Protective Role of CXC Receptor 4/CXC Ligand 12 Unveils the Importance of Neutrophils in Atherosclerosis

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Abstract—The CXC ligand (CXCL)12/CXC receptor (CXCR)4 chemokine–receptor axis controls hematopoiesis, organ development, and angiogenesis, but its role in the inflammatory pathogenesis of atherosclerosis is unknown. Here we show that interference with Cxc12/Cxcr4 by a small-molecule antagonist, genetic Cxcr4 deficiency, or lentiviral transduction with Cxcr4 degrakine in bone marrow chimeras aggravated diet-induced atherosclerosis in apolipoprotein E-deficient (Apoe−/−) or LDL receptor–deficient (Ldlr−/−) mice. Chronic blockade of Cxcr4 caused leukocytosis and an expansion of neutrophils and increased neutrophil content in plaques, associated with apoptosis and a proinflammatory phenotype. Whereas circulating neutrophils were recruited to atherosclerotic lesions, depletion of neutrophils reduced plaque formation and prevented its exacerbation after blocking Cxcr4. Disrupting Cxcl12/Cxcr4 thus promotes lesion formation through deranged neutrophil homeostasis, indicating that Cxcl12/Cxcr4 controls the important contribution of neutrophils to atherogenesis in mice (Circ Res. 2008;102:209-217.)

Key Words: atherosclerosis ■ cardiovascular disease ■ chemokines ■ leukocytes ■ vascular inflammation

Chemokines are small chemotactic peptides instrumental in attracting leukocytes and progenitor cells to specific tissues.1,2 Whereas most inducible chemokines regulate inflammatory processes, the CXC chemokine stromal cell–derived factor-1α/CXCL12 is more constitutively and ubiquitously expressed and crucial for the engraftment, homeostasis, and mobilization of bone marrow (BM) cells in their stromal niche.3–5 Accordingly, short-term disruption of the CXCL12/CXCR4 ligand–receptor axis induces a release of hematopoietic stem cells and leukocytes including neutrophils from the BM.6,7 Mice deficient in Cxcl12 or Cxcr4 die in utero, displaying severe defects in B lymphopoiesis, myelopoiesis, embryonic organ vascularization, and cardiovascular8,9 Hematopoietic growth factors can mediate deployment of Cxcl12 from platelets or its secretion from perivascular fibroblasts, supporting neovascularization through recruitment and/or retention of CXCR4+ hemangiocytes or accessory cells.10,11 After arterial injury, Cxcl12 expression in smooth muscle cells (SMCs) and its presentation by platelets have been implicated in the recruitment of circulating progenitor cells to neointimal lesions.12,13 Conversely, neutralization of Cxcl12 or deficiency in BM-Cxcr4 inhibited SMC progenitor recruitment and neointimal hyperplasia in Apoe−/− mice.12,13 An upregulation of Cxcl12 has been observed in transplant arteriopathy or in hypoxia, and blocking Cxcl12 or Cxcr4 reduced neointima formation and SMC progenitor recruitment in transplant arteriosclerosis or during neovascularization of ischemic tissues.10,11,14

The expression of CXCL12 is also detectable in primary atherosclerotic plaques,15 and reduced CXCL12 plasma levels have been associated with unstable coronary artery disease, suggesting antiinflammatory or plaque-stabilizing properties of CXCL12.16 Owing to the embryonic lethality after genetic deletion, a role of Cxcl12/Cxcr4 in the development, stability, and cellular homeostasis of primary atherosclerosis has not yet been studied in vivo. Here we used the specific CXCR4 antagonist AMD3465 and Apoe−/− mice reconstituted with BM deficient in Cxcr4 or Ldlr−/− mice lentivirally transduced with Cxcr4 degrakine to dissect compartmental contributions of Cxcr4 to diet-induced atherosclerosis.

Materials and Methods

For details, see the online data supplement (including supplemental Figure I), available at http://circres.ahajournals.org. Apoe−/− and Ldlr−/− mice17,18 (both C57BL/6 background) were fed an atherogenic diet. Apoe−/− mice received osmotic pumps for continuous treatment with AMD3465 or PBS, were treated with neutrophil-depleting anti-polymorphonuclear leukocyte (PMN) antibody (Ab) or isotype control, or were reconstituted with Cxcr4−/−, wild-type (WT), or lys-EGFP+ BM. Ldlr−/− mice were reconstituted with BM...
transduced with LV.Empty or LV.CXCR4deg. The extent of atherosclerosis was assessed by staining aortic roots and thoracoabdominal aortas for lipid deposition using oil red O. Immunohistochemistry/immunofluorescence/transmission electron microscopy was performed on aortic roots. Aortic tissue was used for RT-PCR analysis. Neutrophils isolated from BM or peripheral blood were used in respiratory burst, phagocytosis, and calcium mobilization assays or labeled with fluorescent beads for adoptive transfer in Apoe<sup>−/−</sup> mice.

**Results**

**Interference With Cxcr4/Cxcl12 Aggravates Atherosclerosis**

To explore the role of Cxcr4 in atherosclerosis, Apoe<sup>−/−</sup> mice fed an atherogenic diet were continuously treated with the CXCR4 antagonist AMD3465 or vehicle (controls) via osmotic minipumps for 12 weeks (n=6 each). a. Atherosclerotic plaques were quantified in the aortic root (left) and thoracoabdominal aorta (right) after oil red O staining. Apoe<sup>−/−</sup> mice reconstituted with WT (n=6) or Cxcr4<sup>−/−</sup> (n=3) BM were fed a high-fat diet for 12 weeks. b. Oil red O–stained plaques were quantified in the aortic root (left) and thoracoabdominal aorta (right). Ldlr<sup>−/−</sup> mice were repopulated with BM transduced with empty vector (n=10) or CXCR4 degrakine (n=11), and aortic root plaques were stained using oil red O, a through c (middle), Representative images. d through f, The relative content of MOMA-2<sup>+</sup> macrophages (d) or SMCs (e) and CD3<sup>+</sup> T-cell numbers per plaque area (%) were determined by immunofluorescence microscopy. *P<0.05, **P<0.01, ***P<0.005.

