CXCL12 Promotes the Stabilization of Atherosclerotic Lesions Mediated by Smooth Muscle Progenitor Cells in Apoe-Deficient Mice

Shamima Akhtar, Felix Gremse, Fabian Kiessling, Christian Weber, Andreas Schober

Objective—Unstable atherosclerotic lesions are prone to rupture, which leads to atherothrombosis. CXCL12 promotes the mobilization and neointimal recruitment of smooth muscle progenitor cells (SPCs), and thereby mediates vascular repair. Moreover, treatment with SPCs stabilizes atherosclerotic lesions in mice. We investigated the role of CXCL12 in the treatment of unstable atherosclerotic lesions.

Approach and Results—Intravenous injection of CXCL12 selectively increased the level of Sca1+Lin−PDGFR-β+ SPCs in the circulation as determined by flow cytometry. Macrophage-rich lesions were induced by partial ligation of the carotid artery in Apoe−/− mice. Repeated injection of CXCL12 reduced the macrophage content, increased the number of smooth muscle cells, increased the fibrous cap thickness, and increased the collagen content in these lesions. However, CXCL12 did not alter the lesion size or the luminal diameter of the carotid artery as determined by planimetry and micro-computed tomography, respectively. Recruitment of bone marrow–derived SPCs to the lesions was increased after treatment with CXCL12 in chimeric mice that expressed SM22-LacZ in bone marrow cells as determined by quantification of the number of lesional β-galactosidase-expressing cells. CXCL12 expression was upregulated in atherosclerotic arteries after CXCL12 treatment. Silencing of arterial CXCL12 expression during atherosclerosis promoted lesion formation and reduced the lesional smooth muscle cell content in CXCL12-treated mice.

Conclusions—Systemic treatment with CXCL12 promotes a more stable atherosclerotic lesion phenotype and enhances the accumulation of SPCs in these lesions without promoting atherosclerosis. Thus, CXCL12-induced SPC mobilization appears a promising approach to treat unstable atherosclerosis. (Arterioscler Thromb Vasc Biol, 2013;33:XX-XX.)

Key Words: atherosclerosis • chemokine • plaque modification • vascular biology

The most devastating clinical manifestations of atherosclerosis are atherothrombotic events, such as myocardial infarction and stroke.1,2 Plaque rupture is the major cause of symptomatic coronary thrombosis and is characterized by a deep injury to the fibrous cap that exposes the thrombogenic lipid core to the bloodstream.3 Moreover, the rupture of atherosclerotic lesions and subsequent asymptomatic mural thrombosis accelerate atherosclerosis progression.3 Interestingly, the vulnerability of lesions to rupture depends on the lesion composition, but not on the lesion size or the degree of luminal obstruction.1 A large lipid core and a thin fibrous cap characterized by increased leukocyte infiltration and diminished collagen-producing smooth muscle cells (SMCs) are hallmarks of rupture-prone lesions.4,5 Apoptosis of SMCs in atherosclerotic lesions leads to thinning of the fibrous cap, loss of collagen, and intimal inflammation.6 Moreover, increased p53 expression in advanced atherosclerosis may promote lesion rupture by promoting SMC apoptosis in the fibrous cap.7 Accordingly, SMCs are typically absent from the site of rupture in thrombosed coronary atherosclerotic lesions in humans.8 Therefore, the formation of the fibrous cap by SMCs is crucial for the stabilization of atherosclerotic lesions. Smooth muscle progenitor cells (SPCs) have been successfully administered to atherosclerotic mice to therapeutically increase the SMC content in atherosclerotic lesions, and thereby produce a more stable lesion phenotype.9 Although SPCs are rarely found in developing atherosclerotic lesions and fibrous caps, interventions that enhance SPC recruitment may be a promising strategy in lesion stabilization.10,11

In contrast to atherosclerosis, vascular repair after injury is associated with the neointimal accumulation of circulating SPCs, which restores the integrity of the vessel wall in response to extensive apoptosis of medial SMCs.12-15 The chemokine CXCL12 is upregulated in the injured artery via lysosphatidic acid, and the transient increase in circulating...
CXCL12 induces a transient mobilization of a subtype of Sca-1<sup>+</sup>Lin<sup>−</sup> SPCs. Additionally, CXCL12 immobilized on surface-adjacent platelets mediates the recruitment of SPCs to the injured vessel. The effects of CXCL12 on neointima formation can be completely blocked by targeting its receptor CXCR4, indicating that the other CXCL12 receptor, CXCR7, is not involved in SPC-mediated vascular repair. In addition, CXCL12-induced SPC mobilization modulates lesion formation in allograft vasculopathy in mice and humans. In atherosclerosis, upregulation of endothelial CXCL12 expression via the microvesicle-mediated transfer of miR-126 reduces lesion formation, whereas blocking CXCR4 promotes atherosclerosis through the increased release of neutrophils into the circulation. Plasma levels of CXCL12 are reduced during atherosclerosis through the increased release of neutrophils into the circulation.

Results

CXCL12 Treatment Mobilizes SPCs

We performed a dose–response study to determine whether intravenous injection of CXCL12 mobilizes Sca-1<sup>+</sup>Lin<sup>−</sup> SPCs into the circulation in C57BL/6 mice. The level of circulating Sca-1<sup>+</sup>Lin<sup>−</sup> SPCs was determined after administration of 100 ng and 500 ng of CXCL12. Injection of 100 ng of CXCL12 did not affect the level of circulating SPCs; however, at 4 hours after injection of 500 ng of CXCL12, the level was increased by 6-fold compared with the baseline (Figure IA in the online-only Data Supplement). The level of peripheral Sca-1<sup>+</sup>Lin<sup>−</sup> cells was significantly higher at 4 hours after injection of 500 ng of CXCL12 compared with the levels after injection of 100 ng of CXCL12 or PBS (Figure IA and IB in the online-only Data Supplement). After this mobilization of SPCs at 4 hours, the level of SPCs steadily decreased at 8 hours and 24 hours after treatment with 500 ng of CXCL12 (Figure IA in the online-only Data Supplement). CXCL12 treatment mainly increased the number of PDGFR-<b>β</b> cells in the peripheral Sca-1<sup>+</sup>Lin<sup>−</sup> cell population (Figure IC in the online-only Data Supplement). The levels of circulating leukocyte subsets and platelets were not significantly altered at 4 hours after CXCL12 treatment (Table I in the online-only Data Supplement), indicating that CXCL12 selectively increased the level of Sca-1<sup>+</sup>Lin<sup>−</sup> SPCs in the circulation. The plasma and BM concentrations of CXCL12 were compared to assess whether CXCL12 injection altered the concentration gradient of CXCL12 between the circulation and the BM. The plasma CXCL12 concentration peaked at 4 hours after injection of 500 ng of CXCL12 and returned to the baseline level after 24 hours (Figure ID in the online-only Data Supplement). However, the CXCL12 concentration in the BM, which was >3-fold higher than the plasma concentration at baseline, was not altered at 4 hours after injection of 500 ng of CXCL12 (Figure ID in the online-only Data Supplement). Notably, the plasma CXCL12 concentration almost reached the BM CXCL12 concentration at 4 hours after CXCL12 injection (Figure ID in the online-only Data Supplement). These results indicate that injection of 500 ng of CXCL12 temporarily abrogates the CXCL12 concentration gradient between the circulation and the BM.

