Hyperlipidemia-Triggered Neutrophilia Promotes Early Atherosclerosis

Maik Drechsler, MSc; Remco T.A. Megens, PhD; Marc van Zandvoort, PhD; Christian Weber, MD; Oliver Soehnlein, MD, PhD

Background—Inflammation and activation of immune cells are key mechanisms in the development of atherosclerosis. Previous data indicate important roles for monocytes and T lymphocytes in lesion formation, whereas the contribution of neutrophils remains to be firmly established. Here, we investigate the effect of hypercholesterolemia on peripheral neutrophil counts, neutrophil recruitment to atherosclerotic lesions, and the importance of neutrophils in atherosclerotic lesion formation in Apoe<sup>−/−</sup> mice.

Methods and Results—Hypercholesterolemia induces neutrophilia, which was attributable to enhanced granulopoiesis and enhanced mobilization from the bone marrow. The degree of hypercholesterolemia-induced neutrophilia was positively correlated with the extent of early atherosclerotic lesion formation. In turn, neutropenic mice display reduced plaque sizes at early but not late stages of atherosclerotic lesion formation. Flow cytometry of enzymatically digested aortas further shows altered cellular plaque composition in neutropenic mice with reduced numbers of inflammatory monocytes and macrophages. Aortic neutrophil infiltration peaks 4 weeks after the start of a high-fat diet and decreases afterward. The recruitment of neutrophils to large arteries was found to depend on CCR1, CCR2, CCR5, and CXCR2, which contrasts to peripheral venous recruitment, which requires CCR2 and CXCR2 only. The involvement of CCR1 and CCR5 corresponded to the endothelial deposition of the platelet-derived chemokine CCL5 in arteries but not in veins.

Conclusions—Our data provide evidence that hypercholesterolemia-induced neutrophilia is multifactorial and that neutrophils infiltrate arteries primarily during early stages of atherosclerosis. Collectively, these data suggest an important role of neutrophils in the initiation of atherosclerosis. (Circulation. 2010;122:1837-1845.)

Key Words: atherosclerosis ■ cholesterol ■ leukocytes ■ inflammation ■ neutrophil

Dyslipidemia activates both endothelial cells and cells of the myeloid lineage, thereby representing a major risk factor for atherosclerosis.1,2 Hypercholesterolemia increases circulating inflammatory monocytes counts and renders these cells more prone for emigration into atherosclerotic lesions. The causal contribution of monocytes to atherosclerotic plaque formation, progression, and destabilization has been studied extensively.4 Recent evidence from human atherosclerosis specimens and murine models of atherosclerosis, however, suggests the presence of neutrophils and neutrophil-derived mediators in atherosclerotic lesions.5-7 For instance, neutrophil granule proteins such as α-defensins, azurocidin, and LL-37 have been detected within plaques.8 In particular, the function of monocytes, macrophages, and dendritic cells is regulated by neutrophil-derived mediators in terms of recruitment, phagocytic capacity, and cytokine release.9-12 Evidence is accumulating that hyperlipidemia not only activates mononuclear cells but also primes circulating neutrophils. In particular, a positive correlation between plasma triglycerides and low-density lipoprotein and neutrophil reactive oxygen species formation has been shown.13 Recently, Mazor et al14 found an increased rate of superoxide release and CD11b surface expression, which was positively correlated with the severity of hyperlipidemia. In addition, circulating neutrophils contained less myeloperoxidase, whereas plasma myeloperoxidase levels were elevated,14 indicative of granule discharge from neutrophils in patients with hyperlipidemia. Clinical studies correlating systemic neutrophil counts with severity of atherosclerosis in humans further support an association of neutrophils with disease progression.15,16 These indications led us to systematically investigate the effect of hypercholesterolemia on neutrophil homeostasis and phenotype and to dissect the involvement of neutrophils in atherogenesis.

Editorial see p 1786
Clinical Perspective on p 1845

Methods
An expanded Supplementary Methods section is available in the online-only Data Supplement.
Animals

