Original Article

Deficiency of Endothelial Cxcr4 Reduces Reendothelialization and Enhances Neointimal Hyperplasia After Vascular Injury in Atherosclerosis-Prone Mice

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Objective—The Cxcl12/Cxcr4 chemokine ligand/receptor axis mediates the mobilization of smooth muscle cell progenitors, driving injury-induced neointimal hyperplasia. This study aimed to investigate the role of endothelial Cxcr4 in neointima formation.

Approach and Results—β-Galactosidase staining using bone marrow x kinase (Bmx)-CreER<T2> reporter mice and double immunofluorescence revealed an efficient and endothelial-specific deletion of Cxcr4 in Bmx-CreER<T2> compared with Bmx-CreER<T2> Cxcr4<−/−> apolipoprotein E–deficient (ApoE<−/−>) mice (referred to as Cxcr4<EC<WT>ApoE<−/−> and Cxcr4<EC<KO>ApoE<−/−>, respectively). Endothelial Cxcr4 deficiency significantly increased wire injury–induced neointima formation in carotid arteries from Cxcr4<EC<KO>ApoE<−/−> mice. The lesions displayed a higher number of macrophages, whereas the smooth muscle cell and collagen content were reduced. This was associated with a significant reduction in reendothelialization and endothelial cell proliferation in injured Cxcr4<EC<KO>ApoE<−/−> carotids compared with Cxcr4<EC<WT>ApoE<−/−> controls. Furthermore, stimulation of human aortic endothelial cells with CXCL12 significantly enhanced their wound-healing capacity in an in vitro scratch assay, an effect that could be reversed with the CCR4 antagonist AMD3100. Also, flow cytometric analysis showed a reduced mobilization of Sca1<+>Flk1<+>Cd31<+> and of Lin−Sca1<+> progenitors in Cxcr4<EC<KO>ApoE<−/−> mice after vascular injury, although Cxcr4 surface expression was unaltered. No differences could be detected in plasma concentrations of Cxcl12, Vegf, sphingosine 1-phosphate, or Flt3 ligand, all cytokines with an established role in progenitor cell mobilization. Nonetheless, double immunofluorescence revealed a significant reduction in local endothelial Cxcl12 staining in injured carotids from Cxcr4<EC<KO>ApoE<−/−> mice.

Conclusions—Endothelial Cxcr4 is crucial for efficient reendothelialization after vascular injury through endothelial wound healing and proliferation, and through the mobilization of Sca1<+>Flk1<+>Cd31<+> cells, often referred to as circulating endothelial progenitor cells. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: coronary restenosis ■ receptors, CCR ■ receptors, Cxcr4

Balloon angioplasty and stent implantation are widely used techniques to treat patients with obstructive atherosclerosis. As a major limitation, such procedures are associated with a 25% to 40% risk of lumen renarrowing during the first 6 months after intervention.1 This process, also called restenosis, is caused by intervention-associated vascular injury and mediated by inward arterial remodeling and the formation of neointimal lesions consisting mainly of smooth muscle cells (SMCs) and inflammatory leukocytes.2–3 A fraction of the neointimal SMCs driving injury-induced neointimal hyperplasia has been shown to originate from bone marrow–derived or circulating smooth muscle progenitor cells in a Cxcl12/Cxcr4-dependent way.4–7 The chemokine receptor Cxcr4 and its ligand Cxcl12, also called stromal cell–derived factor-1, play an important role in the homing of progenitor cells in the bone marrow and in their mobilization to the periphery under pathological conditions.8 In line with this, either deficiency or blocking of the Cxcl12/Cxcr4 axis reduced smooth muscle progenitor cell recruitment and neointima formation after vascular injury.5,7,9 Of note, these studies induced
a global blockade of Cxcl12/Cxcr4 signaling through the use of Cxcr4 antagonists,9,10 Cxcl12 blocking antibodies,5,7 or bone marrow transplantation with Cxcr4-deficient cells.7 Thus, cell type–specific effects of Cxcr4 on injury-induced neointimal hyperplasia remain unknown. Especially, the role of Cxcr4 in vascular endothelial cells (ECs) is an intriguing open question, because an efficient reendothelialization of a denudated vessel is important in slowing down restenosis and preventing thrombosis.10 Endothelial seeding or implants on the injured vascular wall have been proven to enhance endothelial recovery and significantly reduce neointimal hyperplasia.11,12 Although anti-proliferative agents of drug-eluting stents can reduce neointimal inflammation and SMC proliferation, they also enhance the risk of late stent thrombosis attributable to an impairment in EC proliferation and vascular reendothelialization.13,14

In this study, we investigated the effect of an endothelial-specific Cxcr4 deletion on neointimal hyperplasia after carotid artery injury using bone marrow x kinase (Bmx)-CreERT2 Cxcr4-floxed apolipoprotein E–deficient (Apoe−/−) mice, in which conditional deletion of endothelial Cxcr4 was induced by tamoxifen treatment. We hypothesized that endothelial Cxcr4 deficiency may enhance neointima formation through an impaired reendothelialization of the denudated artery.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Mouse Model

Five- to 7-week-old female Bmx-CreERT2+Cxcr4-floxed Apoe−/− mice (indicated as Cxcr4EC-KOApoe−/−) and littermate Bmx-CreERT2- Cxcr4-floxed Apoe−/− mice (indicated as Cxcr4EC-WTApoe−/−) were treated with tamoxifen to induce Cre-mediated deletion of the floxed Cxcr4 allele in Bmx−/− cells. After a recovery period of minimal 3 weeks after the first tamoxifen injection and 1 week of high-fat diet, wire-mediated injury of the left common carotid artery was induced as previously described.7 Animal experiments were reviewed and approved by local authorities in accordance with the German animal protection law.

Neointimal Lesion Analysis

Serial 4-μm transversal sections of injured carotid arteries were stained with the Elastica-van Gieson staining, Sirius Red, or with specific antibodies and isotype controls, as indicated.

Flow Cytometry

Circulating Sca1+Flk1+Cd31+ mononuclear progenitor cells15-18 and Lin−Sca1+ progenitor cells were quantified after labeling of peripheral blood cells with directly conjugated antibodies.