Macrophage-Rich Lesions Form After Partial Ligation of the Carotid Artery

To induce advanced atherosclerotic lesions with an unstable phenotype, partial ligation of the left carotid artery of Apoe<sup>−/−</sup> mice fed a high-cholesterol diet was performed. At 4 and 6 weeks after partial carotid ligation, atherosclerotic lesions had developed that showed typical signs of advanced lesions, such as a lipid core and a fibrous cap (Figure IIA in the online-only Data Supplement). Although lesion size tended to be larger at 6 weeks after partial carotid ligation than at 4 weeks, the difference was not statistically significant (Figure IIB in the online-only Data Supplement). The diameter of the lumen of the partially ligated left carotid artery was 48% shorter than that of the untreated right carotid artery, as determined by in vivo micro-computed tomography (Figure IIC and IID in the online-only Data Supplement). The relative lesional macrophage content increased by 60% between week 4 and week 6 after partial carotid ligation, atherosclerotic lesions had developed that showed typical signs of advanced lesions, such as a lipid core and a fibrous cap (Figure IIA in the online-only Data Supplement). Although lesion size tended to be larger at 6 weeks after partial carotid ligation than at 4 weeks, the difference was not statistically significant (Figure IIB in the online-only Data Supplement). The diameter of the lumen of the partially ligated left carotid artery was 48% shorter than that of the untreated right carotid artery, as determined by in vivo micro-computed tomography (Figure IIC and IID in the online-only Data Supplement). The relative lesional macrophage content increased by 60% between week 4 and week 6 after partial carotid ligation (Figure III in the online-only Data Supplement), whereas the SMC content decreased by 50% to 70% during the same time period (Figure IV in the online-only Data Supplement). Therefore, the macrophage-rich lesions that developed within 6 weeks after partial carotid ligation showed high vulnerability and caused luminal stenosis.

CXCL12 Treatment Increases the Thickness of the Fibrous Cap

To study the effects of CXCL12 treatment on lesion formation and atherosclerotic luminal narrowing, Apoe<sup>−/−</sup> mice fed a high-cholesterol diet underwent partial carotid ligation,
and 2 weeks later biweekly injections of 500 ng of CXCL12 were commenced for 4 weeks (Figure V in the online-only Data Supplement). Repetitive CXCL12 treatment for 4 weeks increased the number of peripheral Sca-1−Lin− cells; moreover, an increase was also observed after the final CXCL12 injection, indicating that each CXCL12 injection promoted SPC mobilization (Figure VI in the online-only Data Supplement). Similar to the results obtained after a single injection of CXCL12, repetitive treatment with CXCL12 did not significantly affect the levels of circulating leukocyte subsets (Table II in the online-only Data Supplement). Furthermore, treatment with CXCL12 did not significantly affect the lesion or medial area (Figure 1A–1C). The length of the external elastic lamina was similar after CXCL12 and PBS treatment, demonstrating that CXCL12 does not modulate vessel remodeling (Figure 1D). Accordingly, the luminal diameter of the partially ligated carotid artery was similar in PBS- and CXCL12-treated mice, as assessed by micro-computed tomography angiography (Figure 1E). Interestingly, the thickness of the fibrous cap was significantly higher in CXCL12-treated mice than in PBS-treated mice (Figure 1F), whereas the size of the lipid core area was similar (Figure 1G). Cellularity in the fibrous caps was higher in CXCL12-treated mice than in PBS-treated mice (Figure VII in the online-only Data Supplement), whereas the proliferation rate of fibrous cap cells was similar (Figure VIII in the online-only Data Supplement). The apoptosis rate of lesional cells was not statistically different between CXCL12- and PBS-treated mice, as determined by the TUNEL assay (Figure IX in the online-only Data Supplement). The size of the lesion area in the aortic arch was quantified after 6 weeks of a high-cholesterol diet and CXCL12 treatment (Figure 1H). CXCL12 treatment did not affect the lesion size in the aortic arch as determined by oil red O staining of en face–prepared aortas (Figure 1H and 1I). The serum levels of cholesterol and triglycerides were similar in PBS- and CXCL12-treated mice (Table III in the online-only Data Supplement). These results demonstrate that repetitive treatment with CXCL12 in a model of atherosclerosis induced by disturbed flow did not affect the lesion size or the degree of stenosis but increased the thickness of the fibrous cap.

**Effects of CXCL12 Treatment on Lesion Composition**

Next, we analyzed the effects of CXCL12 treatment on the composition of the atherosclerotic lesions by quantitative immunohistochemistry. The lesional SMC content, as determined by SM22, calponin, and α-SMA immunostaining, was significantly higher in CXCL12-treated mice than in PBS-treated mice (Figure 2A and 2B and Figure X in the online-only Data Supplement). Furthermore, the lesional collagen type I area was larger in CXCL12-treated mice than in PBS-treated mice (Figure 2C and 2D). By contrast, the relative lesional macrophage content, as determined by Mac2 immunostaining and flow cytometric analysis of F4/80+ cells, was lower in CXCL12-treated mice than in PBS-treated mice (Figure 2E and 2F; Figure XI in the online-only Data Supplement). The CD3+ T-cell and neutrophil content and the number of neovessels in the lesions were similar in PBS- and CXCL12-treated mice (Figures XII and XIII in the online-only Data Supplement). Thus, CXCL12 treatment reduced the inflammatory response in atherosclerotic lesions and promoted the accumulation of SMCs and collagen, which helped increase the thickness of the fibrous cap, indicative of a more stable lesion phenotype.