Ldlr<sup>−/−</sup> mice (C57BL/6J background Charles River Laboratories, Wilmington, Mass.) were transplanted with bone marrow cells from Cxcr2<sup>−/−</sup>, Cxcr4<sup>−/−</sup>, or respective wild-type litter mates by tail vein injection 24 hours after an ablative dose of whole-body irradiation (2×6.5 Gy). Apoe<sup>−/−</sup>, Ccr1<sup>−/−</sup>, Apoe<sup>−/−</sup>, Cxcr2<sup>−/−</sup>/Apoe<sup>−/−</sup>, Cxcr5<sup>−/−</sup>/Apoe<sup>−/−</sup>, Lysmegfp<sup>−</sup>CD11b<sup>−</sup>/Gr1<sup>−</sup>, CD115<sup>−</sup>/Apoe<sup>−/−</sup> mice<sup>17</sup> were fed a high-fat diet (HFD) containing 21% fat (Altromin, Lage, Germany) for indicated time periods. Platelets were depleted by intraperitoneal injection of 50 μL rabbit anti-mouse platelet serum (Table I in the online-only Data Supplement).<sup>18</sup> Antiplatelet or control rabbit serum was purchased from Accurate Chemical. Neutrophils were depleted by intraperitoneal injection of monoclonal antibody IA8 (100 μg per mouse every other day, BioXCell, West Lebanon, N. Hamp.) during the last 4 weeks of the HFD. Efficiency and specificity of neutrophil depletion were confirmed as reported previously (Table II in the online-only Data Supplement).<sup>18</sup> Mouse strains were backcrossed in the C57Bl/6 background for 10 generations. All animal experiments were approved by the local ethics committee.

Flow Cytometry and ELISA

Staining for flow cytometric analyses was conducted using the indicated combinations of antibodies in Hank’s balanced salt solution with 0.3 mmol/L EDTA and 0.1% BSA. For fluorescence-activated cell sorter analysis, blood was drawn and subjected to red blood cell lysis. Bone marrow cells were obtained by flushing the femur with 0.5 mL RPMI 1640/H11001. Staining for flow cytometric analyses was conducted using the intravital microscopy or ELISA (both R&D Systems, Minneapolis, Minn).

Immunohistochemistry

The extent of atherosclerosis was assessed in aortic roots by Oil Red O staining. Neutrophils in aortic root sections were identified by staining with an antibody to Ly6G (1A8, BD Biosciences, San Jose, Calif).

Intravital Microscopy

Intravital microscopy of the cremaster muscle and the carotid artery was performed in monocyte-depleted Lysmegfp<sup>−</sup>Gr1<sup>−</sup>CD11b<sup>−</sup>/Apoe<sup>−/−</sup> mice<sup>17</sup> as described previously.<sup>10,19</sup> For luminal detection of chemokines presented on the endothelium, Protein G Fluoresbrite YG Microspheres (Polysciences, Eppelheim, Germany) were coupled to polyclonal antibodies to CCL2, CCL3, or CCL5 or an IgG control (all eBioscience, Frankfurt, Germany) and injected intravenously into mice after exposure of the external carotid or the cremaster muscle.

In Vivo 2-Photon Microscopy Imaging

Exposed arteries were visualized in vivo with a LaVision Trismcope multiphoton system (LaVision Biotec, Bielefeld, Germany) in single-beam mode. See the Supplementary Methods section in the online-only Data Supplement for more detailed information.

Statistics

All continuous data are expressed as mean±SD. Statistical calculations were performed with GraphPad Prism 5 (GraphPad Software Inc, San Diego, Calif). Unpaired Student t test, Mann-Whitney test, or Kruskal-Wallis test with posthoc Dunn tests was used as appropriate. Values of P<0.05 were considered significant. It should be noted that the data presented here may be limited by small sample sizes; a nonsignificant difference cannot be interpreted as a lack of association.

Results

Hypercholesterolemia Induces Neutrophilia Positively Correlating With Atherosclerosis

Hypercholesterolemia has been reported to affect counts, phenotype, and function of peripheral blood leukocyte subsets.<sup>2,3,20</sup> Therefore, we analyzed circulating neutrophil counts in Apoe<sup>−/−</sup> mice receiving chow, Apoe<sup>−/−</sup> mice fed an HFD developed...
both leukocytosis and neutrophilia (Figure 1A). It has previously been suggested that the number of circulating neutrophils is positively correlated with the degree of atherosclerosis and atherosclerosis-related diseases. In line with that suggestion, we found that plaque sizes in ApoE−/− mice with early atherosclerotic lesions were highly correlated with circulating neutrophil numbers (Figure 1B and 1C).

We next aimed to elucidate the mechanisms underlying HFD-mediated neutrophilia. Neutrophil homeostasis is regulated at various levels, including granulopoiesis, mobilization from the bone marrow, apoptosis, and subsequent clearance. Therefore, we initially assessed the fraction of mitotic neutrophils in the bone marrow, where neutrophil counts were also elevated (Figure 2A). Cell cycle analysis with the DNA-intercalating dye propidium iodide revealed that the number of neutrophils undergoing mitosis (middle) and serum G-CSF levels in ApoE−/− mice with or without an HFD (right), n=8. Unpaired t test. B. Expression of CXCR2 on bone marrow neutrophils of ApoE−/− mice with or without an HFD (left and middle) and serum levels of mCXCL1 in ApoE−/− mice receiving an HFD or not (right), n=8. Unpaired t test. C. Number of annexin V−/− (AnV−/−) neutrophils in the bone marrow (left), CXCR4 expression of circulating neutrophils (middle), and CXCL12 concentrations in bone marrow lavages (right) of ApoE−/− mice with or without an HFD, n=8. Unpaired t test. MFI indicates mean fluorescence intensity.