In vitro Scratch Assay

Human aortic ECs (HAAoECs) were grown to confluency and stimulated with the indicated compounds for 8 hours before and 20 hours after wound introduction.
the carotid arteries. Immunostaining for Cd31 revealed that the degree of reendothelialization reached only 49% in the Cxcr4EC-KO Apo−/− mice 3 weeks after vascular injury, which was significantly lower than the 64% reendothelialization efficiency in the Cxcr4EC-WT Apo−/− controls (Figure 4A). The role of CXCR4 in the wound-healing capacity of ECs was further investigated in a scratch migration assay in vitro using HAoECs, which express CXCR4 and respond to the CXCR4 ligand CXCL12 as shown by a decreased CXCR4 surface expression in response to CXCL12 stimulation (Figure VII in the online-only Data Supplement). Interestingly, stimulation of HAoECs with CXCL12 significantly enhanced their migration into the scratched area, which could be reversed with the CXCR4 antagonist AMD3100 (Figure 4B). CXCL12 could not enhance basal proliferation of HAoECs in vitro (Figure 4C). Nevertheless, staining of carotid artery sections for Cd31 in combination with the proliferation marker Ki67 revealed a significant reduction in proliferating ECs in injured carotid arteries from Cxcr4EC-KO Apo−/− mice compared with those from Cxcr4EC-WT Apo−/− controls (Figure 4D).

Together, these results demonstrate that the chemokine receptor Cxcr4 plays an important role in the migration and proliferation response of ECs after vascular denudation in vivo.

Figure 1. Efficient deletion of endothelial Cxcr4 in Cxcr4EC-KO Apo−/− mice. A, β-Galactosidase activity in the carotid artery from tamoxifen-treated Bmx-CreERT2 ROSA reporter mice. Scale bar, 100 μm. B and C, Quantification of Cxcr4+ endothelium in carotid arteries from Cxcr4EC-WT Apo−/− and Cxcr4EC-KO Apo−/− mice 3 weeks after injury, based on staining of carotid artery sections for Cd31 (red), Cxcr4 (green), and DAPI (blue). The graph displays the Cxcr4+Cd31+ luminal length as percentage of the total Cd31+ luminal length. Represented are means±SEM; n=8 to 13; ***P<0.001; 2-tailed t test. Representative images are presented in (C), showing more Cxcr4+ endothelial cells (yellow, indicated with arrows) in Cxcr4EC-WT Apo−/− vs Cxcr4EC-KO Apo−/− mice. Scale bars, 100 μm.

Toxicosclerosis.

Endothelial Cxcr4 Deficiency Reduces the Mobilization of Sca1+Flk1+Cd31+ Cells

Previous studies have shown that endothelial progenitor cells (EPCs) contribute to an efficient reendothelialization of denuded vessels.21 Although there are no unique markers for defining EPCs, Sca1+Flk1+ expression is regularly being used for flow cytometric analysis of circulating EPCs in mice.15–18 Mouse Sca1+Flk1+ mononuclear cells were also Cd31+ and showed an FSC/SSC pattern distinct from typical monocytes (Figure 5A). Furthermore, Cd45+ mononuclear leukocytes infiltrated into neointimal lesions of carotid arteries 3 weeks after injury were initially Flk1−, and also Cd45+ vascular cells were Flk1−. In contrast, F4/80+ neointimal macrophages were found to be Flk1+ (Figure VIII A in the online-only Data Supplement), corresponding with an earlier report of Flk1 expression on non-SMCs in injury-induced neointimal lesions in carotid arteries from rat and rabbit.22 Flk1 expression on lesion macrophages could also be detected by coinmunostaining of injury-induced neointimal lesions for Flk1 and the macrophage marker Mac2, however with variable efficiency probably attributable to the low immunofluorescent Flk1 signal revealed by immunostaining (Figure VII B in the online-only Data Supplement). Furthermore, Flk1 expression on macrophages seems context dependent because only a small percentage of thioglycollate-elicited peritoneal macrophages was found to be Flk1+ by flow cytometric analysis (Figure VIII C in the online-only Data Supplement).

Quantification of circulating Sca1+Flk1+Cd31+ cells did not reveal differences between Cxcr4EC-WT Apo−/− and Cxcr4EC-KO Apo−/− mice at baseline conditions (Figure 5B). Vascular injury significantly increased the level of circulating Sca1+Flk1+Cd31+ cells in Cxcr4EC-WT Apo−/− but not Cxcr4EC-KO Apo−/− mice (Figure 5B). Comparably, Cxcr4EC-KO Apo−/− mice showed a significantly reduced mobilization of circulating Lin−Sca1+ progenitor cells after injury (Figure 6A and 6B). No significant differences were observed in the number of Sca1+Flk1+Cd31+ or Lin−Sca1+ progenitor cells in the bone marrow before or after injury (Figure IX in the online-only Data Supplement).

Because the Cxcl12/Cxcr4 axis plays an important role in the mobilization of progenitor cells from the bone marrow to
the periphery, and the Cxcr4 receptor has also been implicated in mediating progenitor cell recruitment to injured carotid arteries, we questioned whether the reduced mobilization of Sca1^+Flk1^+Cd31^+ progenitors in the Cxcr4 EC-KO Apoe^−/− mice could be related to a potential deletion of Cxcr4 in these cells mediated by the Bmx-Cre transgene. Analysis of Cxcr4 expression on the surface of circulating Sca1^+Flk1^+Cd31^+ cells by flow cytometry showed them to be clearly Cxcr4 positive, without significant differences in the Cxcr4 expression level, represented by the geometric mean fluorescence intensity between Cxcr4 EC-WT Apoe^−/− and Cxcr4 EC-KO Apoe^−/− mice (Figure XA in the online-only Data Supplement). Thus, these data indicate that the reduced level of circulating Sca1^+Flk1^+Cd31^+ cells in injured Cxcr4 EC-KO Apoe^−/− is not caused by a Cxcr4 deficiency in these progenitors themselves. Compara

