objectives
Here we assess the intrinsic functions of the chemokine receptor CXCR4 in remodeling after myocardial infarction (MI) using Cxcr4 heterozygous (Cxcr4+/−) mice.

Background
Myocardial necrosis triggers complex remodeling and inflammatory changes. The chemokine CXCL12 has been implicated in protection and therapeutic regeneration after MI through recruiting angiogenic outgrowth cells, improving neovascularization and cardiac function, but the endogenous role of its receptor CXCR4 is unknown.

Methods
MI was induced by ligation of the left descending artery. Langendoff perfusion, echocardiography, quantitative immunohistochemistry, flow cytometry, angiogenesis assays, and cardiomyocyte analysis were performed.

Results
After 4 weeks, infarct size was reduced in Cxcr4+/− mice compared with wild-type mice and in respective bone marrow chimeras compared with controls. This was associated with altered inflammatory cell recruitment, decreased neutrophil content, delayed monocyte infiltration, and a predominance of Gr1low over classic Gr1high monocytes. Basal coronary flow and its recovery after MI were impaired in Cxcr4+/− mice, paralleled by reduced angiogenesis, myocardial vessel density, and endothelial cell count. Notably, no differences in cardiac function were seen in Cxcr4+/− mice compared with wild-type mice. Despite defective angiogenesis, Cxcr4+/− mouse hearts showed no difference in CXCL12, vascular endothelial growth factor or apoptosis-related gene expression. Electron microscopy revealed lipofuscin-like lipid accumulation in Cxcr4+/− mouse hearts and analysis of lipid extracts detected high levels of phosphatidylserine, which protect cardiomyocytes from hypoxic stress in vitro.

Conclusions
CXCR4 plays a crucial role in endogenous remodeling processes after MI, contributing to inflammatory/progenitor cell recruitment and neovascularization, whereas its deficiency limits infarct size and causes adaptation to hypoxic stress. This should be carefully scrutinized when devising therapeutic strategies involving the CXCL12/CXCR4 axis. (J Am Coll Cardiol 2011;58:2415–23) © 2011 by the American College of Cardiology Foundation

In addition to governing hematopoetic cell trafficking, the CXCR4 ligand SDF-1α/CXCL12 has been shown to promote tissue regeneration by mediating recruitment of progenitor cells in ischemic areas (1–3). Therefore, the
interaction between CXCL12 and CXCR4 is increasingly exploited to enhance the efficacy of stem cell therapy after myocardial infarction (MI) (4). Exogenous CXCL12 applied by myocardial injection or overexpressed in transplanted cardiomyocytes, as well as overexpression of CXCR4 in mesenchymal stem cells, induces therapeutic angiogenic/progenitor cell homing (5–7), increasing capillary density and improving cardiac function after MI (8,9). On the other hand, CXCL12 directly activates the cell-survival factor protein kinase B (PKB/Akt) via CXCR4, which may ultimately determine the fate of afflicted tissues (13). We therefore studied the function of CXCR4 in cardiac remodeling after MI in genetically modified mice to evaluate a potential relevance for unwanted effects of pharmacologic compounds (14). However, mice deficient in Cxcr4 display profound defects in the hematopoietic and nervous systems and die perinatally. They have severely reduced B-lymphopoiesis, myelopoiesis in fetal liver, and a virtual absence of myelopoiesis in bone marrow (15). Therefore, we chose to assess the effects of reduced CXCR4 expression after MI in mice heterozygous for CXCR4 (Cxcr4+/−), which appear normal and are viable and fertile (15), although CXCR4 surface expression on bone marrow–derived mononuclear cells from Cxcr4+/− mice is significantly lower compared with that in wild-type BL6/J mice (16).

Methods

For the mouse model of MI and details of other methods (e.g., reverse transcriptase polymerase chain reaction analysis [Online Table 1]), please see the Online Appendix.

Results

Analysis of MI size and inflammatory cell content. Four weeks after MI, the infarct size was reduced in Cxcr4+/− mice by 42% compared with Cxcr4+/− littermates (Fig. 1A). As evident by tetrazolium/Evans blue staining, the area at risk 1 day after MI showed no difference in the 2 groups (Online Fig. 1A), indicating that the reduced infarct size likely reflects an enhanced wound contraction and alterations in reparative pathways rather than a difference in the initial extent of cardiomyocyte injury. Moreover, myofibroblast infiltration (2,600 ± 283/mm² vs. 1,011 ± 165/mm² in controls, p < 0.001) and collagen content in the infarcted area were significantly higher in Cxcr4+/− mice than in wild-type controls (Fig. 1B), indicating a more stable and robust scar formation.