Analysis of the cellular plaque composition by quantitative immunofluorescence revealed that the relative content of MOMA-2<sup>+</sup> macrophages was slightly increased, whereas smoothelin<sup>+</sup> SMC content was significantly decreased in aortic roots of AMD3465-treated Apoe<sup>−/−</sup> mice or Ldlr<sup>−/−</sup> chimeras with Cxcr4 degrakine-transduced BM versus controls (Figure 1d and 1e and supplemental Figure IIa and IIb). Moreover, AMD3465 treatment reduced the number of CD3<sup>+</sup> T cells in aortic root plaques (Figure 1f). Similarly, AMD3465 treatment did not alter the relative content of macrophages (97.2±0.9% versus 94.6±0.7% in controls) or CD3<sup>+</sup> T cells (2.0±0.6% versus 1.1±0.5% in controls) among all inflammatory plaque cells. Although mast cells could not be detected within plaques, their numbers did not differ between control- and AMD3465-treated Apoe<sup>−/−</sup> mice in the aortic root adventitia (36.9±12.9 versus 28.0±6.7 cells/mm<sup>2</sup>, respectively; supplemental Figure Iic). Thus, enlarged atherosclerotic plaques of AMD3465-treated mice were not attributable to an expansion in the content of cell types commonly incriminated in atherogenesis but possibly attributable to an increase in amorphous material.

**Effects of AMD3465 on Leukocyte Homeostasis**

Disruption of the CXCR4/CXCL12 axis has been described to induce a generalized leukocytosis and mobilization of hematopoietic progenitor cells, and short-term bolus treatment with the CXCR4 antagonist AMD3100 causes an acute release of neutrophils from the BM. Here we monitored long-term effects of antagonizing Cxcr4 on blood cell counts. Whereas the mobilization of lineage<sup>−</sup>sca-1<sup>+</sup> progenitors peaked at 3 days after initiating AMD3465 treatment, their percentage remained mildly elevated as compared with base-
line or controls at 10 days (Figure 2a). Continuous treatment of Apoe<sup>−/−</sup> mice with AMD3465 induced a pronounced peripheral blood leukocytosis within 2 days, which was sustained throughout the study period (Figure 2b), and an expansion in the relative number of circulating neutrophils, which further increased over time (Figure 2c and 2d). This was accompanied by an appearance of more immature band neutrophils in peripheral blood (6.1±2.1% with AMD3465 treatment versus 2.5±1.0% in controls at 21 days; *P*<0.05). Notably, relative neutrophil numbers increased during diet-induced disease progression per se in Apoe<sup>−/−</sup> mice (Figure 2c). A moderate elevation of relative monocyte numbers was in line with previous findings<sup>22</sup> and only slightly further increased (supplemental Figure IIIa), whereas relative numbers of lymphocytes were reduced in AMD3465-treated Apoe<sup>−/−</sup> mice (50.3±11.2% versus 67.5±2.4% in controls at 21 days; *P*<0.05). In addition, elevated leukocyte counts occurred in Cxcr<sup>4−/−</sup> versus WT mice in association with an increase in peripheral blood neutrophils but not monocytes (Figure 2e and supplemental Figure IIIb and IIIc).

We further analyzed the cellular composition in the BM. As for peripheral blood, the relative number of neutrophils was significantly increased, whereas the relative number of lymphocytes was reduced and that of monocyte precursors was unaffected by AMD3465 treatment in Apoe<sup>−/−</sup> mice (Figure 2f and supplemental Figure IIIId and IIIe). Accordingly, AMD3465 treatment for 10 weeks increased the number of enhanced green fluorescent protein (EGFP)<sup>+</sup> BM neutrophils in chimeric Apoe<sup>−/−</sup> mice repopulated with BM expressing EGFP under control of the lysozyme M locus (lys-EGFP) in myelomonocytic cells, especially in mature neutrophils (Figure 2g).<sup>23</sup>

**Effects of AMD3465 on Peripheral Neutrophil Migration and Function**

Peripheral blood neutrophils mobilized by AMD3465 did not differ in Cxcr4 expression from that of controls, indicating that the release of neutrophils from the BM does not rely on Cxcr4 downregulation (data not shown). The phagocytic capacity of neutrophils isolated from peripheral blood or BM was unaffected in AMD3465-treated mice, as assessed by flow cytometric analysis of microparticle internalization (91.2±2.2% versus 85.6±5.7% microparticle<sup>+</sup> cells in peripheral blood; 85.6±2.1% versus 87.1±0.8% in BM) or microscopy of cytospins (data not shown). The phorbol 12-myristate 13-acetate–induced production of reactive oxygen species (ROS) by neutrophils was not compromised in AMD3465-treated mice (1.8±0.5-fold increase versus 1.1±0.1-fold in controls). The calcium influx induced by CXCL1 at different concentrations and the adhesion on tumor necrosis factor–activated microvascular endothelial cells under flow conditions in vitro did not differ between neutrophils from AMD3465- versus control-treated Apoe<sup>−/−</sup> mice or between human neutrophils pretreated with or without AMD3465 (data not shown and supplemental Figure IIIId and IIIg). Thus, AMD3465-mobilized neutrophils are functional with preserved phagocytic and adhesive capacity, calcium signals, and respiratory burst.