To explore potential mechanisms underlying the observed effects on progenitor cell mobilization, we quantified plasma concentrations of different stimuli known to be involved in this process. Cxcl12 is an important ligand for Cxcr4, and ECs were previously shown to produce Cxcl12 through a Cxcl12/Cxcr4-positive feedback loop. However, no differences were observed in the Cxcl12 level in plasma or bone marrow from Cxcr4 EC-WT Apoe^−/− and Cxcr4 EC-KO Apoe^−/− mice after vascular injury (Figure 7A). Plasma concentrations of Vegf, Flt3 ligand, and the sphingolipid sphingosine 1-phosphate, which can all influence progenitor cell mobilization, were unaltered at different time points after injury (Figure 7B–7D), whereas plasma levels of GM-CSF remained below detection levels. Similarly, plasma concentrations of Mif, an alternative ligand of Cxcr4 able to mediate EPC chemotaxis in vitro through Cxcr4, were unchanged until 10 days after injury. Surprisingly, Cxcr4 EC-KO Apoe^−/− mice showed a significant increase in Mif plasma levels at later stage (Figure 7E), which can thus also not explain the decreased mobilization of Sca1^+Flk1^+Cd31^+ progenitors. However, immunostaining of injured carotid artery sections for Cxcl12 and Cd31 revealed a significantly lower Cxcl12 concentrations of different stimuli known to be involved in this process. Cxcl12 is an important ligand for Cxcr4, and ECs were previously shown to produce Cxcl12 through a Cxcl12/Cxcr4-positive feedback loop. However, no differences were observed in the Cxcl12 level in plasma or bone marrow from Cxcr4 EC-WT Apoe^−/− and Cxcr4 EC-KO Apoe^−/− mice after vascular injury (Figure 7A). Plasma concentrations of Vegf, Flt3 ligand, and the sphingolipid sphingosine 1-phosphate, which can all influence progenitor cell mobilization, were unaltered at different time points after injury (Figure 7B–7D), whereas plasma levels of GM-CSF remained below detection levels. Similarly, plasma concentrations of Mif, an alternative ligand of Cxcr4 able to mediate EPC chemotaxis in vitro through Cxcr4, were unchanged until 10 days after injury. Surprisingly, Cxcr4 EC-KO Apoe^−/− mice showed a significant increase in Mif plasma levels at later stage (Figure 7E), which can thus also not explain the decreased mobilization of Sca1^+Flk1^+Cd31^+ progenitors. However, immunostaining of injured carotid artery sections for Cxcl12 and Cd31 revealed a significantly lower Cxcl12...
level in the vascular endothelium of \( \text{Cxr}^{4\text{EC-KO} \text{Apoe}^{-/-}} \) mice after injury (Figure 7F).

Together, our results demonstrate a reduced injury-induced mobilization of Sca1'Flk1'Cd31' progenitor cells on endothelial Cxcr4 deficiency, which may contribute to the observed reduction in reendothelialization. Although the underlying mechanisms of decreased mobilization remain currently unclear, it is possible that reduced immobilization of adluminal Cxcl12 on injured vascular endothelium of \( \text{Cxr}^{4\text{EC-KO} \text{Apoe}^{-/-}} \) mice may contribute indirectly to reduced progenitor cell mobilization.

**Discussion**

In this study, we showed that deficiency of endothelial Cxcr4 significantly interfered with the recovery of injured endothelium and enhanced neointima formation in a mouse model of carotid artery injury. Reduced reendothelialization...
of injured carotid arteries from Cxcr4<sup>EC-KO</sup>Apo<sup>e−/−</sup> mice was associated with a decreased endothelial proliferation in vivo, a reduced migratory wound-healing capacity of vascular ECs in vitro, as well as with a lower mobilization of circulating Sca1<sup>+</sup>Flk1<sup>+</sup>Cd31<sup>+</sup> progenitors after injury. Efficient reendothelialization of a denudated vessel has been shown to be important in slowing down neointimal hyperplasia. For example, overexpression of endostatin, a selective inhibitor of EC proliferation, significantly reduced endothelial recovery after endothelial denudation of the mouse femoral artery; and 4 weeks after injury, the degree of reendothelialization showed a significant inverse correlation with neointima formation. Comparably, sequestration of the potent EC mitogen Vegf significantly reduced endothelial proliferation and reendothelialization, and enhanced neointimal hyperplasia after endothelial denudation, whereas opposite results were obtained with Vegf overexpression or local treatment. Also, endothelial seeding or implants on the injured vascular wall or local treatment with culture-modified mononuclear cells displaying an endothelium-like phenotype have been proven to enhance endothelial recovery and significantly reduce neointimal hyperplasia in a rabbit and pig injury model. These data, together with our observation that enhanced neointima formation in the absence of...
endothelial Cxcr4 is associated with a reduced reendothelialization level, underscore the notion that vascular endothelium and injury-induced endothelial repair are key modulators of neointimal hyperplasia after arterial injury.

Migration and proliferation of ECs from adjacent noninjured endothelium are important factors driving vascular reendothelialization. Blocking the CXCR4 receptor with the antagonist AMD3100 significantly reduced the CXCL12-triggered wound-healing capacity of ECs in an in vitro scratch migration assay (Figure 4B). The relatively small, yet significant, effect of CXCL12 stimulation on the EC regeneration ability could be related to the heterogeneous expression level of CXCR4 on HAoEC, with >60% of cultured ECs presenting only a relatively small level of surface CXCR4 (Figure VII in the online-only Data Supplement). Furthermore, deficiency of endothelial Cxcr4 resulted in a significant reduction of endothelial proliferation at the site of injury (Figure 4D). Because CXCL12 could not directly enhance proliferation of human ECs in vitro, we speculate that effects of endothelial Cxcr4 deficiency on EC proliferation in injured vessels are controlled by additional factors released from the site of injury. Alternatively, it is possible that MIF, an alternative ligand for CXCR4, either by direct interaction or by binding its receptor CXCR2 in heterodimerization with CXCR4, for example, through joint interaction with CD74.30 Such mitogenic effect of recombinant MIF on human coronary artery ECs was previously shown in vitro, although receptor involvement was not examined.31

In addition to stimulating reendothelialization through migration and proliferation of vascular ECs, recruitment of EPCs to the injured site has been associated with efficient reendothelialization of denudated arteries, and transplantation or mobilization of EPCs has been linked with enhanced vascular repair after injury.21 For example, systemic injection or local treatment of injured arteries with purified EPCs significantly enhanced reendothelialization and reduced neointimal thickening in mouse or rabbit injury models.17,32–34 These beneficial effects were further enlarged when the EPCs were pretreated with the protein kinase GSK3-β, which improved the adhesion capacity of the injected EPCs.34 Furthermore, cilostazol treatment of rats significantly reduced neointimal hyperplasia after balloon carotid denudation, which was associated with an enhanced reendothelialization in vivo and an increased mobilization, proliferation, and differentiation capacity of EPCs in vitro.35 Also, enhanced mobilization of

**Figure 5.** Less circulating Sca1+Flk1+Cd31+ cells in injured mice with an endothelial-specific Cxcr4 deficiency. A, Gating strategy of Sca1+Flk1+Cd31+ progenitor cells by flow cytometry, including the appropriate fluorescence minus one controls. Representative dot plots are shown. B, Quantification of Sca1+Flk1+Cd31+ progenitor cells in the peripheral blood of Cxcr4EC-WT Apoe−/− vs Cxcr4EC-KO Apoe−/− mice before or at different time points after carotid artery injury, as indicated. The graph represents means±SEM; n=12 to 20 (for baseline, 1 day, 3 weeks) and n=5 to 6 (for 5 and 10 days); *P<0.05 and ***P<0.001; 1-way or 2-way ANOVA with Bonferroni post-test, as appropriate.
mononuclear cells expressing endothelial cell lineage markers (eg, Flk1) on treatment with G-CSF36,37 or GM-CSF38 was linked with an improved endothelial repair and reduced neo-intima formation after vascular injury in rat, mouse, and rabbit, respectively. In conclusion, increased levels of circulating EPCs have been associated with a higher reendothelialization degree of denuded vessels.