We next analyzed the mobilization and recruitment of inflammatory cells after MI. The MI-induced and transient expansion of neutrophils in the circulation (Fig. 1C) and infiltration of the infarcted area with neutrophils (Fig. 1D) were severely reduced in Cxcr4+/− mice 1 day after MI. Thus, the initial inflammatory response differed markedly in Cxcr4+/− mice, indicating a prominent role of CXCR4 in post-infarction neutrophil recruitment. Further, peripheral blood monocyte levels did not differ between Cxcr4+/− mice and wild-type mice after MI (Online Fig. 1B), whereas the myocardial infiltration with monocytes/macrophages presented a slight delay in Cxcr4+/− mice compared with wild-type mice (Online Fig. 1C).

Analysis of monocyte subsets revealed fewer circulating Gr-1high cells and relative expansion of Gr-1low cells in peripheral blood of Cxcr4+/− mice compared with wild-type mice after MI (Fig. 1E). These data correspond to diminished infiltration with proinflammatory tissue-degrading Gr-1high monocytes 4 days after MI, whereas Gr-1low monocytes, known to promote wound healing and collagen deposition (17), were increased in the hearts of Cxcr4+/− mice compared with wild-type mice (Fig. 1F). Thus, the inflammatory reaction after MI in Cxcr4+/− mice is shifted to an earlier termination of the acute response and onset of a repair process involving Gr-1low monocytes. Notably, whereas CXCR4 expression on Gr-1low monocytes from Cxcr4+/− mice (specific mean fluorescence intensity, 31.5 ± 3.0) was reduced by 43% compared with that on Gr-1low monocytes from wild-type mice (specific mean fluorescence intensity, 61.9 ± 7.4), the low CXCR4 expression on Gr-1high monocytes did not differ between wild-type mice and Cxcr4+/− mice (specific mean fluorescence intensity, 6.8 ± 3.5 vs. 5.2 ± 1.5). This suggests a strong adaptation of Gr-1low cells to reduced CXCR4 expression and a possible role of other receptors in their recruitment.

Analysis of cardiac function after MI. Echocardiography (Online Table 2) and Langendorff (Table 1) measurements surprisingly failed to reveal changes in ventricular function and contractility after MI in Cxcr4+/− mice compared with wild-type mice. However, we observed a slightly decreased baseline ejection fraction as well as a moderate increase in the post-MI ejection fraction in Cxcr4+/− mice compared with wild-type mice. Moreover, the difference in ejection fraction before and after MI was significantly decreased in Cxcr4+/− mice compared with wild-type mice (7.6 ± 1.2% vs. 16.8 ± 2.4%, p < 0.01), implying a protective or adaptive mechanism in the hearts of Cxcr4+/− mice.

Moreover, coronary perfusion was markedly decreased in Cxcr4+/− mice compared with wild-type mice, as determined by coronary flow measurements in isolated perfused hearts. Coronary flow was already reduced under baseline conditions in Cxcr4+/− mice compared with wild-type mice (Table 1, Fig. 2A). Ligation of the left anterior descending artery decreased coronary flow by approximately 50% in
both groups (Figs. 2A and 2B). Four weeks after MI, the recovery of coronary perfusion was significantly impaired in Cxcr4−/− mice (Online Table 2, Fig. 2A).

To assess whether defective cardiac angiogenesis and neovascularization after MI contribute to the differences in coronary blood flow, myocardial endothelial cells and vessels were quantified. As determined by flow cytometry, the number of myocardial endothelial cells was intrinsically reduced in Cxcr4−/− mice compared with wild-type mice (Fig. 2C). Similarly, neovascularization after MI was impaired in Cxcr4−/− mice compared with wild-type mice, as evident by reduced formation of CD31+ blood vessels in infarcted myocardium (Fig. 2D). This might contribute to the defective recovery of coronary flow after MI in Cxcr4−/− mice compared with wild-type mice without evidence of disturbed endothelial permeability, as shown by perfusion of the cremasteric artery with albumin–fluorescein isothiocyanate. This indicates that the endogenous defect in angiogenesis was not restricted to the heart.