Neutrophils Infiltrate Large Arteries Predominantly During Early Stages of Atherosclerosis

Previous studies have reported the presence of neutrophils in human and murine atherosclerotic lesions. The mechanisms by which neutrophils promote atherogenesis, however, have not been systematically investigated. To address the involvement of neutrophils at various stages of atherosclerotic lesion formation, we first investigated the prevalence of neutrophils in the aorta at several time points after the start of an HFD. Fluorescence-activated cell sorter analysis of enzymatically digested lungs were not affected by the HFD in association with lower levels of CXCL12 in bone marrow lavage fluids (Figure 2C), suggesting a reduced neutrophil clearance under an HFD. Collectively, these data indicate that a disturbance of the CXCR2-mCXCL1 and CXCR4-CXCL12 axes contributes to HFD-induced neutrophilia. Moreover, a large pool of margined neutrophils can be found in the lung circulation, and mobilization from this location may contribute to HFD-induced neutrophilia. Because myeloperoxidase concentration and the number of neutrophils in enzymatically digested lungs were not affected by the HFD (Figure I in the online-only Data Supplement), such a mechanism seems unlikely.

Figure 2. HFD-induced neutrophilia is based on stimulated granulopoiesis and mobilization from the bone marrow and reduced clearance. A. Absolute numbers of bone marrow neutrophils (left) and of bone marrow neutrophils undergoing mitosis (middle) and serum G-CSF levels in ApoE−/− mice with or without an HFD (right), n=8. Unpaired t test. B. Expression of CXCR2 on bone marrow neutrophils (left and middle) and serum levels of CXCR1 in ApoE−/− mice receiving an HFD or not (right), n=8. Unpaired t test. C. Number of annexin V−/− (AnV−/−) neutrophils in the bone marrow (left), CXCR4 expression of circulating neutrophils (middle), and CXCL12 concentrations in bone marrow lavages (right) of ApoE−/− mice with or without an HFD, n=8. Unpaired t test. MFI indicates mean fluorescence intensity.
early infiltration of neutrophils comes from intravital microscopic observations of the external carotid artery of monocyte-depleted Lysmegfp/egfpApoe−/− mice. In this model, only neutrophils are fluorescent (Figure II in the online-only Data Supplement) and were clearly found to adhere to the lumen of the external carotid artery (Figure 3B). Confirming previous reports,5,6 we further detected neutrophils by immunohistochemical analyses of aortic roots of Apoe−/− mice having received an HFD for 4 weeks by staining for the neutrophil-specific marker Ly6G (Figure 3C). These data suggest a transmural rather than a transluminal route of arterial neutrophil infiltration. This notion is further corroborated by observations using intravital 2-photon microscopy of monocyte-depleted Lysmegfp/egfpApoe−/− mice. After adhesion, neutrophils spread out and migrate into a subendothelial location (Figure 3D and Movie I in the online-only Data Supplement).