**Neutrophil Recruitment Into Atherosclerotic Lesions**

The contribution of neutrophils to atherogenesis has not been conclusively explored to date. Because an increase in circulating neutrophils may entail their accumulation in lesions of AMD3465-treated Apoe<sup>−/−</sup> mice, we evaluated the neutrophil content in aortic root plaques by immunostaining for specific esterase. The number of neutrophils was significantly increased in aortic root plaques and adventitia of AMD3465-treated Apoe<sup>−/−</sup> mice (Figure 3a and 3b), concomitant with an increase in the relative content of neutrophils among all inflammatory plaque cells (4.2±0.2% versus 0.9±0.4% in controls; *P*<0.001) and in the levels of neutrophil elastase mRNA (supplemental Figure IVA). The presence of neutrophil granulocytes with segmented nuclei and typical morphology was also evident by hematoxylin/eosin staining and transmission electron microscopy of plaques (Figure 3c and 3d). This was corroborated by increased neutrophil numbers in aortic root plaques of Apoe<sup>−/−</sup> chimeras with lys-EGFP<sup>+</sup>.
BM after 10 weeks of AMD3465 treatment. Highly EGFP+ neutrophils were negative for the macrophage marker MOMA-2 (Figure 3e). The recruitment of circulating neutrophils was investigated in adoptive transfer experiments. Isolated neutrophils were labeled with fluorescent latex beads (LX), yielding an efficiency of 13.7±0.1% and a specificity of 94.4±1.1% (Figure 3f, left), and transferred into Apoe−/− mice with established plaques after 8 weeks of diet. Indeed, LX+ neutrophils were detectable within lesions of the aortic root and arch (Figure 3f), confirming their recruitment from the circulation. In line with findings that Cxcr4 is required for the return of senescent neutrophils to the BM, fewer LX+ neutrophils were seen in the BM of AMD3465-treated Apoe−/− mice versus controls 3 days after transfer (Figure 3g), whereas splenic sequestration did not differ (1.7±0.4% versus 1.5±0.1% LX+ splenocytes). To identify receptors crucial for neutrophil recruitment to plaques after systemic blockade of Cxcr4, we performed adoptive transfer of Cxcr2−/− LX+ neutrophils. Absence of Cxcr2 impaired LX+ neutrophil recruitment (0.4±0.4% of sections LX+ versus 3.9±1.8% in controls), indicating that neutrophils use Cxcr2 to enter plaques.

**AMD3465 Enhances Plaque Inflammation and Alters the Immune Balance**

Given the presence of neutrophils in atherosclerotic lesions, we analyzed the expression of inflammatory mediators. Myeloperoxidase (MPO) stored in neutrophil granules has been correlated with cardiovascular disease.25,26 Whereas few Ly-6G+ neutrophils were found in colocalization with MPO in lesions of controls, multiple neutrophils that stained for MPO were detectable in AMD3465-treated Apoe−/− mice (Figure 4a). Similarly, abundant expression of neutrophil gelatinase-associated lipocalin (NGAL) colocalized with matrix metalloproteinase (MMP)-9 in aortic roots of AMD3465-treated Apoe−/− mice but not controls (Figure 4b and 4c).
addition, transcript levels for proinflammatory interferon (IFN)-γ, the Cxcr2 ligand Cxcl1, and tissue factor but not C3 complement were substantially upregulated in aortas of AMD3465-treated Apoe−/− mice (supplemental Figure IVb and IVc). Notably, the number of lesional apoptotic TUNEL+ cells, particularly in luminal plaque areas, and the size of necrotic cores in atherosclerotic lesions was markedly increased in AMD3465-treated Apoe−/− mice (Figure 5a through 5c). In these mice, double immunofluorescence revealed costaining of TUNEL+ cells with Ly-6G+ neutrophils and to a lesser extent with MOMA-2+ macrophages (Figure 5d). The ratio of macrophages within superficial plaque layers (<40 μm) relative to the more central plaque areas was 1.4±0.6 in controls versus 2.9±0.7 in AMD3465-treated Apoe−/− mice, indicating an augmented recruitment of monocytes subsequent to neutrophils into luminal areas. These data imply that neutrophil accumulation may promote plaque growth and a more vulnerable phenotype. To examine whether blocking Cxc4 with AMD3465 alters systemic immune responses, the intracellular expression of IFN-γ and interleukin (IL)-10 was analyzed by flow cytometry in CD4+ iliac lymph node cells or splenocytes, revealing a shift toward cells expressing the proinflammatory cytokine IFN-γ in AMD3465-treated Apoe−/− mice (Figure 5e and 5f).