Moreover, to distinguish the influence of the CXCR4 heterozygous background and its role in circulating cells, bone marrow chimera experiments were performed after lethal irradiation. Four weeks after MI, the infarction area was significantly reduced in wild-type mice transplanted with Cxcr4−/− bone marrow and in Cxcr4−/− mice transplanted with wild-type bone marrow compared with the control group (Fig. 3A). In addition to the effects attributable to reduced leukocyte infiltration, these data suggest the existence of an additional intrinsic mechanism that can substantially influence scar formation in our model. Notably, neovascularization after MI was impaired in both groups compared with controls, as evident by CD31+ staining in infarcted myocardium (Fig. 3B), whereas heart function, as assessed by
echocardiography and Langendorff perfusion showed no significant differences between the groups (Fig. 3C). The reduction of neovascularization was more pronounced in Cxcr4−/− mice transplanted with wild-type bone marrow and correlated with decreased coronary flow (Fig. 3D). This may reflect that the vascularization of the scar is based mostly on vessel formation around pre-existing collaterals, which may explain the markedly reduced neovascularization in Cxcr4−/− mice despite reconstitution with wild-type bone marrow.

The myocardial infiltration with neutrophils, as well as blood leukocyte subsets (Fig. 3E) after MI in Cxcr4−/− mice transplanted with wild-type bone marrow emulate the pattern

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type (n = 3–4)</th>
<th>Cxcr4−/− (n = 3–5)</th>
<th>Wild Type (n = 4–5)</th>
<th>Cxcr4−/− (n = 5–6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDP, mm Hg</td>
<td>86.7 ± 8.8</td>
<td>78.3 ± 17.0</td>
<td>40.0 ± 10.1</td>
<td>50.0 ± 11.5</td>
</tr>
<tr>
<td>Increase after dobutamine (Δ)</td>
<td>40.3 ± 4.0</td>
<td>46.2 ± 5.2</td>
<td>13.8 ± 4.3</td>
<td>10.3 ± 2.6</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>3.189 ± 99</td>
<td>3.058 ± 363</td>
<td>1.540 ± 97</td>
<td>1.590 ± 94</td>
</tr>
<tr>
<td>Increase after dobutamine (Δ)</td>
<td>2.410 ± 629</td>
<td>1.941 ± 187</td>
<td>770 ± 91</td>
<td>704 ± 74</td>
</tr>
<tr>
<td>dP/dt min, mm Hg/s</td>
<td>−2.695 ± 244</td>
<td>−2.011 ± 411</td>
<td>−1.267 ± 111</td>
<td>−1.280 ± 79</td>
</tr>
<tr>
<td>Increase after dobutamine (Δ)</td>
<td>−1.756 ± 544</td>
<td>−1.407 ± 314</td>
<td>545 ± 28</td>
<td>484 ± 68</td>
</tr>
<tr>
<td>Coronary flow, ml</td>
<td>3.9 ± 0.2</td>
<td>2.0 ± 0.3*</td>
<td>3.2 ± 0.3</td>
<td>1.2 ± 0.3*</td>
</tr>
<tr>
<td>Increase after brief ischemia (Δ)</td>
<td>3.4 ± 0.2</td>
<td>2.8 ± 0.1</td>
<td>1.8 ± 0.4</td>
<td>0.2 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *p < 0.05 versus wild type.

dP/dt = derivative of pressure increase (maximum) and decay (minimum); LVDP = left ventricular developed pressure; MI = myocardial infarction.
in Cxcr4<sup>−/−</sup> mice, indicating a shift toward an earlier termination of the acute response and earlier onset of a repair process.

The role of CXCR4 for early outgrowth cells trafficking and function. Because early outgrowth cells (EOCs) contribute to post-infarction neangiogenesis, the effect of Cxcr4 on EOC function was studied. Despite endothelial-like properties of both Cxcr4<sup>−/−</sup> EOCs and wild-type EOCs, the function of Cxcr4<sup>−/−</sup> EOCs was impaired, as shown in chemotaxis or Matrigel assays in vitro and in vivo. (Online Fig. 2). For more details, see the Online Appendix.

Myocardial apoptosis after MI. No difference in myocardial apoptosis after induction of MI was observed in Cxcr4<sup>−/−</sup> mice and wild-type mice after MI, as assessed by quantifying TUNEL (deoxyuridine-5′-triphosphate biotin nick end labeling)-positive cells and by reverse transcriptase polymerase chain reaction for Bax and Bcl2 expression (Online Fig. 3). To evaluate a potential contribution of the PS fractions to cardioprotection in Cxcr4<sup>−/−</sup> mice, isolated cardiomyocytes were pre-incubated with the PS fractions 1, 2, or 3 for 3 h, and the response to hypoxic stress was analyzed. As evident by dihydroethidium staining to monitor radical formation, all PS fractions protected cardiomyocytes against hypoxic injury (Fig. 4C). The triglyceride fraction obtained from Cxcr4<sup>−/−</sup> mouse hearts contained 10% more unsaturated fatty acids than wild-type mouse hearts, and 6 different unsaturated fatty acids appeared in the triglyceride fraction of Cxcr4<sup>−/−</sup> hearts. No differences were noted for saturated/unsaturated fatty acid content in phosphatidylcholine or phosphatidylethanolamine. The fatty acid composition of the 3 PS fractions is detailed in Online Table 3.
Discussion