**Arterial Neutrophil Recruitment Partially Depends on Platelet-Derived Chemokines**

To further elucidate the involvement of chemokine receptors in neutrophil accumulation in the aorta, we quantified neutrophil counts in digested aortas of atherosclerotic mice with deficiencies in Ccr1, Ccr2, and Ccr5. The involvement of CXCR2 and CXCR4 was studied in bone marrow chimeras. Interestingly, Ccr1−/−Apoe−/−, Ccr2−/−Apoe−/−, Ccr5−/−Apoe−/−, and Cxcr2−/−→Ldlr−/− mice exhibited reduced aortic neutrophil numbers compared with Apoe−/− or wild type→Ldlr−/−, respectively (Figure 4A). Cxcr4−/−→Ldlr−/− mice, on the other hand, displayed higher neutrophil counts (Figure 4A), likely attributable to increased peripheral neutrophil count in this model.5 To further corroborate our findings, we used a model of intravital microscopy using short-term treatment with specific chemokine receptor antagonists, hence not skewing peripheral neutrophil counts. Using monocyte-depleted Lysmegfp/egfpApoe−/− neutrophil-reporter mice, we could validate the involvement of CCR1, CCR2, CCR5, and CXCR2 in neutrophil adhesion (Figure 4B) to large arteries. This contrasts to the reported recruitment pattern of neutrophils, which have been described to use primarily CXCR2 and in some circumstances CCR2 for tissue infiltration.26,27 Because these data stem primarily from microcirculatory recruitment models, we compared the recruitment of neutrophils to large arteries with postcapillary venous recruitment behavior. Intraperitoneal challenge of chemokine receptor–deficient atherosclerotic mice (Figure III in the online-only Data Supplement) or Apoe−/− mice pretreated with chemokine receptor antagonists with recombinant mouse (rm)TNF (Figure 4C) confirmed the involvement of CCR2 and CXCR2 in neutrophil extravasation, as has been reported.26,27 Furthermore, we found CCR2 and CXCR2 to be involved in the adhesion and extravasation of neutrophils in the cremaster muscle of monocyte-deplete Lysmegfp/egfpApoe−/− mice locally injected with rmTNF (Figure 4D). Taken together, these data suggest a microvascular recruitment pattern that largely differs from that in large arteries such that CCR1 and
CCR5 seem to have important roles in arterial but not venous recruitment. Because the expression of CCR1 and CCR5 was not significantly altered in Apoe<sup>/−/−</sup> mice receiving an HFD (Figure IV in the online-only Data Supplement), we consequently hypothesized that the endothelial presentation of CCL3 and CCL5, both of which interact with CCR1 and CCR5, may differ between the vascular beds. Hence, we examined the luminal immobilization of fluorescent beads conjugated with antibodies to CCL2, CCL3, or CCL5 in vivo as a measure of locally presented chemokines. Although CCL2 was clearly detected in the carotid artery and postcapillary venules of the cremaster muscle compared with the respective IgG control, in agreement with the involvement of CCR2 in neutrophil recruitment at either site, CCL5 was detectable primarily in the carotid artery but not the veins of cremaster muscles (Figure 5A and 5B). CCL3, on the other hand, was not found at either vascular bed. Hence, differential presentation of the CCR1 and CCR5 ligand CCL5 may be the underlying cause of the different neutrophil recruitment pattern.

Platelets are a major source for CCL5, and endothelial deposition of CCL5 has recently emerged as a therapeutic option. To dissect the importance of platelets in endothelial CCL5 deposition in our model, we selectively depleted platelets (Table I in the online-only Data Supplement). Compared with control animals, thrombocytopenic mice exhibited lower amounts of CCL5 deposited in carotid arteries. This observation was corroborated in mice treated with a P-selectin antagonist or an inhibitor of platelet glycoprotein IIb/IIIa (ReoPro), which displayed reduced CCL5 deposition (Figure 5C). Similarly, the involvement of platelet-derived CCL5 in arterial neutrophil recruitment was further evaluated in platelet-depleted mice, which exhibit reduced numbers of neutrophils adhering to the carotid artery (Figure 5D).

**Neutrophils Fuel Early Atherosclerotic Lesion Formation**

To link aortic neutrophil infiltration to atherogenesis, we selectively depleted Apoe<sup>/−/−</sup> mice of neutrophils (Table II in the online-only Data Supplement) at various time points. Using this approach, we found a significant reduction in the degree of atherosclerotic lesion formation of aortic roots (Figure 6A) at early time points only. Specifically, depletion of neutrophils during the first 4 weeks of an HFD reduced atherosclerotic lesions by 49%, whereas no effect was seen when mice were rendered neutropenic after 3 or 11 months of an HFD (Figure 6A). Neutrophils have been implicated in the recruitment of monocytes, macrophages, and dendritic cells, all of which play important roles in atherogenesis and progression. Therefore, we investigated the cellular composition of digested aortas in neutropenic mice and mice with normal white blood cell count after 1 month of an HFD. Depletion of neutrophils reduced the amount of CD45<sup>+</sup> cells in aortic lysates, much of which was attributed to reduced neutrophil recruitment.
phil counts (Figure 6B). In addition, the prevalence of CD45<sup>+</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> inflammatory monocytes and CD45<sup>+</sup>F4/80<sup>+</sup>Gr1<sup>+</sup>CD11b<sup>hi</sup> macrophages was significantly reduced. Furthermore, neutropenic mice exhibited a higher relative content of CD3<sup>+</sup> T lymphocytes. In contrast, aortic counts of dendritic cells and resident Gr1<sup>+</sup> monocytes were not affected.

Discussion

Atherosclerosis is currently viewed as a chronic inflammatory disease of the arterial vessel wall with prominent roles for endothelial cells, T lymphocytes, and monocyte-derived cells. Hypercholesterolemia is a major risk factor activating these cell types, resulting in enhanced expression of cell...
adhesion molecules, chemokines, and proinflammatory cytokines and ultimately promoting arterial infiltration with immune cells. Recent work suggests a prominent role for neutrophils in atherosclerosis, and this study is the first to link early stages of atherosclerosis to hypercholesterolemia-induced neutrophilia (Figure V in the online-only Data Supplement).