Depletion of Neutrophils Protects From Exacerbated Atherosclerosis
To examine whether neutrophil mobilization and recruitment causally contributes to lesion progression, Apoe−/− mice were treated with an Ab to neutrophils (anti-PMN Ab),27 which was verified to reduce circulating neutrophil counts in peripheral blood within 3 hours (Figure 6a) and during chronic administration after 4 weeks without affecting monocyte counts (6.0±0.8% versus 6.0±1.5% in control mice). No bacterial infections were observed during long-term PMN depletion. Treatment of Apoe−/− mice fed an atherogenic diet with anti-PMN Ab for 4 weeks impaired plaque development in aortic roots (Figure 6b and 6c), clearly demonstrating that neutrophils promote atherogenesis in Apoe−/− mice. Interestingly, analysis of the cellular plaque composition revealed that the relative macrophage content was reduced in plaques of anti-PMN Ab-treated Apoe−/− mice (23.3±5.0% versus 36.8±3.9% in controls; *P=0.055), corroborating a functional link to neutrophils, which precede macrophage recruitment.
Moreover, neutrophil depletion with anti-PMN Ab for 4 weeks reversed the exacerbation of atherosclerotic lesion formation in AMD3465-treated ApoE^{-/-} mice (Figure 6d and 6e). This indicates that a derangement of neutrophil homeostasis with increased numbers of circulating cells is a crucial mechanism accounting for the aggravation of atherosclerosis following interference with Cxcr4.

**Discussion**

Our data demonstrate that long-term disruption of the Cxcr4/Cxcl12 axis by the small molecule antagonist AMD3465 or deficiency in BM Cxcr4 aggravated diet-induced atherosclerotic lesion development in ApoE^{-/-} mice and Ldlr^{-/-} mice. Interfering with Cxcr4 caused a severe unbalance in hematopoiesis with an expansion of BM neutrophils and their mobilization into peripheral blood. These neutrophils can be recruited to atherosclerotic lesions, leading to secretion of inflammatory mediators and thereby promote plaque growth and instability. By demonstrating that a depletion of circulating neutrophils reduced diet-induced plaque formation and prevented its exacerbation following Cxcr4 blockade, we provide conclusive evidence that neutrophils crucially contribute to atherogenesis and identify a protective role for Cxcr4/Cxcl12 through controlling neutrophil homeostasis and their recruitment to plaques.

Chronic inflammation is recognized as a key force driving the development of atherosclerosis, and epidemiological studies have correlated peripheral blood leukocyte counts with coronary artery disease. However, neutrophils as the first line of immune defense have to date not been implicated in atherosclerosis, as mononuclear cell infiltrates of plaques almost exclusively contain macrophages and T cells. Conversely, activated neutrophils have been identified at sites of plaque rupture and erosion in patients with acute coronary syndromes. Here we have made the surprising discovery that neutrophils are detectable within diet-induced atherosclerotic lesions of ApoE^{-/-} mice, predominantly localized in luminal plaque regions but also in adventitial layers. Correlating with lesion size, neutrophils were clearly more abundant in plaques of AMD3465-treated ApoE^{-/-} mice. Adaptively transferred LX^{+} neutrophils were actively recruited to atherosclerotic lesions from the circulation, implying that influx of neutrophils can occur in chronically inflamed arteries. Notably, the depletion of circulating neutrophils attenuated plaque formation in ApoE^{-/-} mice and prevented AMD3465-induced lesion progression, indicating an active involvement of neutrophils in atherogenesis and disease exacerbation. This collides with findings that neutrophils can serve as predictors of complex coronary stenosis or myocardial infarction and that the appearance of band neutrophils in peripheral blood (‘left shift’) correlates with coronary atherosclerosis.

Neutrophils endocytose foreign material, produce potent ROS, and release a variety of proteolytic enzymes, such as elastase, and MPO, which help to clear infections but can also participate in tissue degradation and destruction. The levels of MPO and its increased activity in patients with a genetic polymorphism, have been associated with cardiovascular disease. Here we show that MPO expression in plaques of ApoE^{-/-} mice was colocalized with neutrophils and substantially increased by AMD3465 treatment. In parallel, neutrophil MMPs NGAL and MMP-9 were markedly upregulated. NGAL inhibits MMP-9 inactivation to augment its proteolytic activity and prolongs effects on collagen degradation and destabilization of the plaque architecture. Moreover, increased mRNA levels for tissue factor in aortas of AMD3465-treated mice may originate from neutrophils or monocytes and may contribute to atherosclerosis by triggering endothelial dysfunction. Although no differences in C3 mRNA levels were detected, likely reflecting unaltered complement-producing plaque macrophages in controls versus AMD3465-treated ApoE^{-/-} mice, C3-induced activation of neutrophils encompasses release of tissue factor and may thereby promote atherosclerosis.

In atherosclerotic lesions of AMD3465-treated ApoE^{-/-} mice, the number of apoptotic cells was increased particularly in lumen-near areas, possibly corresponding to a decay of infiltrating neutrophils or macrophages. Although the relative macrophage content was unaltered overall, their increase in superficial plaque regions of AMD3465-treated mice implies their enhanced recruitment and subsequent death. This could be explained by a function of neutrophils and their secretory products as a prerequisite for subsequent monocyte recruitment, which is supported by an inhibition of monocyte arrest in carotid arteries after neutrophil depletion in vivo and by sequential perfusion experiments, where neutrophils precede monocyte arrest (O.S., unpublished data). In contrast, signals required for cell migration or survival may be devoid when interfering with CXCR4. The overload of phagocytic capacities for the clearance of apoptotic cells by residual macrophages may in turn lead to secondary necrosis, promoting inflammation and plaque instability. Indeed, the substantial necrotic core in the plaques of AMD3465-treated ApoE^{-/-} mice displayed increased expression of proinflammatory IFN-γ, which may favor the development of vulnerable plaques by inhibiting SMC growth and collagen production, as reflected by reduced SMC and collagen content in these mice. AMD3465 treatment also entailed a shift toward systemic inflammation in lymph node cells and splenocytes, which is in line with findings that AMD3100 increased pulmonary IFN-γ expression in a model of allergic lung inflammation.