**CXCL12 Treatment Increases the Lesional Accumulation of BM-Derived SPCs**

To determine whether CXCL12 treatment increases the recruitment of BM-derived SPCs into atherosclerotic lesions, we studied Apoe−/− mice that had received BM from Apoe−/− mice that expressed β-galactosidase under the control of the SMC-specific promoter, SM22. β-galactosidase–expressing SPCs were barely detectable by immunostaining in the lesions of PBS-treated mice and in the right carotid arteries of CXCL12-treated mice (Figure 3A). However, the number of β-galactosidase–expressing SMCs was significantly increased in the lesions of CXCL12-treated mice, primarily in the fibrous cap (Figure 3A–3C). Combined immunostaining of β-galactosidase and cell-specific markers revealed β-galactosidase colocalized with calponin, but not with CD31.

![Figure 1](https://example.com/figure1.png)
or F4/80 (Figure XIV in the online-only Data Supplement). Moreover, β-galactosidase activity as determined by X-gal staining was absent in the lesions of PBS-treated mice, whereas prominent X-gal staining was detected in the lesions of CXCL12-treated mice (Figure 3C). The rate of apoptosis was not markedly different between β-galactosidase+ and β-galactosidase− lesional cells (Figure XV in the online-only Data Supplement). Double staining of β-galactosidase and proliferation markers, such as Ki67 and PCNA, did not show any cellular colocalization (data not shown). Taken together, CXCL12 treatment promotes the accumulation of BM-derived SPCs in atherosclerotic lesions (mainly in the fibrous cap), which differentiate into SMCs and contribute to the increased thickness of the fibrous cap observed in CXCL12-treated mice.

Role of CXCL12-Induced Endothelial CXCL12 Expression in Plaque Stabilization

CXCL12 is required for the local recruitment of SPCs to injured arteries.16–18 To study whether systemic treatment with CXCL12 increases the lesional CXCL12 content as a prerequisite for the efficient recruitment of SPCs, we analyzed CXCL12 expression by immunostaining. In PBS-treated mice, CXCL12 was detectable in a subset of the cells within the lesions and in a subset of the endothelial cells covering the lesions, as determined by double immunostaining for CXCL12 and CD31 (Figure 4A). After CXCL12 treatment, almost all of the lesional endothelial cells expressed CXCL12, and prominent CXCL12 staining was detectable in the cell layers underneath the endothelium (Figure 4A). Moreover, the number of CXCL12-immunopositive endothelial cells was substantially higher in CXCL12-treated mice than in PBS-treated mice (Figure 4A). Lesion formation in the carotid artery was accompanied by substantial upregulation of Cxcl12 mRNA (Figure 4B). In addition, Cxcl12 mRNA expression was higher in the lesions, but not in the nonligated right carotid arteries, of CXCL12-treated mice than of PBS-treated mice (Figure 4B). CXCL12-induced Cxcl12 mRNA expression was also observed in murine endothelial cells in

**Figure 2.** CXCL12 treatment induces a stable lesion phenotype. The relative content of SMCs (A), collagen type I (B), and macrophages (C) was determined in lesions of partially ligated carotid arteries from ApoE−/− mice treated with PBS or CXCL12 for 4 weeks by immunostaining with SM22, collagen type I (Col-1), and Mac2, respectively. Nuclei were counterstained with DAPI. Arrows delineate the lesions. Scale bars indicate 200 μm. *P<0.05, **P<0.01, n=6 to 7 mice per group.

**Figure 3.** CXCL12 treatment promotes the recruitment of bone marrow (BM)-derived smooth muscle progenitor cells into atherosclerotic lesions. A, Immunostaining of β-galactosidase (β-gal) was performed in sections of partially ligated carotid arteries from ApoE−/− mice that were repopulated with BM cells from SM22-LacZ/ApoE−/− mice 5 weeks before treatment with PBS (left) or CXCL12 (middle) for 4 weeks. β-Galactosidase immunostaining was not detected in unligated right carotid arteries of CXCL12-treated mice (right). Arrows indicate the luminal side of the vessel wall. B, β-galactosidase+ cells were quantified in carotid lesions after treatment with PBS or CXCL12 for 4 weeks. C, Distribution of β-galactosidase+ cells in the fibrous cap (FC). *P<0.05, **P<0.01, n=4 to 5 mice per group. D, β-Galactosidase activity was determined by X-gal staining in lesions from ApoE−/− mice that had received SM22-LacZ/ApoE−/− BM and been treated with PBS or CXCL12. Arrows indicate cells with β-galactosidase activity (blue). Scale bars indicate 100 μm (A) and 50 μm (D).
vitro (Figure 4C). Notably, expression levels of Hif1a mRNA, which induces CXCL12 expression, were significantly higher in the lesions of CXCL12-treated mice (Figure 4B) and in CXCL12-stimulated endothelial cells (Figure 4C) than in their PBS-treated controls.

To further confirm the role of lesional CXCL12 expression in SPC recruitment and lesion stabilization, partially ligated carotid arteries of CXCL12-treated mice were perivascularly treated with small interfering RNA targeting CXCL12 or a nontargeting small interfering RNA control. The lesion size and lesional SMC content, as determined by elastic Van Gieson staining and SM22 immunostaining, respectively, were reduced, whereas the lesional macrophage content (Mac2+ immunostaining) was increased in mice treated with CXCL12-specific small interfering RNA compared with mice treated with control small interfering RNA (Figure 5).

Taken together, repetitive CXCL12 administration increases lesional CXCL12 expression presumably via upregulation of HIF-1α, and thereby promotes the lesional accumulation of SMCs.