Similar results were obtained in our study, in which a lower endothelial recovery was observed in injured Cxcr4EC-KOApoe−/− mice displaying a significantly lower level of circulating Sca1 +Flk1+Cd31+ progenitors compared with Cxcr4EC-WTApoe−/− controls (Figure 5). Circulating EPCs are poorly defined, and their definition is currently intensely debated.18 Gating of Sca1 +Flk1+ cells, which we found to be also Cd31+, has recurrently been used in mice to quantify such circulating EPCs.15–17 Furthermore, induced pluripotent stem cell–derived Flk1+ cells were recently shown to prevent injury-induced neointimal thickening in mice through enhanced reendothelialization,39 supporting a role for Flk1+ progenitor cells in injury-induced endothelial repair. However, whether EPCs contribute to endothelial repair through differentiation into ECs and integration in the injured endothelium, or rather to paracrine effects on resident ECs through secretion of stimulatory molecules, is still heavily debated.40

Figure 6. Less circulating Lin−Sca1+ progenitor cells in injured mice with an endothelial-specific Cxcr4 deficiency. A, Gating strategy of Lin−Sca1+ progenitor cells by flow cytometry, including the appropriate fluorescence minus one controls. Representative dot plots are shown. B, Quantification of Lin−Sca1+ cells in the peripheral blood of Cxcr4EC-WTApoe−/− vs Cxcr4EC-KOApoe−/− mice before or at different time points after carotid artery injury, as indicated. The graph represents means±SEM; n=11 to 21 (for baseline, 1 and 10 days) or n=5 to 6 (for 3 weeks); *P<0.05; 1-way or 2-way ANOVA with Bonferroni post-test, as appropriate.

Cxcr4 is an important chemokine receptor regulating the mobilization of EPCs and other progenitor cells to peripheral sites of injury through injury-induced upregulation of Cxcl12.41 In the context of carotid artery injury in mice, antibody-mediated blocking of Cxcr4 on EPCs significantly reduced their adhesion rate to injured arteries ex vivo and in vivo17,23 and abolished the capacity of infused EPCs to promote endothelial recovery and reduce neointimal hyperplasia after vascular injury.17,42 In contrast, overexpression of Cxcr4 enhanced the adhesion and Cxcl12-induced migration ability of EPCs in vitro and significantly increased the capacity of EPCs to promote injury-induced reendothelialization.43 However, Cxcr4EC-KOApoe−/− mice did not show a reduced Cxcr4 expression on circulating Sca1+Flk1+Cd31+ cells (Figure X in the online-only Data Supplement). This indicates that Bmx is not expressed on their precursor cells at the time of tamoxifen treatment, and that differences in the attracting chemokine concentration instead of altered chemokine receptor levels on Sca1+Flk1+Cd31+ progenitors underlie their reduced levels in peripheral blood. Although the concentration of Cxcl12 in plasma and bone marrow was not changed after injury (Figure 7A), endothelial Cxcl12 levels at the injured site were significantly reduced in Cxcr4EC-KOApoe−/− mice compared with Cxcr4EC-WTApoe−/− controls (Figure 7F). This may
reflect a reduced immobilization of Cxcl12 on Cxcr4-deficient endothelium or could be related to a decreased endothelial Cxcl12 expression, given that Cxcl12 and endothelial apoptotic bodies generated after endothelial injury trigger endothelial Cxcl12 expression through a Cxcr4-mediated positive feedback loop.24 The decreased endothelial Cxcl12 staining suggests that a local reduction in luminally exposed Cxcl12 on the endothelium of injured arteries from Cxcr4EC-KOApoe−/− mice may contribute to reduced reendothelialization through a reduction in adhesion and mobilization of endothelial cell progenitors.17,23,44 This is further supported by recent findings that Cxcl12 can indeed mediate EPC migration, adhesion, and survival,17,41–44 and that treatment of mice with a Cxcl12 blocking antibody significantly reduces circulating Sca1+Flik1+EPC numbers and reendothelialization after carotid artery injury.17 Furthermore, the importance of locally increased Cxcl12 availability in the recruitment of bone marrow–derived cells to sites of injury has previously been shown in different injury models.41,44,45 Also, a previous report demonstrated an association between (endothelial) progenitor cells adhering to the luminal side of atherosclerotic vessels and enhanced luminal Cxcl12 expression after treatment of mice with endothelial apoptotic...
bodies. Together, these data support a role for luminally exposed Cxcl12 on the endothelium in the recruitment of Sca1-Flik1-Cd31+ progenitors to injured vessels.

Which molecular mechanisms underlie the decreased mobilization of Sca1-Flik1-Cd31+ cells in the blood of injured Cxcr4EC-KOApoe−/− mice remains currently unclear. Of note, the apparent absence of differences in plasma and bone marrow Cxcl12 levels could be related to the specificity of the ELISA-based measurement for the Cxcl12-α isoform, whereas differences in local endothelial Cxcl12 levels were revealed with an antibody recognizing both the α- and β-isoform. Therefore, it could be hypothesized that reduced progenitor cell mobilization in Cxcr4EC-KOApoe−/− mice is caused by reduced plasma levels of Cxcl12-β or even another Cxcl12 isoform. Such isoform-specific role of Cxcl12 has previously been suggested in the context of cerebral ischemia, where leukocyte infiltration was associated with endothelial Cxcl12-β but not -α.46 Furthermore, it is conceivable that endothelial Cxcr4 deficiency modifies progenitor cell recruitment in stress conditions by directly or indirectly affecting local or systemic levels of mobilizing factors, other than Cxcl12. For example, Cxcl12/Cxcr4 signaling can induce production of Vegf47,48 and the sphingolipid sphingosine 1-phosphate through mTOR. Both mediators are produced in ECs and can mediate progenitor cell mobilization.49,50 However, we could not detect differences in plasma levels of Vegf or sphingosine 1-phosphate after injury of Cxcr4EC-KOApoe−/− versus Cxcr4EC-WTApoe−/− mice, and plasma concentrations of Flt3 ligand, with a known role in progenitor cell mobilization, remained unchanged. Surprisingly, Mif, an alternative Cxcr4 ligand able to mediate EPC chemotaxis through Cxcr4 in vitro,25 showed unaltered plasma levels up to 10 days after injury but was significantly increased in plasma of injured Cxcr4EC-KOApoe−/− mice at later stage. Because Mif was shown to be mostly expressed by neointimal foam cells at 2 weeks after injury, compared with a mainly SMC-dependent expression of Mif 1 day after injury,8 it can be hypothesized that the increased macrophage content of neointimal lesions in injured Cxcr4EC-KOApoe−/− mice contributes to the observed increase in plasma Mif levels at later stage. It is possible that endothelial Cxcr4 deficiency affects plasma levels of other signaling molecules mediating progenitor cell mobilization from the bone marrow in conditions of stress, as, for example, endothelial NO synthase, angiopoietin-1, or the chemokinones Il-8 and Gro-β, among others. Alternatively, a decrease in Cxcl12-dependent recruitment and survival of progenitor cells at the injured site could cause a reduced secretion of such mobilization factors from the recruited cells, as observed after skin wounding. In addition, a different inflammatory context in the neointima could contribute to the observed reduction in progenitor cell mobilization in Cxcr4EC-KOApoe−/− mice through a modified expression of mobilizing proteins. Future studies, using mice with an endothelial-specific Cxcl12 deficiency, are required to investigate the role of endothelial Cxcl12 expression in the injury-induced mobilization and recruitment of Sca1-Flik1-Cd31+ progenitors in more detail.