The Cxcl12/Cxcr4 axis is critical for retention and release of hematopoietic cells from the BM, and mice deficient in Cxcl12 or Cxcr4 show severe defects in BM myelopoiesis. The bicyclam Cxcr4 antagonist AMD3100 induces generalized leukocytosis, releasing hematopoietic progenitors into peripheral blood and has been used together with granulocyte colony-stimulating factor for autologous reconstitution of hematopoiesis after myeloablation. Although CXCR4 regulates the steady-state release of BM neutrophils into peripheral blood, where they exhibit moderate levels of CXCR4 expression and function, their high Cxcr2 expression entails a release and strong chemotactic activity toward inflammatory Cxcr2 ligands. Continuous steady-state exposure to Cxcl12 in the BM stromal niche leads to an incomplete desensitization, which is enhanced by heterologous stimulation with inflammatory Cxcl11 to induce neutrophil...
release. This antilogy has been refined by findings that CXCR2-mediated chemotaxis of neutrophils and their mobilization from BM is impeded by CXCL12 but enhanced by blocking CXCR4. This is also relevant for neutrophil release from the BM by CXcr4 antagonism with the macrolytic N-pyridinylmethylene cyclam AMD3465, which is 10-fold more effective than AMD3100, or in Cxcr4−/− mice. Surface levels of Cxcr4 were unaltered in AMD3465-mobilized neutrophils, in line with findings that CXCR4 expression and function did not differ in AMD3100-mobilized and steady-state BM cells, indicating that mobilization did not depend on CXCR4 cleavage or downregulation. Likewise, adhesion receptors crucial for vascular recruitment were unaffected in AMD3100-mobilized cells. Thus, AMD3465 may synergize with inflammatory chemokines, eg, Cxcl1, expressed in atherosclerotic lesions to potentiate neutrophil release from the BM. Subsequently, their recruitment into plaques may be mediated by Cxcr2, implying that CXCR2 may modulate atherosclerosis through several pathways, including neutrophil, as well as monocyte recruitment. During senescence, neutrophils upregulate CXCR4 for CXCL12-dependent homing to the BM. Indeed, BM homing of LX− neutrophils was diminished in AMD3465-treated Apoe−/− mice, adding to increased peripheral numbers. Given the atherogenic function of macrophage inhibitory factor (MIF) as a dual agonist of CXCR2 and CXCR4, the striking effects of MIF inhibition limiting athroprogession and even mediating plaque regression can be explained by blocking MIF actions on both Cxcr2 and Cxcr4. Thus, MIF is expected to retain a potent and predominant Cxcr2 activity in atherogenesis, when the Cxcr4/Cxcl12 axis is blocked.

Granulo- and lymphopoiesis may be coupled by developing in a common BM niche. This notion was corroborated by findings that an inflammatory reduction in CXCL12 can contribute to an expansion of immature, proliferating neutrophils replacing lymphocytic cells in the BM. Indeed, prolonged AMD3465 treatment led to an expansion of myeloid neutrophils and a slight increase in monocytes concomitant with a reduction of lymphocytes in the BM. Short-term AMD3100 treatment can increase the number of circulating angiogenic cells in peripheral blood. Here we confirm that AMD3465 acutely mobilized progenitor cells to the peripheral circulation. In Apoe−/− mice, transplantation of mononuclear BM cells accelerated atherosclerotic plaque progression in the context of hind limb ischemia, and transfer of BM or spleen-derived endothelial progenitor cells increased aortic lesion size and altered plaque composition, with larger lipid cores and thinner fibrous caps. Hence, caution seems warranted when attempting therapy with CXCR4 antagonists, which can mobilize hematopoietic cells but may inhibit the recruitment of plaque-stabilizing progenitor subsets and may thus promote atherosclerosis. An attenuation of lesion formation was seen in Apoe−/− mice transplanted with WT or Cxcr4−/− Apoe−/− BM, supporting the notion that the secretion of Apoe by BM-derived macrophages protects Apoe−/− mice from diet-induced atherosclerosis.

After vascular trauma and in hypoxic tissues, a delivery of CXCL12 by activated platelets and its expression in pericytes has been implicated in vascular repair and neoangiogenesis by attracting or retaining progenitors or myeloid assistance. After arterial injury in Apoe−/− mice or in transplant arteriopathy, interference of Cxcl12/Cxcr4 reduced neointimal hyperplasia by diminishing recruitment of BM-derived SMC progenitors to neointimal lesions as confirmed by reduced neointima formation in AMD3465-treated mice (A.S., unpublished data). Thus, protective mechanisms limiting neointimal hyperplasia contrast those underlying an exacerbation of primary atherosclerosis. Moreover, differences may arise from characteristics of vessel wall architecture, the extent of apoptosis, and shear stress exposure in smaller arteries versus the aorta.

Notably, reduced CXCL12 plasma levels have been associated with unstable coronary artery disease in a clinical study, suggesting antiinflammatory or plaque-stabilizing properties of CXCL12 in human atherosclerosis. These effects have been linked to a CXCL12-mediated attenuation of MMP-9 expression, which in turn is upregulated in plaques of AMD3465-treated mice. In conjunction, therapeutic interventions aimed at enhancing CXCL12 expression/activity may help to maintain plaque stability in acute coronary syndromes.