### Long-Term Effects of CXCL12 Treatment on the Lesion Phenotype

To assess the long-term effects of CXCL12 treatment on atherosclerosis, lesion size and composition were studied in Apoe−/− mice 2 weeks after the termination of CXCL12 or PBS treatment. Similar to the lesions observed immediately after the termination of CXCL12 treatment, these lesions exhibited a thicker fibrous cap in CXCL12-treated mice than in PBS-treated mice, whereas the size of the lipid core area was not significantly different (Figure 6A–6C). In addition, the lesional SMC and collagen-I content, as determined by SM22 and collagen-I immunostaining, respectively, was higher in

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**Figure 4.** Systemic CXCL12 treatment induces lesional CXCL12 expression. **A**, Double immunostaining for CXCL12 and CD31 in lesions of partially ligated carotid arteries after 4 weeks of PBS or CXCL12 treatment (n=3 mice per group). **B**, Expression levels of Cxcl12 and Hif1a mRNA were determined in partially ligated left (LC) and untreated right (RC) carotid arteries of Apoe−/− mice treated with PBS or CXCL12 for 4 weeks. **C**, Expression levels of Cxcl12 and Hif1a mRNA were quantified in mouse endothelial cells in vitro after treatment with PBS or CXCL12 by qRT-PCR (n=3 per group). Scale bars indicate 100 µm. *P<0.05, **P<0.01, ***P<0.0001.

**Figure 5.** Role of lesional CXCL12 expression in lesion formation during CXCL12 treatment. **A**, Lesion size was determined in elastic Van Gieson-stained carotid sections 6 weeks after partial ligation in Apoe−/− mice treated systemically with CXCL12 and perivascularly with nontargeting small interfering RNA (siRNA [siCTRL]) or an siRNA targeting CXCL12 (siCXCL12). **B**, The lesional macrophage (B) and SMC (C) content were assessed by Mac2 and SM22 immunostaining, respectively. Scale bars indicate 200 µm. *P<0.05, **P<0.01. n=4 mice per group.
CXCL12-treated mice than in PBS-treated mice 2 weeks after the termination of treatment (Figure 6D and 6E). The lesional macrophage content, as determined by Mac2 immunostaining, remained lower in CXCL12-treated mice than in PBS-treated mice even 2 weeks after the termination of treatment (Figure 6F). Moreover, the size of the aortic lesion was not significantly different in CXCL12- and PBS-treated mice 2 weeks after the termination of treatment (Figure 6G).

To study the potential effects of CXCL12 treatment on tumor formation, whole body micro-computed tomography scans were performed 2 weeks after the termination of treatment. Malignancies, particularly of the brain, liver, lung, and lymph nodes, were not detectable in PBS- or CXCL12-treated mice (Figure XVIA– XVIC in the online-only Data Supplement). In addition, histological analysis of the kidney, spleen, and intestine showed no distortion of the normal tissue architecture that is indicative of malignancies (Figure XVIND–XVIF in the online-only Data Supplement). Together, these data support the hypothesis that the beneficial effects of CXCL12 treatment on lesion composition are long-lasting and persist after interruption of the treatment.

**Discussion**

We found that repetitive, transient treatment with CXCL12 stabilizes lesions in a mouse model of advanced, stenotic atherosclerosis, and this stabilization is sustained after the termination of treatment. The stabilization of the atherosclerotic lesions by CXCL12 was associated with the mobilization of SPCs and increased lesional accumulation of BM-derived SPCs. Therefore, our results indicate that inducing lesional SPC recruitment by repetitive treatment with CXCL12 is a promising approach to treat vulnerable lesions.

Shortly after wire injury of the carotid artery, transiently elevated CXCL12 levels in the circulation mobilize PDGFR-β⁺ SPCs into the peripheral circulation.¹⁶,¹⁷ Similarly, CXCL12 treatment induced the mobilization of Sca1⁺Lin⁻PDGFR-β⁺ SPCs concomitant with an increase in plasma CXCL12 levels. The physiological CXCL12 concentration gradient between the circulation and the BM, which is crucial for the retention of stem cells in the BM, was reduced after injection of CXCL12 because the BM levels of CXCL12 were not elevated. Several reports have demonstrated that reversal of the CXCL12 gradient, attributable to increased circulating CXCL12 levels and reduced levels of CXCL12 in the BM, mobilizes mature and immature hematopoietic cells into the circulation.²⁸,²⁹,³² Although levels of white blood cells, including lymphocytes and monocytes, tended to increase after CXCL12 treatment, the mobilization of SPCs was not accompanied by a significant increase in the level of circulating mature hematopoietic cells. This rather specific mobilization of progenitor cells after CXCL12 injection may be because the CXCL12 concentration gradient was only mildly modulated in comparison with studies that reported CXCL12-mediated mobilization of white blood cells.²⁹,³² Furthermore, neutrophilia has been observed during chronic application of a CXCR4 antagonist, whereas the level of Sca-1⁺Lin⁻ cells increases during short-term CXCR4 inhibition and, subsequently, decreases during long-term CXCR4 inhibition.³⁴ Therefore, continuous, long-term disruption of the CXCL12 gradient may have different effects on the mobilization of BM-derived cells compared with the changes induced by intermittent CXCL12 injections.
The local recruitment of SPCs after vascular injury requires HIF-1α-mediated upregulation of CXCL12 and its deposition on the luminal surface.\(^7,33\) CXCL12 treatment increased the transcription of CXCL12 in atherosclerotic arteries and endothelial cells, which might promote the recruitment of SPCs. Notably, autoinduction of CXCL12 in endothelial cells via increased microRNA126-controlled CXCR4 signaling has been described.\(^23\) CXCL12 upregulated HIF-1α mRNA levels in the atherosclerotic vessel wall and endothelial cells, which may underlie CXCL12-triggered CXCL12 expression. Silencing of CXCL12 expression in the vessel wall of CXCL12-treated mice decreased the lesional SMC content; this supports the hypothesis that lesional CXCL12 expression is crucial for the recruitment of SPCs mobilized by CXCL12. Therefore, we conclude that the increase in CXCL12, both in the circulation and on the lesion surface after repetitive CXCL12 injections, coordinates the mobilization and subsequent lesional recruitment of SPCs.