Although long-term treatments with Cxcr4 antagonists have been shown to stimulate EPC mobilization in mice, these antagonists also interfered with the adhesion, migration, and proliferation capacity of EPCs in vitro. This can explain why systemic treatment of mice with Cxcr4 antagonists did not affect the reendothelialization degree after carotid artery injury in mice, in contrast to BmxCreER2-mediated EC-specific Cxcr4 deficiency studied in this article.

Furthermore, the observed decrease in injury-induced neointima formation in mice treated with Cxcr4 antagonists was associated with a reduced mobilization of Lin−Sca1+ progenitor cells in the peripheral blood,43,56 a phenomenon that was also observed on treatment of mice with a Cxcl12 blocking antibody. Similarly, Cxcr4EC-KOApoe−/− mice displayed a lower injury-induced mobilization of Lin−Sca1+ cells (Figure 7). This was not associated with a decreased surface expression of Cxcr4, again suggesting that the reduced endothelial Cxcl12 expression in Cxcr4EC-KOApoe−/− mice at the site of injury is directly or indirectly involved in the reduced mobilization of Lin−Sca1+ progenitor cells. Lin−Sca1+ progenitors recruited through the Cxcl12/Cxcr4 axis have been shown to contribute to injury-induced neointimal hyperplasia through differentiation in neointima-driving SMCs at the site of injury.57 Thus, the reduced Lin−Sca1+ mobilization in the Cxcr4EC-KOApoe−/− mice may underlie the reduced number of neointimal SMCs (Figure 3A), providing a protective effect on itself. However, the overall increase in neointima formation in these mice indicates that this protective effect is surmounted by the reduced reendothelialization efficiency in injured Cxcr4EC-KOApoe−/− mice, which allows for a higher infiltration of inflammatory macrophages (Figure 3C), shown to contribute to injury-induced neointimal hyperplasia.57,58

In summary, this study reveals an important protective role of endothelial Cxcr4 and the Cxcl12/Cxcr4 axis in injury-induced neointima formation by promoting efficient reendothelialization, mediated by endothelial migration and proliferation in vivo, as well as by mobilization of protective Sca1−Flik1−Cd31+ progenitors. Our study was performed in Apoe−/− mice on a high-cholesterol diet, which was suggested as a more valid model for characterizing the development of restenotic lesions after mechanical irritation, such as angioplasty.59 However, as a limitation, it cannot be excluded that part of the observed effects might specifically be related to hypercholesterolemic conditions, requiring further investigation. Furthermore, although endothelial Cxcr4 deficiency did not reduce the luminal area in our injury model, it remains to be investigated whether such role in true restenosis could be revealed after more severe vascular injury with increased damage to the medial layer, as observed after angioplasty. Also, future studies are required to clarify the role of endothelial Cxcl12 in injury-induced neointimal hyperplasia and restenosis in more detail.

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References


**Significance**

This study demonstrates that endothelial expression of the chemokine receptor Cxcr4 plays a protective role after vascular injury by promoting efficient reendothelialization through endothelial proliferation and migration and by supporting the mobilization of protective endothelial progenitor cells. Endothelial Cxcr4 deficiency is associated with a reduced endothelial Cxcl12 level, which may play an important role in the observed reduction in injury-induced progenitor cell mobilization. As a result of inefficient reendothelialization, injury-induced neointimal lesions become bigger and show a higher inflammatory phenotype in the absence of endothelial Cxcr4. Although further investigations are required to clarify the role of endothelial Cxcl12 in more detail, our findings indicate a local increase of endothelial Cxcr4 signaling as a potential therapeutic strategy to reduce injury-induced neointimal hyperplasia.
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Figure I. Efficient Bmx-CreER\textsuperscript{TZ}-mediated Cxcr4 deletion in endothelium of injured vessels. Quantification of Cxcr4\textsuperscript{+} endothelium in carotid arteries from Cxcr4\textsuperscript{EC-WT} Apoe\textsuperscript{\textminus\textminus} and Cxcr4\textsuperscript{EC-KO} Apoe\textsuperscript{\textminus\textminus} mice 3 weeks after injury, based on costaining of carotid artery sections for Cd31 and Cxcr4. The graphs display the Cxcr4\textsuperscript{+}Cd31\textsuperscript{+} luminal length as % of the total Cd31\textsuperscript{+} luminal length, restricted to endothelium covering neointimal lesions (A) vs areas without neointimal hyperplasia (B). Represented are means ± SEM; n=8-13; ***P<0.001; two-tailed t-test.
Figure II. No differences in plasma lipid levels upon endothelial Cxcr4 deficiency. Plasma triglyceride and cholesterol concentrations were measured in Cxcr4EC-WT Apoe<sup>+</sup> and Cxcr4EC-KO Apoe<sup>+</sup> mice 3 weeks after carotid artery injury. The graph shows means ± SEM; n=8-13.
Figure III. No significant differences in leukocyte or platelet numbers upon endothelial Cxcr4 deficiency. Numbers of leukocytes (including monocytes, lymphocytes and neutrophils) and platelets were quantified in the peripheral blood of Cxcr4EC-WT Apoe−/− and Cxcr4EC-KO Apoe−/− mice at baseline and at different time points after injury, as indicated. “Baseline” was measured at least 3 weeks after the first tamoxifen injection, but before the start of high-fat diet. The graphs represent means ± SEM; n=13-22 (for baseline, 1 day and 3 weeks); n=3-6 (for 5 and 10 days).
Figure IV. No significant differences in leukocyte subset frequencies upon endothelial Cxcr4 deficiency. A-D, Analysis of monocytes and Gr1^{high} vs Gr1^{low} monocyte subsets (A), T-cells and CD4^{+} vs CD8a^{+} T-cell subsets (B), B-cells (C) and neutrophils (D) by flow cytometry of peripheral blood from Cxcr4^{EC-WT}Apoe^{+/-} and Cxcr4^{EC-KO}Apoe^{+/-} mice at baseline and at different time points after injury, as indicated. "Baseline" was measured at least 3 weeks after the first tamoxifen injection, but before the start of high-fat diet. The graphs represent means ± SEM; n=5-13 (for baseline, 1 day and 3 weeks); n=2-3 (for 10 days).
Figure V. Endothelial Cxcr4 deficiency enhances injury-induced neointima formation, without significant effects on vessel dimensions in the absence of injury. A, Shown are representative images of carotid artery sections from Cxcr4<sup>EC-WT</sup> Apoe<sup>−/−</sup> vs Cxcr4<sup>EC-KO</sup> Apoe<sup>−/−</sup> 4 weeks after vascular injury, positioned ~164 µm from the bifurcation. Scale bars = 200 µm. B, Quantification of luminal area, medial area and the area within the external elastic lamina (aEEL) in carotid artery sections from uninjured Cxcr4<sup>EC-WT</sup> Apoe<sup>−/−</sup> vs Cxcr4<sup>EC-KO</sup> Apoe<sup>−/−</sup> mice. Graphs represent means ± SEM; n=5-6.
A