Here we have provided the first evidence that protective effects of the Cxcl12/Cxcr4 axis in atherosclerosis are attributable to the control of myeloid cell homeostasis. A mobilization of neutrophils, eg, in the course of systemic immune responses or inflammation, may initiate vascular damage during atherogenesis, whereas the continuous inflammation in the local plaque environment may exacerbate the mobilization, recruitment, and intraleisonal activity and eventually decay of neutrophils to aggravate disease progression in ill alliance with other inflammatory mediators. The interplay of CXCL12 with other chemokines and their receptors will have to be carefully scrutinized when devising strategies directed at the prevention of athroprogession and at targeting plaque destabilization in human disease. Notably, our data shift the current paradigm defining inflammatory atherogenesis by challenging and revising the long-standing and widely acknowledged belief that neutrophils are absent and of marginal relevance in atherosclerosis.

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Disclosures

None.

References


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Material and Methods

Lentivirus vector construction, production and cell transfection
The lentiviral expression vector pRRl-cPPT-CMV.CXCR4degrakine (LV.CXCR4deg) was constructed by inserting the CXCR4 degrakine construct into pRRl-cPPT-CMV-PreSIN (LV.Empty) by digestion from pHSPG-CXCL12-HA-VpuC (kindly provided by Dr. Su, University of North Carolina) and virus was produced using transient calcium phosphate co-transfection of 293T cells with the target gene vector together with pMDL/RRE, pRSV-REV and pVSV-G. Viral supernatants were passed through 0.45 µm filters and concentrated by ultracentrifugation. HEK293 cells were transduced with serially diluted viral supernatant, after 48 hours total genomic DNA was isolated and the number of vector DNA copies was determined using PCR analysis with pRRl-cPPT-CMV.PreSIN vector as calibration standard (primers: forward GTGCAGCAGCAGAACAATTTG, reverse CCCCAGACTGTGGATCAA). Transductions were performed by incubating murine BM or FDCP-mix hematopoietic stem cells with LV.Empty or LV.CXCR4deg (m.o.i. = 15) in DMEM with DEAE-dextran (10 µg/mL) at 37°C for 24 hours. The effectiveness of CXCR4 degrakine was confirmed in transduced FDCP-mix cells, which displayed a reduced adhesion to carotid arteries of Apoe<sup>-/-</sup> mice compared to empty vector-controls (Online Data Supplement Figure 1a).

Ex vivo perfusion
Carotid arteries were explanted from Apoe<sup>-/-</sup> mice after an atherogenic diet for 6 weeks, transferred onto the stage of an epifluorescence microscope (Zeiss Axiotech, 20x water immersion) and perfused at 4µL/min with calcein-AM labeled FDCP-mix cells transduced with empty vector control or CXCR4degrakine. Arrest was analyzed after 10 min of perfusion.

Mouse model of diet-induced atherosclerosis
Apoe<sup>-/-</sup> and Ldlr<sup>-/-</sup> mice (both C57BL/6 background) were bred in the local animal facilities. Apoe<sup>-/-</sup> mice were fed an atherogenic diet containing 21% fat (Altromin). Mice were anesthetized and implanted with a primed 28-day osmotic pump (model 2004, Alza Corp) subcutaneously.
placed via transverse midscapular incision for continuous treatment with AMD3465 (in PBS, pump-rate of 0.005mg/h; provided by AnorMED Inc., Langley, Canada) or sham-operation. Hearts, aortas and arteries were harvested by *in situ* perfusion fixation with 4% paraformaldehyde, 20 mM EDTA and 5% sucrose. *Cxcr4*+/− mice (C57BL/6 background) were kindly provided by Dr. D. Littman (New York University School of Medicine). Some *Apoe*−/− mice were treated with a neutrophil-depleting anti-PMN Ab (from Accurate Chemical or RB6-8C5, provided by O.S.) by daily *IP* injections of serum (100 µl) diluted in PBS. The efficiency of neutrophil depletion was confirmed by flow cytometry and differential blood smear counts. Cholesterol and triglyceride levels were determined by standard laboratory analysis and revealed no significant differences between any of the mouse groups investigated, neither did their weight differ (data not shown).

**Bone marrow repopulation**

BM transplantation was performed as described1,4. Embryonic livers of *Cxcr4*+/− mice were aseptically removed after 16 days and homogenized to gain *Cxcr4*+/+ and *Cxcr4*−/− fetal stem cell suspensions, as identified by genotyping. Femurs and tibias were aseptically removed from donor lysozyme M (lys)-enhanced green fluorescent protein (EGFP) mice5 kindly provided by Dr. T. Graf (Albert Einstein College, New York). Marrow cavities were flushed, cells were resuspended in PBS, and 5×10⁶ donor cells were administered to 6-8 week old *Apoe*+/− mice 24 hours after an ablative dose of whole-body irradiation (2×6.5 Gy). Four weeks after reconstitution chimeric lys-EGFP*Apoe*−/− displayed normal peripheral blood cell counts (data not shown) with high EGFP expression by Gr-1+/CD115− neutrophils (Online Data Supplement Figure 1b) and were placed on an atherogenic diet for another 10 weeks. To induce BM aplasia in female *Ldlr*−/− mice (14 weeks of age), mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation, using an Andrex Smart 225 X-ray source (YXLON International, Copenhagen, Denmark) with a 6 mm aluminium filter, one day before transplantation. BM cell suspensions were isolated from femurs and tibias of male *Ldlr*−/− mice. Single cell suspensions were prepared by passing the cells through a cell strainer and 10⁷ cells/well were plated for viral transductions with either LV.Empty or LV.CXCR4deg. After 24 hours, cells were washed with PBS and subsequently injected into irradiated recipients. After 6 weeks, mice were fed a Western-type diet, containing 0.25% cholesterol and 15% cacao butter.
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(SDS, Sussex, UK) for 10 weeks. Effectiveness of CXCR4 degrakine was confirmed by a 49.5±6.5% reduction in Cxcr4 expression on CD3⁺ T cell in Apoe⁻/⁻ mice 16 weeks after transduction (not shown). Animal experiments were approved by local authorities and complied with German animal protection law and Dutch government guidelines.