SPC recruitment to injured arteries by lysosphatidic acid-induced upregulation of CXCL12 increases the neointimal accumulation of SMCs and neointima formation.\(^16,18\) A similar effect of CXCL12 on circulating SPCs has been implicated in the development of allograft vasculopathy.\(^21,22\) By contrast, protective roles of CXCL12 and CXCR4 in atherosclerosis have previously been reported. Chronic application of a CXCR4 antagonist and genetic deficiency of CXCR4 in BM cells enhance the formation of atherosclerotic lesions.\(^3,33\) Moreover, CXCL12 upregulation through microRNA-126–mediated suppression of RGS16 in early atherosclerosis reduces lesion formation and the accumulation of vascular progenitor cells.\(^23\) By contrast, coronary artery disease risk alleles at chromosome 10q11 are associated with a high plasma CXCL12 level, indicating that circulating CXCL12 is atherogenic, although plasma CXCL12 levels were not significantly different in patients with and without coronary artery disease.\(^8\) We studied the effect of CXCL12 treatment on lesion formation in a mouse model of advanced, unstable atherosclerosis induced by an acute flow disturbance. Our results indicate that intermittent increases in circulating CXCL12 levels after repeated bolus injections do not aggravate atherosclerotic lesion formation or modulate the degree of stenosis. However, the lesion phenotype was substantially changed by CXCL12 treatment, resulting in reduced infiltration of inflammatory cells and lesional accumulation of SMCs attributable, at least in part, to the recruitment of BM-derived SPCs. Moreover, the lesional collagen content was increased after CXCL12 treatment, which may be caused by the increased number of collagen-producing SMCs.\(^1\) In addition, BM-derived SPCs and CXCL12 induce collagen synthesis in differentiated medial SMCs.\(^10,19\) The expansion of lesional SMC content and collagen deposition likely promote fibrous cap thickening, and thereby stabilize atherosclerotic lesions. This improvement in the vulnerability of plaques after CXCL12 treatment is consistent with the finding that patients with unstable angina have lower CXCL12 levels than those with stable angina.\(^25\) The effect of CXCL12 treatment on plaque composition was observed even 2 weeks after the termination of injections, suggesting that intermittent treatment with CXCL12 might be sufficient to stabilize plaques.

In addition, our finding that silencing of CXCL12 expression in atherosclerotic arteries reduced lesion size indicates that upregulation of CXCL12 during atherosclerosis has alternative antiatherogenic effects, besides the recruitment of SPCs observed after CXCL12 treatment.

SPCs play a key role in vascular repair after injury and in neointima formation.\(^7\) Although SPCs are rarely found in atherosclerotic lesions, the induction of SPC apoptosis limits atherosclerosis and reduces the expression of proinflammatory genes, indicating that SPCs have proinflammatory effects in atherosclerosis.\(^10,11\) By contrast, treatment with CD34+ SPCs derived from human umbilical cord blood and expanded ex vivo reduces early atherosclerosis by diminishing the lesional macrophage content.\(^9\) Additionally, injection of CD34+ SPCs results in a stable lesion phenotype, characterized by an increased SMC and collagen content.\(^9\) In agreement with the results of Zoll et al,\(^7\) we found that the induction of lesional SPC recruitment by CXCL12 treatment reduced the macrophage content. Although the lesion size was not significantly altered by CXCL12-induced SPC recruitment, the treatment duration might have been too short to observe any effects on lesion growth. In addition, functional differences may exist among endogenous SPCs that are recruited during atherosclerosis, ex vivo expanded CD34+ SPCs, and CXCL12-responsive SPCs. Moreover, CXCL12 treatment may not only direct SPCs to the vessel wall but also affect the function and phenotype of SPCs.

In summary, we have provided evidence for a novel therapeutic strategy to improve atherosclerotic lesion stability via intermittent treatment with CXCL12, which mediates the mobilization and recruitment of BM-derived SPCs.

Acknowledgments

We thank Kathrin Heyll and Anna Thiemann for excellent technical assistance.

Sources of Funding

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (to A. Schober, Scho1056/3-1), by a DAAD-Siemens scholarship (to S. Akhtar), by the DZHK (German Center for Cardiovascular Research), and by the BMBF (German Ministry of Education and Research).

Disclosures

None.

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**Significance**

CXCL12 mediates vascular repair by mobilizing and recruiting smooth muscle progenitor cells. This study demonstrates in a murine model of vulnerable atherosclerosis that systemic treatment with CXCL12 promotes sustained lesion stabilization by smooth muscle progenitor cells without affecting lesion growth and vessel stenosis. CXCL12 treatment mobilizes smooth muscle progenitor cells into the circulation and upregulates CXCL12 expression in atherosclerotic arteries, which may play an important role in the lesion-specific recruitment of smooth muscle progenitor cells. Moreover, our findings indicate that the upregulation of CXCL12 expression during arteriosclerosis has a strong atheroprotective effect. Together, a transient treatment course with CXCL12 appears to be a promising therapeutic strategy in patients with vulnerable atherosclerotic lesions.
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Arterioscler Thromb Vasc Biol. published online February 7, 2013;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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**Supplemental Figures**