B

C

FMO: 491
Mac2: 746.7 ± 35.7

FMO: 28.0
Mac2: 70.30 ± 4.041

F4/80
Mac2
Mac2/DAPI
Figure VI. Correlation of macrophage markers Mac2 and F4/80 in neointimal macrophages. A, Flow cytometric analysis of carotid arteries from Apoe−/− mice, 3.5 weeks after vascular injury. Expression of F4/80 and Mac2 was analyzed on Cd45− vascular cells, Cd45+ mononuclear cells and Cd45+ macrophages. n=3 mice. B, F4/80 immunostaining of macrophages within pericardium after myocardial infarction, as positive control for F4/80 staining. C, Considerable overlap between F4/80 (green) and Mac2 (red) immunostaining of injured carotid arteries, 4 weeks after vascular injury. A first set of pictures was taken after F4/80 staining, and only then Mac2 immunostaining was performed on the same section, to prevent spill-over of the strong Mac2 signal in the weak F4/80 signal. Scale bars = 50 µm
Figure VII. Heterogeneous CXCR4 expression on human aortic endothelial cells. 
HAoECs, untreated or stimulated with 100 ng/ml CXCL12 for 22 h as indicated, were analyzed for surface CXCR4 expression using flow cytometry and an appropriate isotype control. Different cell populations were identified in the FSC-SSC blot (A), showing dissimilar levels of CXCR4 expression on their cell surface (B-E). Shown is 1 representative experiment of 2, with n=2-4. Graph represent means ± SEM. Representative histograms are shown. *P<0.05 and **P<0.01; one-way ANOVA with Newman-Keuls post-test.
Figure VIII. Neointimal macrophages are Flk1+. A, After 1 week of high-fat diet, wire-mediated vascular injury was induced in Apoe−/− mice. After 3.5 weeks, expression of F4/80 and Flk1 was analyzed by flow cytometry on Cd45 vascular cells, Cd45 mononuclear cells and Cd45 macrophages from carotid arteries. N=3 mice. B, Colocalization of Flk1 (green) and the macrophage marker Mac2 (red) was analyzed by immunostaining of injured carotid arteries, 4 weeks after vascular injury. A first set of pictures was taken after Flk1 staining, and only then Mac2 immunostaining was performed on the same section, to prevent spill-over of the strong Mac2 signal in the weak Flk1 signal. Considerable overlap between Flk1 (green) and Mac2 (red) could be detected in some (upper panels) but not all sections (lower panels), which is most likely attributed to the in general very low immunofluorescent Flk1 signal revealed by immunostaining. Flk1+ endothelium of small vessels in the surrounding adventitia are indicated with arrows. Scale bars = 100 µm. C, Flow cytometric analysis of F4/80 and Flk1 expression on cells from the peritoneal lavage of ApoE−/− mice, 4 days after intraperitoneal thioglycollate injection. N=2 mice.
Figure IX. No differences in the number of Sca1\(^+\)Flk1\(^+\)Cd31\(^+\) or Lin\(^-\)Sca1\(^+\) progenitor cells in the bone marrow upon endothelial Cxcr4 deficiency. Quantification of leukocytes and progenitor cells in the bone marrow of Cxcr4\(^{EC-WT}\) Apoe\(^{−/−}\) and Cxcr4\(^{EC-KO}\) Apoe\(^{−/−}\) mice at baseline conditions and 10 days after vascular injury. A, Absolute leukocyte numbers. B-C, Relative and absolute content of Sca1\(^+\)Flk1\(^+\)Cd31\(^+\) progenitors (B) and Lin\(^-\)Sca1\(^+\) progenitors cells (C). “Baseline” was measured at least 3 weeks after the first tamoxifen injection, but before the start of high-fat diet. Graphs represent means ± SEM; n=5-6.
Figure X. Comparable expression of Cxcr4 on circulating Sca1\textsuperscript*Flk1\textsuperscript*Cd31\textsuperscript* and Lin\textsuperscript*Sca1\textsuperscript* progenitor cells upon endothelial Cxcr4 deficiency. Cxcr4 expression on the surface of circulating Sca1\textsuperscript*Flk1\textsuperscript*Cd31\textsuperscript* progenitors (A) and Lin\textsuperscript*Sca1\textsuperscript* progenitors (B) was analyzed by flow cytometry in Cxcr4\textsuperscript{EC-WT}Apoe\textsuperscript{-/-} and Cxcr4\textsuperscript{EC-KD}Apoe\textsuperscript{-/-} mice before and at different time points after vascular injury, as indicated. Displayed is the geometric MFI (gMFI) of surface Cxcr4 after subtraction of the FMO control. The graphs represent means ± SEM; n=4-10 for (A) and n=9-12 for (B). Representative histograms are shown.
SUPPLEMENTAL MATERIALS AND METHODS

Nomenclature
Throughout the manuscript, the letter format of all gene and protein notations was chosen to conform with internationally agreed gene/protein nomenclature guidelines: all letters of human genes/proteins are in uppercase, whereas for mouse genes/proteins, only the first letter is in uppercase and the remaining letters are in lowercase. Gene names are in italics.