Atherosclerotic lesion quantification and immunohistochemical analysis

The extent of atherosclerosis was assessed on aortic roots and on thoracoabdominal aortas by staining for lipid deposition with oil-red-O as described and quantified by computerized image analysis (Diskus Software, Hilgers, Königswinter) and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Briefly, atherosclerotic lesions were measured on a constant number of 5-µm transversal sections through the heart and aortic roots and an average of lipid deposition was calculated from 3-10 sections. The thoracoabdominal aorta was opened longitudinally, and the percentage of lipid deposition was calculated by dividing the stained area by the total thoracoabdominal aortic surface. The relative content of macrophages, SMCs, and CD3⁺ T cells was determined by mAb staining for MOMA-2 (MCA519), CD3 (MCA1477, Serotec, Kidlington, Oxford, UK), smoothelin (N-15, Santa Cruz) and detection with FITC-conjugated antibody (Sigma, St Louis, MO) or alkaline phosphatase substrate (Vector Laboratories). Neutrophils were detected by specific esterase staining (Naphthol AS-D chloroacetate, Sigma) and collagen stained by Masson-trichrome (Sigma). Mast cells were detected by Toluidin Blue Staining (Sigma). Apoptotic nuclei were detected by terminal deoxynucleotidyl nick-end labeling (TUNEL-kit, Roche). Nuclei were counter-stained by 4',6-Diamidino-2-phenylindol (DAPI). Images were recorded with a Leica DMLB fluorescence microscope and CCD camera. After subtracting isotype controls, specific immunostaining was expressed as percentage of plaque area (SoftImagingSystems). CD3⁺ and TUNEL⁺ cells were quantified and expressed as cells/plaque area.

Transmission electron microscopy

Aortic roots were fixed in 3% glutaraldehyde (in 0.1 M B2 buffer [pH 7.4; 13 mM NaH₂PO₄ x H₂O; 87 mM Na₂HPO₄ x 2H₂O]) for 14 hours, washed with 0.1 M B 2 buffer overnight, followed by 1 hours in 1% OsO₄ (in 17% sucrose buffer [pH 7.4; 88 ml 0.1 M B 2 buffer; 12 ml aqua dest., 17 g sucrose]), rinsed with water and dehydrated with ethanol (30% - 100%) and
propylenoxide (100%) and embedding in Epon, polymerized (48 h at 60°C), cut into 80-100 nm thick slices and contrasted with uranyl acetate and lead citrate. Electron microscopy was performed (Philips EM 400 T) and images were recorded with a CCD-Kamera (MORADA; Olympus Soft Imaging Solutions, Münster).

**Reverse transcriptase-PCR**

RNA was isolated from thoracoabdominal aortas using TRIzol (Invitrogen). cDNA was reverse transcribed from 1 µg of DNAase-treated total RNA (Omniscript RT Kit, Qiagen) and RT-PCR was performed using 20 ng cDNA, Taq polymerase (Promega) and specific primers for *Ifn-γ* (5’-GCTGTTTCTGGCTTTACTGC-3’, 5’-TCACCATCCTTTTGCCAGTTC-3’), *elastase-2* (5’-TAAATTTCCCGTACGTGCAG-3’, 5’-GGGTGATGGTCTGTTTGTG-3’), *Cxcl1* (5’-AGCACCACCGCTCGTTCTCTGTG-3’, 5’-ACGCCTCGCGATCTTTGAGTGG-3’), TF (5’-CAGGAAGCAGTACAGTGTGC-3’, 5’-GGTGTTCTCTCCTTTCCTGTGC-3’), *C3* (5’-GGCTGTAGTCAGTTGGACAACCA-3’, 5’-CAGCCACATCAAGTGCAAGAAACG-3’), *aldolase* (5’-AGCTGTCTGGCACATCGCTCAACG-3’, 5’-CACATACTGCCAGCGCTTCAAGA-3’), or *Gapdh* (5’-CCACAGCCTTTGGCAGCAG-3’, 5’-ACTCAAGATGGTCAGC-3’). Products were separated by agarose gel electrophoresis and analyzed by comparison to *Gapdh* or *aldolase*.

**Cell culture, flow cytometry of blood leukocytes and labeling of isolated neutrophils**

Murine multipotent factor-dependent cell-Paterson (FDCP)-mix cells (clone A4, kindly provided by Dr. A. Whetton, University of Manchester) were cultured as described⁴. Mouse SV40-immortalized microvascular endothelial cells (SVECs) were generated as described⁷. Human neutrophils were isolated by Ficoll gradient centrifugation and subsequent erythrocyte lysis⁷. Whole blood was subjected to red cell lysis using Pharmlyse (BD Biosciences) and washed in DMEM with 2mM EDTA and 0.5% BSA. Staining for flow cytometric analysis was conducted using combinations of antibodies from BD Biosciences (Gr-1, CD11b, CD3, CD19, CD45, Ifn-γ, Il-10), eBiosciences (CD115, CD4, CD8) in HBSS with 0.3 mM EDTA and 0.1% BSA. Probes were analyzed in a FACSCanto II (BD Biosciences). Neutrophils were enriched from BM by magnetic depletion of B220⁺, CD115⁺ and I-A⁺ (MHCII)⁺ cells using AutoMACS (Miltenyi), resulting in a purity of 74.4±1.4% and minimal contamination with monocytes or B cells (<1%, Online Data Supplement Figure 1c). After labeling with fluorescent latex beads (LX)⁹,
13.7±0.1% of neutrophils had phagocytically internalized LX, as they were inaccessible to reactivity with anti-FITC mAb.