Hyperlipidemia exhibits apparent effects on homeostasis of bone marrow-derived cells, with increases in circulating numbers of lymphocytes, platelets, monocytes, and progenitor cells. In most cases, hyperlipidemia-induced perturbations in leukocyte homeostasis are multifactorial. For instance, hypercholesterolemia-associated mononcytosis resulted from continued bone marrow production of inflammatory monocytes, increased survival of these cells in the periphery, and impaired conversion to resident monocytes. Here, we show that hypercholesterolemia induces neutrophilia, which is positively associated with atherosclerotic plaque burden. Clinical studies have previously proposed a positive correlation between circulating neutrophil counts and the risk for cardiovascular events. Neutrophil homeostasis is regulated at various levels, including production, mobilization, and clearance, much of which is regulated by an equilibrium of chemokines, cytokines, and growth factors. Our study shows that hypercholesterolemia induces G-CSF, the key cytokine in regulating granulopoiesis. G-CSF itself is induced primarily by increased levels of TNF and IL-17; the latter is the effector of a neutrophil clearance feedback loop. Our study and recent findings by others have provided evidence of increased TNF and IL-17 levels in the plasma of atherosclerotic mice. G-CSF not only stimulates proliferation of myeloid precursors but also reduces bone marrow CXCL12 levels, hence reducing the clearance of aged neutrophils. Finally, hypercholesterolemia enhances serum CXCL1 levels, which promotes neutrophil mobilization via CXCR2. Hence, hyperlipidemia disturbs the tightly regulated cytokine system controlling neutrophil homeostasis at various levels, ultimately increasing peripheral neutrophil counts.

Neutrophil extravasation involves the coordinated and well-regulated interaction of selectins, cell adhesion molecules, and chemokines. It has previously been reported that the majority of transient leukocyte endothelial contacts in atherosclerosis are attributable to neutrophils, which interact with endothelial selectins. Here, we extend these observations, describing that neutrophils adhere to the lumen of large arteries at early stages of atherosclerosis. The importance of molecules such as P-selectin, $\beta_2$-integrins, and intracellular adhesion molecule-1 in neutrophil adhesion and recruitment is without doubt. Hence, the vast reduction of atherosclerotic lesions in mice lacking P-selectin, the common $\beta_2$-integrin chain CD18, or its counterreceptor intracellular adhesion molecule-1 may be explained at least in part by reduced neutrophil extravasation. In addition, leucotriene B4 is a potent chemoattractant for neutrophils, and deletion of its high-affinity receptor BLT1 reduces atherosclerosis, specifically at early stages of atherosclerosis. In light of the data presented here, this stage-specific effect may be attributed to the importance of neutrophils in initial phases of atherosclerotic lesion formation. In addition, the deletion of chemokine receptors CXCR2 and CCR2 results in reduced atherosclerotic burden. Given the importance of both of these receptors in transmitting chemotactic signals for neutrophils, the atherosclerotic phenotype in these mice may be attributed in part to diminished arterial neutrophil infiltration. However, our data also describe an important role of CCR1 and CCR5 in adhesion and extravasation of neutrophils to large arteries. It has previously been shown that the use of monocytic CCR1 and CCR5 in arterial recruitment is due partially to chemokines that are deposited by activated platelets. This mechanism is important primarily in early stages of atherosclerosis and hence led us to investigate the importance of platelet-derived chemokines in neutrophil adhesion and recruitment. Indeed, we found that CCL5 deposited by activated platelets induces neutrophil adhesion through engagement of CCR1 and CCR5. The contribution of CCR1 and CCR5 to the extent of lesion formation may differ because a prevailing role of CCR5 in atherosclerosis has been reported. CXCR4, on the other hand, seems to have a mere homeostatic effect, as has previously been demonstrated.