Our data demonstrate the double-edged effects of CXCR4 on myocardial remodeling after MI and point to a variety of possible mechanisms with major clinical implications. Compared with wild-type mice, Cxcr4−/− mice revealed smaller and stable MI scars due to an attenuation of the acute inflammatory recruitment of neutrophils, a shift toward a more regenerative monocyte response, and better adaptation of cardiomyocytes to hypoxic stress. This was balanced by impaired EOC function, myocardial neovascularization, and coronary flow recovery, overall amounting to a lack of improvement in ventricular function. Given the major efforts to exploit the CXCL12/CXCR4 axis therapeutically to promote angiogenesis and cellular regeneration, our data provide important insights into endogenous function of CXCR4 after MI.

First, we found an altered inflammatory pattern in Cxcr4−/− mouse hearts compared with wild-type hearts. (C) In in vitro hypoxia experiments, pre-incubation of isolated cardiomyocytes with PS fractions protects against hypoxic injury. *p < 0.01 versus control, §p < 0.05 versus ischemia, n = 4.
(20,21) by reducing the release of reactive oxygen species, proteases, and inflammatory mediators. Recently, CXCR4 was identified as a central regulator of neutrophil homeostasis directing their release from bone marrow under stress conditions (20). Although complete disruption or deficiency of CXCR4 caused an expansion of less mature neutrophils in the circulation in the chronic context of atherogenesis (22), we found that an acute mobilization of neutrophils was blocked by the potent CXCR4 antagonist AMD3645 (23). Similarly, we observed that MI caused an acute expansion of circulating neutrophils and their myocardial recruitment, which was attenuated in Cxcr4+/− mice. This is in line with a recent study that failed to detect neutrophil mobilization after various forms of stimulation or infection when CXCR4 signaling was abrogated (20). Thus, our data confirm a role of CXCR4 in injury- or stress-induced neutrophil mobilization, allowing their subsequent recruitment.

Monocytes play an important and finely tuned role in cardiac repair (17). We found that after MI, overall monocyte/macrophage infiltration into the myocardium was delayed in Cxcr4+/− mice owing to a reduced infiltration with Gr-1high inflammatory monocytes during the initial phase, in a process that may be governed by neutrophil secretory products (24,25). Preventing Gr-1high monocytosis results in a delayed or inefficient removal of apoptotic cells and necrotic tissue but does not impede healing (17,26). Conversely, Gr-1low monocytes, which promote healing via myofibroblast accumulation and collagen deposition, were more prevalent and recruited earlier after MI in Cxcr4+/− mice. This shift to a more robust repair may contribute to smaller and stable scar formation. Interestingly, we found that Gr-1high monocytes from Cxcr4+/− mice did not display reduced CXCR4 expression. Whereas one may hypothesize that the reparative Gr-1 cells use additional receptors to compensate for lower CXCR4 levels in recruitment, these data generally imply an important role of other receptors, namely, CCR2, in the recruitment of Gr-1high monocytes.

Despite reduced MI size, ventricular function was not significantly improved in Cxcr4+/− mice. This could be due to the reduced basal coronary flow and to the impaired coronary flow recovery in Cxcr4+/− hearts 4 weeks after MI. As an underlying mechanism, we studied the function of EOCs as important contributors to neovascularization after MI. The SDF-1/Cxcr4 interaction is crucially involved in the mobilization and recruitment of stem and progenitor cells to the heart after MI (5,27). Despite appropriate acquisition of typical endothelial differentiation markers, splenic EOCs from Cxcr4+/− mice showed deficient chemotaxis toward CXCL12 (but not vascular endothelial growth factor) and reduced tube formation in vitro. Accordingly, myocardial vessel density, endothelial cell content, vessel invasion in Matrigel and arterial branching in vivo was impaired in Cxcr4+/− mice. This is in keeping with a previous study showing that EOCs from Cxcr4+/− mice were also significantly impaired to restore blood flow in ischemic nude mice compared with wild-type EOCs in the hindlimb ischemia model (16). Although we show in vitro that these effects are mostly due to a dysfunction of EOCs, we cannot exclude that a decrease in surrounding vascular density also plays a supportive role in our in vivo models through reducing the number of circulating cells available at the site of injury. Conversely, a lack of functional improvement in Cxcr4+/− mice cannot be explained by a modulation of cardiomyocyte contractility by CXCR4 because CXCL12 has been shown to exert negative inotropic effects (28) so that one would rather expect improved ventricular function on inhibition of Cxcr4 deficiency.