**Adhesion assays and ex vivo perfusion**

Arrest of calcein-AM (Molecular Probes)-labeled neutrophils on SVECc activated with TNF-α (100 U/mL, 12 h) was quantified in parallel-wall chambers under flow conditions (1.5 dynes/cm², 5 min)⁷. Carotid arteries were explanted from Apoe⁻/⁻ mice after an atherogenic diet for 6 weeks, transferred onto the stage of an epifluorescence microscope (Zeiss Axiotech, 20x water immersion) and perfused at 4µL/min with calcein-AM labeled FDCP-mix cells transduced with empty vector control or CXCR4degrakine. Arrest was analyzed after 10 min of perfusion⁷.

**Respiratory burst, phagocytosis assay and calcium mobilization**

Neutrophils were isolated from peripheral blood or BM of mice treated with AMD3465 (100µg s.c. for 3 days) after Lympholyte Mammal (Cedarlane) density gradient centrifugation with subsequent lysis of erythrocytes, resulting in a purity of 84.6±2.9%. For analysis of ROS generation, 10⁶ neutrophils were incubated in 300 µl of HBSS containing 1 mM MgCl₂, 1.3 mM CaCl, 5.6 mM D-glucose with 10 µM luminol (37°C, 15 min, Pierce). Cells were left untreated or stimulated with of 3.2 µM PMA and chemiluminescence was monitored in a Luminescence Image Analyzer LAS-3000 (Fuji) und Multi-Gauge Software for 10 min. For phagocytosis measurements, cells were resuspended in RPMI-40 supplemented with 50% FCS containing 0.5µm FITC⁺ microspheres (37°C, 15 min; Molecular Probes). After rigorous washing, endocytosed microspheres were quantitated by flow cytometry. Isotpye control and anti-FITC-Ab staining confirmed intracellular localization of the spheres⁹. For calcium mobilization assays, neutrophils were labeled with Fluo-4-AM (Molecular Probes). After the addition of CXCL1 at various concentrations (10 ng/ml, 100 ng/ml, 1 µg/ml), the mean fluorescence intensity (MFI) was determined as a measure of cytosolic Ca²⁺ concentrations using a BD FACSaria⁷.

**Statistical Analysis**

Data represent mean±SEM and were compared by 2-tailed Student’s t test (InStat software, GraphPad). Differences with P<0.05 were considered to be statistically significant.
References


Online Data Supplement Figure 1

Online Data Supplement Figure 1: Arrest of FDCP-mix cells transduced with lentiviral vectors as indicated in *ex vivo* perfused carotid arteries (a). Peripheral blood neutrophils (defined as Ly-6G/C+CD115- cells) from lys-EGFP bone marrow-chimeric Apoe−/− mice display a strong expression of EGFP, as shown by flow cytometry. Inserted values indicate percentage of gated cells (b). Isolation of neutrophils from bone marrow results in minimal monocyte contamination, as shown by flow cytometric analysis of CD115+ cells. Inserted values indicate percentage of gated cells (c). *P<0.05.
Online Data Supplement Figure 2: The relative content of MOMA-2⁺ macrophages (a) and SMCs (b) per plaque area was determined by immunofluorescence microscopy in Ldlr⁻/⁻ mice repopulated with BM transduced with empty vector or CXCR4 degrakine. Mast cells (arrows) could be detected by Toluidin Blue staining (c) in the adventitia of aortic root plaques of control and AMD3465-treated Apoe⁻/⁻ mice. *P<0.05.
Online Data Supplement Figure 3

Online Data Supplement Figure 3: The relative number of monocytes (a) was determined by flow cytometric analysis in peripheral blood of vehicle (control, squares) or AMD3465-treated (triangles) Apoe<sup>−/−</sup> mice at the indicated time points after pump implantation. Monocytes were distinguished by staining for CD115 (M-CSF receptor). Leukocyte counts (b) and the relative number of monocytes (c) were determined in peripheral blood of wild-type and Cxcr<sup>4+/−</sup> mice by standard cytometry and flow cytometric analysis. The relative BM-content of neutrophils (d,e) and monocytes (e) was analyzed in vehicle- and AMD3465-treated Apoe<sup>−/−</sup> mice by flow cytometry; representative dot plots are shown, inserted values indicate percentage of gated cells (d). Calcium influx was analyzed by flow cytometry in Fluo4-AM-labeled human neutrophils with or without pre-treatment with AMD3465 (f) after stimulation with CXCL1 at 100 ng/mL (arrows). Representative traces are shown The arrest of neutrophils isolated from Apoe<sup>−/−</sup> mice treated with vehicle or AMD3465 for 4 weeks to TNF-α-activated microvascular endothelial cells (SVECs) (g) was analyzed in multiple fields (n=3).
Online Supplement Figure 4: The mRNA expression levels of esterase (a), \textit{Ifn-\gamma} (b), \textit{Cxcl1}, \textit{TF} and \textit{C3} (c) were analyzed in aortas from vehicle or AMD3465-treated \textit{ApoE}^{-/-} mice in comparison to \textit{GAPDH} or \textit{aldolase} by RT-PCR.