Mouse models
The Cxcr4-floxed knock-in mouse model\textsuperscript{1}, kindly provided by Prof. Y. Zou (Columbia University, New York, US), and the Bmx-Cre\textsuperscript{ERT2} transgenic mouse line were crossed with C57Bl/6 Apoe\textsuperscript{-/-} mice to create an atherosclerosis-prone background. The presence of the Cre transgene was detected using the primers 5'-AAA TAC CTT CAG TTT TCATCT-3' (Cre-F) and 5'-TTG CGA ACC TCA TCA CTC GTT-3' (Cre-R). The presence of a wild-type or loxP-flanked Cxcr4 allele was detected using the primers 5'-CAC TAC GCA TGA CTC GAA ATG-3' (5X4 FS) and 5'-GTG TGC GGT GGT ATC CAG C-3' (3X4 FS), as previously described\textsuperscript{1}. The inclusion of a third primer 5'-GTG CTC CTC GGA ATG AAG AG-3' allowed to discriminate between a wild-type (330 bp), floxed (430 bp) or deleted (200 bp) Cxcr4 allele after tamoxifen-induced Cre expression. The Cre-reporter mouse line Gt(ROSA)26Sor\textsuperscript{tm1Sor} (Jackson Laboratory), expressing a lac\textsubscript{Z} reporter gene that is transcriptionally silenced by an upstream floxed stop sequence, was crossed with the Bmx-Cre\textsuperscript{ERT2}+ mouse line to allow investigation of the cellular expression pattern of the Bmx-Cre\textsuperscript{ERT2} transgene through analysis of β-galactosidase activity after tamoxifen treatment. Five- to 7-week old female Bmx-Cre\textsuperscript{ERT2}+ Cxcr4-floxed Apoe\textsuperscript{-/-} mice (indicated as Cxcr4EC-KOApoe\textsuperscript{-/-}) and littermate Bmx-Cre\textsuperscript{ERT2}− Cxcr4-floxed Apoe\textsuperscript{-/-} controls (indicated as Cxcr4EC-WTApoe\textsuperscript{-/-}) were treated with tamoxifen (1mg/20g mouse/day, i.p., tamoxifen (Sigma-Aldrich) dissolved in Miglyol (Caelo)) for 5 consecutive days to induce Cre-mediated deletion of the floxed Cxcr4-allele in Bmx\textsuperscript{*} cells. After a recovery period of minimal 3 weeks after the first tamoxifen injection, a first blood analysis was performed, later referred to as the “baseline control”. Then, the mice were fed a western-type diet containing 21% fat and 0.15% cholesterol (Altromin). After the first week of high-fat diet, wire-induced injury of the common carotid artery was performed as previously described\textsuperscript{2}. The mice were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine i.p. and an endothelial denudation of the left common carotid artery was induced by a 1 cm insertion of a flexible 0.36-mm guide wire through a transverse arteriotomy of the external carotid artery, followed by 3 rotational passes. Peripheral blood analyses were performed at different time points after injury, as indicated. Differential blood cell counts were determined by routine laboratory assays (Animal facility, University Hospital Aachen). At the indicated time points after injury, the mice were sacrificed and perfused in situ with a 4% paraformaldehyde solution. The injured carotid arteries were carefully isolated, overnight fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Animal experiments were reviewed and approved by local authorities in accordance with the German animal protection law.
Neointimal lesion quantification and immunohistochemistry

Plasma cholesterol and triglyceride levels were quantified using enzymatic assays (Cobas, Roche) according to the manufacturer’s protocol. Serial 4 µm transversal sections from the paraffin-embedded carotid arteries were collected on glass microscopy slides. Within a standardized distance (0 to 320 µm) from the bifurcation, carotid artery sections (9 sections per mouse; each separated 40 µm apart) were stained using the Elastica-van Gieson (EVG) stain and areas of lumen, neointima (between lumen and internal elastic lamina), media (between internal and external elastic laminae) and area within the external elastic lamina (aEEL) were measured by planimetry using Diskus Software (Hilgers). For each mouse, data from these 9 sections were averaged to represent lesion formation along this standardized distance. Neointimal macrophages, smooth muscle cells and endothelial cells were visualized by immunofluorescent staining for Mac2 (M3/38, Cedarlane), Sma (1A4, Dako) or Cd31 (M-20, Santa Cruz Biotechnology), respectively, followed by a FITC- or Cy3-conjugated secondary antibody staining (Jackson ImmunoResearch). Endothelial expression of Cxcr4 and Cxcl12 was quantified after co-staining for Cd31 and Cxcr4 (2074, Abcam) or Cxcl12 (79018, R&D System), respectively. Proliferation of endothelial cells or macrophages was visualized by co-staining for Cd31 or Mac2, respectively, with the proliferation marker Ki-67 (TEC-3, Dako). F4/80 and Flk1 immunostaining were performed using a primary antibody from AbD Serotec (Clone Cl:A3-1) and R&D Systems (AF644), respectively. Appropriate IgG antibodies were used as isotype controls. Nuclei were counterstained with 4’,6-diamidino-2-phenylindol (DAPI). Collagen content was determined by Sirius Red staining. Images were recorded with a Leica DM2500 fluorescence microscope and CCD camera. The quantification of lesion size, composition and endothelial Cxcr4 and Cxcl12 expression was performed using image analysis software (Diskus Software, Hilgers) without prior knowledge of the genotype. Vessel reendothelialization was calculated as the ratio of the length of luminal Cd31+ endothelial immunostaining to the total luminal circumference. The collagen content was analyzed by Image J software. Mean values were derived from the analysis of 9 sections per mouse for neointimal lesion size and from 3-5 sections per mouse for all other analyses.