**Figure I: Effects of CXCL12 treatment on SPC mobilization and CXCL12 levels.**

(A) Time course of the mobilization of Sca1^+Lin^- cells into the circulation after intravenous injection of 100 ng or 500 ng CXCL12 or PBS into C57BL/6 mice determined by flow cytometry. (B) Representative dot plots showing quantification of Sca1^+Lin^- cells at 4 h after injection of PBS or 500 ng CXCL12. (C) Quantification of Sca1^+Lin^-Pdgfr-β^+ cells in the peripheral blood at 4 h after injection of PBS or 500 ng CXCL12. (D) CXCL12 concentration in the plasma and the BM was measured at different time points following injection of 500 ng CXCL12. *P < 0.05; ***P < 0.0001; n = 3–4 mice per group.
Figure II: Partial ligation of the carotid artery (A) EVG-stained section of the carotid artery obtained from a stenotic area 6 weeks following partial ligation. Scale bar indicates 200 μm. (B) Quantification of EVG-stained lesions of the carotid arteries of Apoe<sup>−/−</sup> mice at 4 or 6 weeks after partial ligation. (C) 3D renderings of a micro-CT angiography scan of the aortic arch (Ao) and the right (RC) and left (LC) carotid arteries from Apoe<sup>−/−</sup> mice 6 weeks after partial ligation of the left carotid artery. Jugular vein, JV. (D) Luminal diameters of the right and left carotid arteries in Apoe<sup>−/−</sup> mice 6 weeks after partial ligation of the left carotid artery were determined in micro-CT angiography scans. ***P < 0.001, n = 4 mice per group.
Figure III: Lesional macrophage accumulation after partial carotid ligation.
The lesional Mac2$^+$ area was quantified in carotid lesions in Apoe$^{-/-}$ mice at 4 or 6 weeks after partial ligation. Scale bars indicate 100 μm. **$P < 0.01$, n = 3–4 mice per group.
Figure IV: SMC content in carotid plaques after partial ligation. SM22 (A), calponin (B), and α-SMA (C) immunopositive areas were determined in carotid lesions in Apoe<sup>−/−</sup> mice at 4 or 6 weeks after partial ligation. Scale bars indicate 100 μm. *P < 0.05, **P < 0.01, n = 3–4 mice per group.
Figure V: Experimental scheme of CXCL12 treatment. Partial ligation of the left carotid artery in Apoe<sup>−/−</sup> mice was performed on day 0 and mice were subsequently fed a HCD. CXCL12 (500 ng) or PBS treatment (intravenously, biweekly) was started in the third week after partial carotid ligation and continued for 4 weeks. Micro-CT angiography was performed at 6 or 8 weeks after partial carotid ligation, after which mice were sacrificed.
Figure VI: Effect of prolonged CXCL12 treatment on SPC mobilization.
Representative dot plots of circulating Sca-1^+Lin^- cells 4 h after the final injection of CXCL12 or PBS in Apoe^−/− mice that were subjected to partial carotid ligation and 4 weeks of treatment with CXCL12 or PBS. (B) Quantification of the level of circulating Sca-1^+Lin^- cells 4 h after the final injection of CXCL12 or PBS. *P < 0.05, n = 5–7 mice per group.
Figure VII: Effect of CXCL12 treatment on cell density in fibrous caps.
The ratio between the number of cells (as determined by counting the DAPI-stained nuclei) and the collagen area (as determined by immunostaining of collagen type I) in the fibrous cap region was studied in the lesions of carotid arteries of CXCL12- and PBS-treated Apoe<sup>−/−</sup> mice at 6 weeks after partial ligation. Arrows delineate the fibrous cap region. *P < 0.05, n = 4–5 mice per group.
Figure VIII: Effect of CXCL12 treatment on the proliferation of fibrous cap cells.
(A) Ki67 immunostaining of carotid artery lesions in CXCL12 and PBS-treated Apoe<sup>−/−</sup> mice at 6 weeks after partial ligation. (B) The percentage of Ki67<sup>+</sup> cells in the lesion fibrous caps was determined. *P < 0.05, n = 7 mice per group. Arrows indicate cells stained for Ki67. Scale bars are 100 µm.
Figure IX: Effect of CXCL12 treatment on cell apoptosis in carotid lesions.

(A) Representative images showing TUNEL+ cells in plaques in carotid arteries of PBS and CXCL12-treated Apoe−/− mice at 6 weeks after partial ligation. Arrows indicate TUNEL+ cells. Scale bars are 100 µm. (B) The percentage of TUNEL+ cells in carotid plaques of PBS- and CXCL12-treated mice was determined. n = 7 mice per group.
Figure X: The lesional SMC content is increased in CXCL12-treated mice.
The lesional SMC content in carotid lesions induced by partial ligation was studied in PBS- and CXCL12-treated Apoe−/− mice after 4 weeks of treatment by immunostaining of calponin (A, n = 6 mice per group) and α-SMA (B, n = 3–4 mice per group). Scale bars are 100 µm. *P < 0.05.
Figure XI: The number of macrophages in plaques is reduced in CXCL12-treated mice. (A) Dot plots illustrating the quantification of macrophages stained with an anti-F4/80 antibody in plaques of carotid arteries from PBS- and CXCL12-treated Apoe⁻/⁻ mice at 6 weeks after partial ligation as determined by flow cytometry. Propidium iodide⁺ cells were gated out to exclude dead cells. (B) Quantification of F4/80⁺ cells in the propidium iodide⁻ cell population of the carotid lesions in PBS- and CXCL12-treated Apoe⁻/⁻ mice. *P < 0.05, n = 4–5 mice per group.
Figure XII: Effect of CXCL12 treatment on lesional neutrophil and CD3$^+$ cell infiltration. (A) Neutrophil-specific esterase staining was performed to determine the lesional neutrophil content. Arrows indicate esterase-positive neutrophils. Scale bars are 50 µm. (B) The number of neutrophils per lesion were quantified in PBS- and CXCL12-treated mice after 4 weeks of treatment. n = 4 mice per group. (C) Immunostaining for CD3 (red) was performed to determine the lesional T-cell content. Arrows indicate CD3$^+$ cells. Nuclei were counterstained with DAPI (blue). Scale bars are 100 µm. (D) Quantification of the percentage of cells that were CD3$^+$ in carotid lesions induced by partial ligation in PBS- and CXCL12-treated Apoe$^{-/-}$ mice. n = 5–6 mice per group.
Figure XIII: Effect of CXCL12 treatment on intra-lesion neovascularization.
Representative images of CD31 immunostaining of sections from carotid arteries at 6 weeks after partial ligation in PBS- and CXCL12-treated Apoe<sup>−/−</sup> mice. Quantification of CD31<sup>+</sup> neovessels in the carotid lesions. Scale bars are 100 µm. n = 4 mice per group.
Figure XIV: BM-derived SPCs differentiate into SMCs in lesions.
Double immunostaining of β-galactosidase (β-gal) and endothelial-specific CD31 (A), SMC-specific calponin (Calp) (B), and macrophage-specific F4/80 (C) was performed in lesions of the carotid artery at 6 weeks after partial ligation in Apoe<sup>−/−</sup> mice that received SM22-LacZ BM cells and were treated with CXCL12. Scale bars are 20 µm.
Figure XV: Rates of apoptosis of SPCs and non-SPC-derived lesional cells.
Apoptosis rates of lesional β-galactosidase (β-gal)$^+$ and β-galactosidase$^-$ cells were determined in carotid lesions at 6 weeks after partial ligation and treatment with CXCL12 by staining of apoptotic cells using the TUNEL assay and β-galactosidase immunostaining. Arrow indicates a TUNEL$^+$ cell. Scale bar is 50 µm. *$P < 0.05$, n= 3 mice per group.
Figure XVI: Screening for malignancies in mice after systemic CXCL12 treatment. Images are of $Apoe^{-/-}$ mice 2 weeks after completion of a 4-week course of CXCL12 treatment and 8 weeks after partial carotid ligation. Micro-CT scans of the brain (A), liver (B) and lung (C). Representative H&E staining of sections from the kidney (D), spleen (E), and intestine (F). Scale bars indicate 500 µm (main figure) and 200 µm (inset).
## Supplemental Tables

Table I: Number of differentiated blood cells in C57BL/6 mice at 4 hours after injection of PBS or CXCL12