Neutrophils infiltrate atherosclerotic vessels primarily during early stages and, in line with this depletion of neutrophils, reduce atherosclerotic lesion burden only in the initial phase. Neutrophils may exert proatherogenic effects via several direct and paracrine mechanisms. Neutrophils produce large amounts of oxygen radicals via NADPH oxidase and myeloperoxidase. Much of the oxygen radicals are secreted extracellularly, where they have been proposed to be key mediators involved in the oxidation of low-density lipoprotein particles by which low-density lipoprotein is entrapped in the subendothelial space. On the other hand, emigrating neutrophils release a wide panoply of granule proteins that act as extracellular regulators of inflammatory processes. Myeloperoxidase, for example, is released from neutrophil primary granules once the neutrophil has emigrated. Its transcytosis across endothelium allows interaction with leukocytes in flow and modification of leukocyte adhesion. Furthermore, myeloperoxidase acts as a leukocyte-derived nitric oxide oxidase, which limits nitric oxide bioavailability, thus promoting impairment of endothelial cell function. In patients with stable coronary artery disease, plasma myeloperoxidase levels have been shown to predict the prevalence and extent of coronary disease and future risk of cardiovascular events. Other granule proteins may serve as a link between the early efflux of neutrophils and the recruitment of inflammatory monocytes. In this context, LL-37 and azurocidin have recently gained attention because they specifically induce recruitment of inflammatory monocytes via involvement of formyl-peptide receptors. Accordingly, we show here that digested aortas of neutropenic mice contain lower numbers of inflammatory monocytes and macrophages, which likely are descendants of inflammatory monocytes.

Although not dealt with in this study, neutrophils may also have important roles during destabilization of advanced plaques. The accumulation of neutrophils in human atherosclerotic plaques is associated with characteristics of rupture-prone lesions. In addition, specimens from ruptured or eroded human plaques show distinct infiltrations with neutrophils. Crucial to plaque destabilization, rupture, or ero-
sion is a thin and collagen-poor fibrous cap, which results from impaired local synthesis or increased breakdown and thus the activity of matrix-degrading proteases. Neutrophils contain large amounts of matrix-degrading proteases, and data from human specimens indicate that these proteases are indeed released by neutrophils in unstable plaques and contribute to plaque destabilization and rupture. Of note, neutrophil infiltration and release of matrix-degrading enzymes into unstable plaques were found to be reduced on statin treatment in a mouse model of plaque disruption.

Conclusions
Hypercholesterolemia in mice results in neutrophilia, which can be attributed to stimulation of granulopoiesis, enhanced bone marrow mobilization, and reduced peripheral clearance (Figure V in the online-only Data Supplement). Increased peripheral neutrophil counts were found to correlate closely with the extent of early atherosclerosis formation. In these initial stages, neutrophils prominently infiltrate arteries through the involvement of CCR1, CCR2, CCR5, and CXCXR2. The use of CCR1 and CCR5 contrasts to peripheral neutrophil recruitment and may be ascribed to endothelial deposition of CCL5 by platelets. Once emigrated, neutrophils promote atherogenesis, as evidenced by reduced plaque sizes in neutropenic mice. Thus, the use of CCR1 and CCR5 in arterial but not venous recruitment may emerge as a feasible option for therapeutic targeting. Clearly, the addition of the neutrophil as a previously not fully appreciated player in atherosclerosis increases the complexity of cellular interactions in disease pathogenesis but also harbors valuable strategies for prevention and treatment.

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Disclosures
None.

References

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Atherosclerosis is a chronic inflammatory disease of large arteries with prominent roles of various leukocyte subsets that are recruited from the bloodstream into the vessel wall. Although current dogma emphasizes the role of monocyte and lymphocyte subsets, we describe here a pivotal role for neutrophils in the early stages of atherosclerosis. Hypercholesterolemia is an important risk factor, and we find evidence that high levels of cholesterol induce neutrophilia by cranking up granulopoiesis and by disturbing the chemokine axes regulating neutrophil mobilization from the bone marrow. We further find that levels of circulating neutrophils correlate with the degree of atherosclerosis, which may be useful as a simple approach to cardiovascular risk prediction. In addition, neutrophils infiltrate arteries prominently through the involvement of CCR1, CCR2, CCR5, and CXCR2. The use of CCR1 and CCR5 contrasts to peripheral neutrophil recruitment and may be ascribed to endothelial deposition of CCL5 by platelets. Once emigrated, neutrophils promote atherogenesis, as evidenced by reduced plaque sizes in neutropenic mice. Thus, the use of CCR1 and CCR5 in arterial but not venous recruitment may emerge as a feasible option for therapeutic targeting. Clearly, the addition of the neutrophil as a previously not fully appreciated player in atherosclerosis increases the complexity of cellular interactions in disease pathogenesis but also harbors valuable strategies for prevention and treatment.
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SUPPLEMENTAL MATERIAL

Supplementary methods

**Immunohistochemistry**

The extent of atherosclerosis was assessed in aortic roots and thoracoabdominal aortas by staining for lipid depositions with oil-red-O, quantified by computerized image analysis (Diskus Software) and Leica Qwin Imaging software (Leica Ltd.). Aortic roots were stained with an antibody to Ly6G (1A8, BD Biosciences). Staining was visualized by cyanine-3-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei were counter-stained by 4',6-Diamidino-2-phenylindol (DAPI). Images were acquired with a Leica DMLB fluorescence microscope and a CCD camera.