Moreover, we performed bone marrow chimera experiments to distinguish the influence of the Cxcr4 heterozygous background and its effect on circulating cells in normally developed wild-type mice. Despite a reconstitution with wild-type bone marrow, a reduction of infarction area and neovascularization persisted in Cxcr4+/− mice. Moreover, a significant, albeit less marked, reduction was observed in wild-type mice reconstituted with Cxcr4+/− bone marrow, indicating that reduced CXCR4 levels on circulating cells (namely, progenitor cells and leukocytes) may also contribute to the effects observed after MI in Cxcr4 heterozygous mice, independently of their abnormal cardiovascular development.

Notably, the reduced basal and neovascularization of Cxcr4+/− hearts without any sign of physiologic dysfunction raises several questions. Diminished blood supply should lead to a series of histopathologic and structural changes of the myocardium with an increase in cardiomyocyte apoptosis, ventricular mass and volume, and progressive decline in left ventricular performance. None of these parameters, however, differ in Cxcr4+/− mice. Moreover, recent data indicate that CXCR4 expression on cardiomyocytes is not essential for cardiac development and has no major role in ventricular remodeling after MI (29). Because Cxcr4+/− myocardium is spared and hypoxic injury seems to be less extensive, compared with that in wild-type mice, we assume that the protective mechanism in Cxcr4+/− myocardium is mostly due to adaptive changes during embryogenesis. Using electron microscopy, we observed lipofuscin-like lipid accumulations, which resembled those found in rat hearts after dietary fish oil feeding (18). A diet enriched with n-3 fatty acids can reduce ischemic damage to the heart (30) and may represent a possible lead to protection, but this clearly requires further investigation into underlying mechanisms.

Another notable difference in the lipid extracts of Cxcr4+/− myocardium is the high levels of PS, generally known as a marker of cell death (31). However, PS supports other cell functions, including mitochondrial membrane integrity and activation of protein kinase C, which is important in hypoxia tolerance during late preconditioning (32), as well as in the inhibition of specific immune responses (33). In our study, the permanently decreased coronary flow in Cxcr4+/− mice may induce a chronic ischemia and thus may force cardiomyocytes to adapt even from early stages of embryonic development. An increase in
cardiac PS seems to be a possible cause mediating this adaptive mechanism because pre-incubation of cardiomyocytes with PS isolated from Cxcr4+/− mice hearts protected cardiomyocytes against hypoxic injury. However, the exact mechanism remains to be established.

Extensive attempts have been made to directly affect the CXCL12/CXCR4 axis (e.g., by direct injection, nanofiber-mediated delivery of CXCL12, or overexpression of CXCL12/CXCR4 in cells transplanted into the myocardium (5–11), aiming to reduce MI size and to improve ventricular function after MI. The double-edged effects of CXCR4 are illustrated by an alteration of the inflammatory response and protection against hypoxic stress, as well as impaired EOC function, neovascularization, and coronary flow recovery. Pharmacologic antagonism of CXCR4 with AMD3100 has been reported to reduce infarct size and to improve ventricular function after MI in rats (12). Although the decrease in MI size is consistent with our findings, an improved contractility has been explained by a suppression of the hypertrophic response in the noninfarct area. This differs from Cxcr4+/− mice, which have intrinsically reduced coronary flow and can be considered as a model for congenitally impaired vascularization and adaptation to hypoxia.

Limitations and Conclusions

Although studies in bone marrow chimeras suggested a role of CXCR4 on cells infiltrating from the circulation in explaining the reduction in infarct size, one notable limitation of our study is clearly the lack of mice with specific and inducible deletion of CXCR4 in either circulating cells or resident myocardial cells to better dissect the underlying mechanisms. In addition, caution should be exerted when extrapolating these results to an inhibition of CXCR4 in the human system. Nevertheless, cell-specific, context-dependent, and long-term effects of CXCR4 interference or CXCL12 application need to be carefully taken into account when devising therapeutic strategies for MI and ischemic cardiomyopathy.

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REFERENCES


Key Words: angiogenesis • chemokine receptor • inflammation • myocardial infarction • myocardial remodeling.

APPENDIX

For an expanded Methods section and supplemental tables and figures, please see the online version of this article.