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>CXCL12 (100 ng)</th>
<th>CXCL12 (500 ng)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells (×10³/µl)</td>
<td>2.5 ± 0.6</td>
<td>3.3 ± 0.6</td>
<td>4.0 ± 0.7</td>
<td>0.3282</td>
</tr>
<tr>
<td>Platelets (×10³/µl)</td>
<td>560.3 ± 54.3</td>
<td>610.3 ± 55.15</td>
<td>457.7 ± 158.9</td>
<td>0.5880</td>
</tr>
<tr>
<td>Lymphocytes (×10³/µl)</td>
<td>1937 ± 528.2</td>
<td>2498 ± 482.8</td>
<td>3119 ± 803.1</td>
<td>0.4529</td>
</tr>
<tr>
<td>Neutrophils (×10³/µl)</td>
<td>430.0 ± 96.0</td>
<td>637.3 ± 125.3</td>
<td>492.0 ± 93.3</td>
<td>0.4185</td>
</tr>
<tr>
<td>Monocytes (×10³/µl)</td>
<td>96.7 ± 6.4</td>
<td>80.0 ± 2.3</td>
<td>131.3 ± 76.2</td>
<td>0.7173</td>
</tr>
<tr>
<td>Eosinophils (×10³/µl)</td>
<td>36.3 ± 16.4</td>
<td>70.33 ± 26.1</td>
<td>89.0 ± 34.2</td>
<td>0.4196</td>
</tr>
</tbody>
</table>

n = 3 mice per group; data represent means ± SEM.
Table II: Number of circulating white blood cells and leukocyte subtypes in Apoe<sup>−/−</sup> mice at 6 weeks after partial ligation and 4 weeks of treatment with CXCL12 or PBS

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>CXCL12 (500 ng)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell count ($\times 10^3/\mu l$)</td>
<td>6.825 ± 0.4</td>
<td>5.375 ± 1.75</td>
<td>0.4488</td>
</tr>
<tr>
<td>Lymphocyte count ($\times 10^3/\mu l$)</td>
<td>4923 ± 486.6</td>
<td>3791 ± 1409</td>
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</tr>
<tr>
<td>Neutrophil count ($\times 10^3/\mu l$)</td>
<td>1268 ± 303.2</td>
<td>1128 ± 132.9</td>
<td>0.6860</td>
</tr>
<tr>
<td>Monocyte count ($\times 10^3/\mu l$)</td>
<td>375.5 ± 82.68</td>
<td>258.0 ± 114.2</td>
<td>0.4365</td>
</tr>
<tr>
<td>Eosinophil count ($\times 10^3/\mu l$)</td>
<td>258.5 ± 93.81</td>
<td>198.0 ± 100.5</td>
<td>0.6753</td>
</tr>
</tbody>
</table>

n = 4 mice per group; data represent means ± SEM.
Table III: Serum cholesterol and triglyceride levels in *Apoe*−/− mice at 6 weeks after partial ligation and 4 weeks of PBS or CXCL12 treatment

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>CXCL12 (500 ng)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>18.06 ± 1.9</td>
<td>18.87 ± 1.4</td>
<td>0.7393</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>0.94 ± 0.1</td>
<td>1.38 ± 0.5</td>
<td>0.4501</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>0.94 ± 0.1</td>
<td>1.38 ± 0.5</td>
<td>0.4501</td>
</tr>
</tbody>
</table>

n = 5-6 mice per group; data represent means ± SEM.
Material and Methods

Mice
C57BL/6 mice (Janvier) were injected with CXCL12 (100 ng or 500 ng, PeproTech) or PBS via the tail vein, and blood was drawn to quantify the Sca-1^−Lin^− PDGFR-β^+ cells.

Partial ligation of the left carotid artery was performed in 6–8-week-old male Apoe^−/− mice. Briefly, a ventral midline incision was made in the neck, and the left carotid artery was exposed. The left external and internal carotid and occipital arteries were ligated to allow blood outflow only via the superior thyroid artery. Following partial ligation, the mice were fed a high-cholesterol diet (HCD) (0.15% cholesterol, Altromin, Lage, Germany) for 6 weeks. Biweekly injections of CXCL12 (500 ng) or PBS via the tail vein were started 2 weeks after ligation. To study the long-term effects of CXCL12, some mice were kept for an additional 2 weeks following the termination of CXCL12 treatment. To assess the role of lesional CXCL12 expression, Apoe^−/− mice were perivascularly treated with a siRNA targeting CXCL12 or a non-targeting siRNA (once weekly, 4 nmol, dissolved in pluronic gel; Dharmaco) during the treatment with CXCL12. Perfusion fixation with paraformaldehyde (4%) was performed at 6 or 8 weeks after partial carotid ligation. All animal experiments were reviewed and approved by the local authorities in accordance with German animal protection law.

Flow cytometry
Smooth muscle cell progenitor cells (SPCs) were quantified after erythrocyte lysis (BD FACS™ lysing solution) in anti-coagulated blood by incubation with an allophycocyanin (APC)-conjugated mouse anti-PDGFR-β/CD140b antibody (eBioscience), a phycoerythrin-conjugated anti-Sca1 (Ly-6A/E) antibody (clone D7, eBioscience) and the biotinylated Mouse Hematopoetic Lineage Flow Panel (CD3, CD45R, CD11b, Ter-119, Ly-6G, CA 88-7774, eBioscience), followed by a streptavidin-phycocythrin-Cy7 conjugate. Sca1^+ Lin^− PDGFR-β^+ cells were quantified by flow cytometry (FACS Canto™ II BD Biosciences) in a mononuclear cell gate.

To quantify lesional macrophages, ligated carotid arteries was harvested after perfusion with ice-cold PBS and kept in RPMI-1640 medium on ice until used. Excised carotid arteries were digested with Liberase™ (100 µg/ml; diluted in RPMI-1640, Roche) for 1 h at 37°C in a shaking incubator. Fetal calf serum was added to stop the digestion, the sample was gently pipetted, and a cell suspension was obtained. The cell suspension was passed through a cell strainer (70 µm, BD Falcon) and stained with a macrophage-specific APC-conjugated anti-F4/80 antibody (clone BM8, eBioscience) for 30 min. Dead cells were identified by propidium iodide (eBioscience) staining and were excluded from the analysis. Data were analyzed using FlowJo software (TreeStar).
ELISA
Plasma was obtained from blood that was anti-coagulated with EDTA by centrifugation at 1000 g for 10 min at 4°C. To measure the level of CXCL12 in the BM, the femurs of C57BL/6 mice were flushed with 500 µl of ice-cold PBS, the eluent was centrifuged at 1000 g for 10 min at 4°C, and the supernatant was used for analysis. The CXCL12 concentration was determined by ELISA (mouse CXCL12 ELISA kit, Ray Biotech, INC) according to the manufacturer’s protocol. The absorbance was measured at 450 nm with a microplate reader (SPECTRAFluor Plus, Tecan).