**Intravital microscopy**

Neutrophil rolling, adhesion and extravasation in the cremaster muscle was observed by intravital microscopy in Lysm^{egfp/egfp} Apoe^{−/−} mice depleted of monocytes by chlorodronate liposome injection to obtain mice with fluorescent neutrophils only (fig. S1). Inflammation was initiated by local injection of rmTNF (50ng/scrotum, Peprotech) and the cremaster muscle was exteriorized for microscopic observation 4h later. Rolling neutrophil flux was determined as the number of neutrophils passing a reference line perpendicular to blood flow within 30 seconds. Neutrophils were considered adherent when no rolling was observed for at least 30 seconds. The following chemokine receptor antagonists were injected i.p. 1h prior to stimulation: J113863 to CCR1 (5mg/kg), RS504393 to CCR2 (5mg/kg) DAPTA to CCR5 (1mg/kg), SB225002 to CXCR2 (5mg/kg, all Tocris Bioscience), AMD3100 to CXCR4 (5mg/kg, Sigma). For analysis of neutrophil interactions with the external carotid artery, Lysm^{egfp/egfp} Apoe^{−/−} mice were fed high fat diet for four weeks.
For luminal detection of chemokines presented on the endothelium, 50µl of Protein G Fluoresbrite® YG Microspheres (Polysciences) were coupled to 50µg of polyclonal antibodies to CCL2, CCL3, or CCL5 or an IgG control (all eBioscience). Beads and antibodies were reacted for 30min at room temperature, washed twice and subsequently injected i.v. into mice after exposure of the external carotid or the cremaster muscle. Antibody/bead complexes were allowed to circulate for 15min and immobilized complexes were detected by intravital microscopy. Platelets were depleted by i.p. injection of 50µl of rabbit anti-mouse platelet serum. Anti-platelet serum and control rabbit serum were purchased from Accurate Chemical. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera and a 10x saline-immersion objective. For image acquisition and analysis Olympus cell software was used.

**Peritonitis**

Mice were injected intraperitoneally with 50ng rmTNF in sterile PBS. After 4h, mice were sacrificed and the peritoneal cavity was lavaged. The number of infiltrated neutrophils was analyzed using flow cytometry. Specific chemokine receptor antagonists were instilled intraperitoneally 1h before administration of rmTNF as described for intravital microscopy above.

**In-vivo Two-Photon microscopy imaging**

Mice were placed in supine position and the right common carotid artery including the bifurcation and part of the internal branch were surgically exposed. Exposed areas were kept moist with saline at all time during the experiment. Exposed arteries were imaged using a LaVision Biotec Trimscope multiphoton system (LaVision Biotec GMBH, Bielefeld, Germany). In short, the LaVision Trimscope was used in single beam mode and coupled into an Olympus BX61WI microscope with an Olympus 20X NA 0.95; water dipping objective
Two-photon excitation was achieved using a pulsed Ti-Sapphire laser (MaiTai HP, Spectra Physics, Mountain View, USA) tuned to 830 nm for visualization of second Harmonic Generation (SHG) signal of collagen, Green Fluorescent Protein (GFP), and autofluorescent signal originating from the elastic laminae. Emitted fluorescent signals were detected with two photomultiplier tubes (PMTs) tuned to corresponding parts of the emission spectra: SHG, 453-497 nm (PMT 1); GFP, 500-550 nm (PMT 2); autofluorescence signal of elastin was detected in both PMT’s. Series of subsequent xy-sections (180x180 pixels) were obtained over time at an acquisition rate of 6Hz using the Imsector Pro 4.0.43 acquisition software (LaVision Biotec GMBH). After image acquisition the obtained time series were analyzed and only xy-sections acquired at comparable z-position and without severe motional artefacts (due to heart- and respiration cycle$^3$), were selected for further analyses. Overall image quality was improved by resizing the pixel matrix 2.5 times using a bilinear scaling technique. Furthermore, 2D Gaussian weighted filtering was performed to improve the signal-to-noise ratio and thus overall image quality.$^3$ All image processing was performed using Image-Pro 3D analyzer 7.0 (Media Cybernetics, Silver Spring, USA).
Table S1: Differential leukocyte counts and platelet counts in mice receiving anti-platelet or control serum. All values are given in count/ml venous blood.

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<td>0.506</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Table S2: Differential leukocyte counts in mice with intact WBC and in neutropenic mice. All values are given in count/ml venous blood.