Flow cytometry

Bone marrow cells were harvested and a single-cell suspension was prepared by filtering over a 70 µm cell strainer (Greiner). Bone marrow cells and EDTA-anti-coagulated whole blood obtained from the retro-orbital plexus of mice were subjected to red blood cell lysis (0.8% NH4Cl, 10 mM KHCO3 and 0.1 M EDTA in Millipore water) at room temperature. After washing with HANKS Complete buffer containing 1x HBSS with 0.3 mM EDTA and 0.1% BSA, cells were stained with combinations of antibodies to Cd45, Cd115, Gr1, Cd3, Cd4, Cd8a, Cd19 and Flk1 (eBioscience or BD Bioscience). B-cells were identified as Cd45+Cd19+; T-cells as Cd45+Cd3+, with Cd4+ and CD8a+ T-cells as separate T-cell subsets; neutrophils as Cd45+Cd115-Gr1high; monocytes as Cd45+Cd115+, with Gr1high and Gr1low monocytes as separate monocyte subsets. Circulating Sca1+Fk1+Cd31+ mononuclear cells were characterized by flow cytometry using an antibody cocktail against Sca1 (Ly6A/E, clone D7, BD Biosciences), Flk1 (Vegfr-2, clone Avas12a1, eBioscience) and Cd31 (clone 390, Abcam)5-10. Lin’Sca1+ progenitor cells were determined after staining with an anti-Sca1 antibody (clone D7, BD Biosciences) and a
Mouse Lineage Panel (Cd3ε, Cd11b, Cd45R, Gr-1, Ter-119, Ly-6G, BD Pharmingen). Cxcr4 surface expression was quantified after staining with an anti-Cxcr4 antibody (clone 2B11/Cxcr4, BD Biosciences) and represented by the geometric mean fluorescence intensity (gMFI).

To analyze surface marker expression on lesional monocytes and macrophages, injured carotid arteries were harvested, perfused with ice-cold PBS and kept in RPMI-1640 medium on ice until used. The carotid arteries were digested with LiberaseTM (100 μg/ml; diluted in RPMI-1640, Roche) for 1 h at 37°C in a shaking incubator. Afterwards, Hanks Complete Buffer was added and the sample was gently pipetted to obtain a cell suspension. The cells were washed with Hanks Complete Buffer and passed through a cell strainer (70 μm, BD Falcon). After treatment with mouse Fc block (2.4G2, BD Pharmingen) to prevent non-antigen-specific binding of antibodies, cells were stained for Cd45, F4/80 (BM8, eBioscience), Flk1 (Avas 12alpha1, BD Pharmingen) and Mac2 (M3/38, Cedarlane).

Thioglycollate-elicited peritoneal macrophages were pretreated with mouse Fc blocking before staining for F4/80 and Flk1.

Stained cells were analyzed after appropriate fluorescence compensation and gating strategies based on the appropriate FMO (fluorescence minus one) controls for directly fluorescence-conjugated primary antibodies. For the non-directly conjugated antibody to Mac2, a staining mix without anti-Mac2 antibody but with inclusion of the fluorescent secondary antibody for Mac2 was used as negative control for Mac2 staining. Flow cytometric analysis was performed using a FACSCanto-II and FACSDiva software (BD Biosciences) and the FlowJo analysis program (Treestar).

**ELISA**

Plasma was obtained from blood that was anti-coagulated with EDTA or heparin by centrifugation at 400 g for 15 min. To measure the level of Cxcl12 in the bone marrow, the femurs of mice were flushed with 1 ml of ice-cold PBS, the eluent was centrifuged at 300 g for 5 min, and the supernatant was used for analysis. Cxcl12 levels in EDTA-plasma and bone marrow was measured using a mouse Cxcl12 ELISA kit from RayBiotech, according to the manufacturer’s protocol. Quantikine ELISA kits from R&D were used to measure the levels of Vegf, Flt3-ligand and GM-CSF in EDTA-plasma. A home-made ELISA was used to measure plasma levels of Mif. The concentration of the sphingolipid sphingosine 1-phosphate (S1P) was measured in heparin-plasma using an ELISA from Echelon.

**β-Galactosidase staining**

β-Galactosidase activity was determined using a whole mount X-gal histochemical staining in which the substrate X-gal produces a blue dye when cleaved by β-galactosidase. Mice were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine i.p., exsanguinated and the vasculature was rinsed in vivo with cold phosphate-buffered saline (PBS) containing 2 mM MgCl2. After in situ perfusion with a cold 2% paraformaldehyde solution, carotid arteries were isolated, fixed for 15 min in the same fixation solution on ice and washed three times in PBS containing 2 mM MgCl2. Then, the carotid arteries were incubated overnight at 37°C in a prewarmed β-gal staining solution, containing 1 mg/ml of the substrate X-gal in an X-gal reaction buffer (PBS containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide,
2 mM MgCl₂, 0.02 % Nonidet P-40 and 0.01% Na-deoxycholate). The next day, carotid arteries were rinsed with PBS until the washing buffer no longer turned yellow. The stained carotid arteries were then embedded in Tissue Tek (SAKURA) for 2 h and cryo-sections were prepared. β-Galactosidase staining was visualized using light microscopy.

**In vitro scratch assay and proliferation assay**

Human aortic endothelial cells (HAoECs) were purchased from PromoCell, plated on cell culture dishes coated with collagen (Biochrom) and cultured in Endothelial Cell Growth Medium MV (PromoCell), according to the manufacturer’s recommendations. For an in vitro scratch assay, cells were grown to confluency and were left untreated or were prestimulated for 8 h with CXCL12 (Recombinant human SDF-1α/CXCL12, 100ng/ml, PeproTech), AMD3100 (1µg/ml, Sigma) or a combination of both, as indicated. Wounds were introduced by dragging a sterile pipette tip across the monolayers to create a cell-free path. After 20 h of migration under ongoing stimulation, microscopic pictures were taken using phase contrast microscopy. For each well, 10 consecutive microscopic pictures were taken at the start and end of the migration assay and were overlayed, and the area and number of cells migrating into the wound area was quantified using Diskus software (Hilgers).

To examine effects on proliferation in vitro, HAoECs were plated in 24-well plates und cultured for the indicated amount of time, without or with stimulation with CXCL12 (100ng/ml, PeproTech) or AMD3100 (1µg/ml, Sigma), as indicated. At specific time points, plates were gently inverted to remove the medium and frozen at -80°C. At the end of the experiment, proliferation was measured in all plates simultaneously using a CyQUANT® Cell Proliferation Assay Kit (Life Technologies).

**Isolation of thioglycollate-elicited peritoneal macrophages**

Thioglycollate-elicited peritoneal macrophages were isolated as described. Mice were injected intraperitoneally with 1 ml of sterile Brewer's complete thioglycollate broth (3% wt/vol). After 4 days, cells were isolated by flushing the peritoneum with ice-cold PBS. Red blood cells were lysed in red blood cell lysis (0.8% NH₄Cl, 10 mM KHCO₃ and 0.1 M EDTA in Millipore water). After washing with HANKS Complete buffer, remaining cells were used for analyzing surface markers through flow cytometric analysis. Macrophage identity was determined by gating F4/80⁺ cells.

**Statistics**

Data are represented as means ± SEM and were analyzed using a t-test, 1-way ANOVA with Newman-Keuls or Bonferroni post-test, or 2-way ANOVA with Bonferroni post-test, as appropriate (GraphPad Prism 5 or 6). P<0.05 was considered statistically significant.
References