Micro-CT angiography
To determine the luminal diameters of carotid arteries, Apoe<sup>−/−</sup> mice were scanned by <i>in vivo</i> dual-energy micro-CT (TomoScope DUO, CT Imaging, Erlangen, Germany) 6 weeks after partial carotid ligation<sup>6</sup>. A blood-pool contrast agent was injected intravenously, and the luminal diameters of the common carotid arteries were quantified within 1 mm of the bifurcation using the Imalytics Research workstation (Philips Technologie GmbH, Aachen, Germany). 3D renderings of the carotid arteries were created using Definiens Developer XD software (Definiens, Munich, Germany).

To screen CXCL12-treated mice for malignancies, micro-CT scans of Apoe<sup>−/−</sup> mice were performed 8 weeks after partial ligation of carotid arteries. The vasculature was visualized by a dual-energy scan directly after injection of an iodine-based contrast agent (ExI A 160 XL, Binitio Biomedical, USA). In addition, a whole-body scan (for each X-ray source, 720 projections were acquired at 65 kV and 0.5 mA) was performed 1 h after injection of the contrast agent to image the liver, brain, lung, and lymph nodes. 3D datasets were reconstructed at an isotropic voxel size of 35 µm using smooth and sharp reconstruction kernels.

BM transplantation
The femurs and tibias of SM22-LacZ/Apoe<sup>−/−</sup> mice (C57BL/6 background) were aseptically excised and flushed with ice-cold, sterile PBS and filtered through a cell strainer (40 µm, BD Falcon). Approximately 1–2 million donor cells were injected via the tail vein into 6–8-week-old Apoe<sup>−/−</sup> mice 24 h after whole-body irradiation with an ablative dose (2x6 Gy). Partial ligation of the left carotid artery was performed 3 weeks after BM transplantation.

Histology
Histomorphometric quantification of the lesional area in serial sections (5 µm thickness, six sections per mouse) of the paraffin-embedded left carotid artery between 200 µm and 1 mm from the bifurcation was performed after staining with EVG. Neutrophils were detected by specific esterase staining (Naphthol AS-D chloroacetate, Sigma). β-galactosidase activity was determined after <i>in situ</i> perfusion with 2% paraformaldehyde by incubating the carotid arteries in X-gal staining
buffer (PBS containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate and 0.01% NP-40, pH 7.4) containing 1 mg/ml X-gal (Invitrogen) overnight at 37°C. Carotid arteries were then embedded in OCT (Optimal Cutting Temperature, Tissue-Tek, Sakura), and cryosections were obtained. Restaining with X-gal (overnight at 37°C) was performed on frozen sections, and nuclei were counterstained with nuclear fast red (Vectorlabs).

**Immunostaining**
Quantitative immunofluorescence staining of SMCs (SM22, rabbit polyclonal, Abcam; α-SMA, mouse monoclonal, Dako; calponin, clone EP798Y, rabbit monoclonal, Abcam), macrophages (Mac-2, clone M3/38, Cedarlane), collagen type I (rabbit polyclonal, Cedarlane), CD31 (goat polyclonal, Santa Cruz), Ki67 (clone TE3, rat monoclonal, Dako) and T-cells (CD3, rabbit polyclonal, Dako) was performed on paraffin-embedded sections. Anti-rabbit Dylight 549-conjugated IgG (goat, KPL, USA), anti-rat FITC-conjugated IgG (donkey, Jackson immunoResearch), and anti-rabbit Cy3-conjugated IgG (donkey, Jackson ImmunoResearch) were used as detection antibodies. Apoptotic cells were detected using the TUNEL assay (Roche) in accordance with the manufacturer’s instructions. Immunostaining for β-galactosidase (rabbit polyclonal, Abcam) was performed on cryosections after heat-mediated antigen retrieval in citrate buffer and incubation with a secondary Dylight 549-conjugated antibody (KPL, USA). The percentage of the lesion area that was positively stained for SM22, α-SMA, calponin, Mac-2, or collagen type I was determined using image analysis software (ImageJ), setting the threshold based on the background of negative control staining. Immunostaining results for Ki67, CD3, β-galactosidase, and apoptotic cells were analyzed by counting the number of Ki67⁺, CD3⁺, β-galactosidase⁺, and TUNEL⁺ cells in the lesions, relative to the number of DAPI-stained nuclei and are expressed as the percentage of the total number of lesional cells. CD31⁺ neovessels were counted within the lesion area and are expressed as the absolute number per lesion.

Double immunostainings for CXCL12 (clone 79018, R&D Systems) and the endothelial cell marker CD31 (goat polyclonal, Santa Cruz) were performed by sequential incubation with the primary antibodies. Similarly, co-immunostaining for β-galactosidase with CD31, the SMC-specific marker calponin and macrophage-specific F4/80 was performed on cryosections by sequential incubation with primary antibodies. Primary antibodies were detected using fluorescently-conjugated secondary antibodies. Non-specific primary antibodies served as negative controls. Digital images were recorded using a fluorescence microscope (Leica DMLB microscope) connected to a CCD camera (JVC) using DISKUS software (Hilgers).

**Blood cell counting and lipid analysis**
Serum samples were analyzed by dry chemistry using a Vitros 250 Analyzer (Ortho Clinical Diagnostics). The blood cell count was determined using an automated hematology analyzer (Celltac MEK-6450K, Nihon Kohden). The differential leukocyte count was obtained manually by a blood film.
**Cell culture**

Endothelial cells were isolated from the aorta of C57BL/6 mice and cultured as described previously. Stimulation with CXCL12 (100 ng/ml) or PBS was performed and RNA isolated after 2 h.

**Statistical analysis**

Data represent the means ± SEM and were compared using a one-way or two-way ANOVA followed by the Newman-Keuls or Bonferroni post-test, respectively, or an unpaired two-tailed Student t-test (Prism, GraphPad, version 5). $P < 0.05$ was considered statistically significant.

**References:**