<table>
<thead>
<tr>
<th></th>
<th>neutrophils</th>
<th>inflammatory monocytes</th>
<th>resident monocytes</th>
<th>T-lymphocytes</th>
<th>B-lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact WBC</td>
<td>9.7x10^7</td>
<td>3.7x10^5</td>
<td>2.2x10^5</td>
<td>1.9x10^5</td>
<td>2.3x10^5</td>
</tr>
<tr>
<td></td>
<td>+/- 2.2x10^3</td>
<td>+/- 0.9x10^3</td>
<td>+/- 0.3x10^5</td>
<td>+/- 0.4x10^5</td>
<td>+/- 0.5x10^5</td>
</tr>
<tr>
<td>neutropenic</td>
<td>0.8x10^7</td>
<td>3.4x10^5</td>
<td>2.2x10^5</td>
<td>1.8x10^5</td>
<td>2.2x10^5</td>
</tr>
<tr>
<td></td>
<td>+/- 0.2x10^3</td>
<td>+/- 0.6x10^3</td>
<td>+/- 0.2x10^3</td>
<td>+/- 0.2x10^3</td>
<td>+/- 0.4x10^3</td>
</tr>
<tr>
<td>p-value</td>
<td>0.002</td>
<td>0.309</td>
<td>0.630</td>
<td>0.309</td>
<td>0.588</td>
</tr>
</tbody>
</table>
Supplemental figures

Figure S1

Figure S1: Lung neutrophil content is not affected by HFD. Absolute numbers of neutrophils in enzymatically-digested lungs (left) and MPO activities in lung homogenates (right) in Apoe−/− mice with or without HFD. n=5. Mann-Whitney.
Figure S2

Figure S2: Monocyte-depleted \textit{Lysm}^{egfp/egfp} \textit{Apoe}^{-/-} mice are neutrophil-reporter mice. In \textit{Lysm}^{egfp/egfp} \textit{Apoe}^{-/-} mice, monocyte subsets and neutrophils are gfp-fluorescent (top). Intravenous injection of chlodenenate-filled liposomes (bottom) but not PBS-filled liposomes (middle) depletes monocytes.
Figure S3

Figure S3: Peritoneal neutrophil recruitment requires CXCR2 and CCR2. Peritoneal neutrophil accumulation in response to rmTNF (50 ng/mouse) in chemokine receptor deficient atherosclerotic mice. *indicates significant difference compared to respective control (shaded). n = 5-7 for each bar. Kruskal-Wallis test with posthoc Dunn.
Figure S4

Figure S4: High-fat diet does not alter neutrophilic chemokine receptor expression. Quantification of CCR1, CCR2, and CCR5 surface expression on neutrophils from Apoe<sup>-/-</sup> mice with or without HFD for four weeks expressed as fold change compared to Apoe<sup>-/-</sup> mice without diet is given to the right. Mann-Whitney.
Figure S5: Neutrophils promote atherosclerotic lesion formation. Hypercholesterolemia in mice results in neutrophilia. Granulopoiesis is stimulated by an increase in TNF and IL-17 which will enhance G-GSF levels. G-CSF also downregulates CXCL-12 in the bone marrow which acts as retention and clearance signal. In combination with enhanced neutrophilic CXCR2 expression in bone marrow neutrophils and increased serum mCXCL1 levels, these changes result in enhanced neutrophil mobilization from the bone marrow and reduced clearance of circulating neutrophils. Enhanced peripheral neutrophil counts were found to closely correlate with the degree of early atherosclerosis formation. In these initial stages, neutrophils prominently infiltrate arteries by involvement of CCR1, CCR2, CCR5, and CXCR2. The utilization of CCR1 and CCR5 contrasts to peripheral neutrophil recruitment and may be ascribed to endothelial deposition of CCL5 by platelets. Once emigrated, neutrophils promote atherogenesis, part of which may be due to the secondary recruitment of inflammatory monocytes that give rise to macrophages.
Supplemental references


**Video S1: Luminally adherent neutrophils infiltrate atherosclerotic arteries.** Neutrophils adhering to the carotid artery in monocyte-depleted $\text{Ly}5^\text{m}^{\text{eGFP}}$, $\text{Apoe}^{-/-}$ mice that had received HFD for 4 weeks were tracked for 10 minutes by 2-photon microscopy. Emitted fluorescent signals were detected with two photomultiplier tubes (PMTs) tuned to corresponding parts of the emission spectra, with green representing neutrophils, blue being collagen, and aquamarine being elastin. Series of subsequent xy-sections (180x180 pixels) were obtained over time at an acquisition rate of 6 Hz. After image acquisition the obtained time series were analyzed and only xy-sections acquired at comparable z-position and without severe motional artefacts were selected. From these, 10 representative frames were merged to a video sequence.